VALIDATION OF SALIVA BIOSPECIMEN: AN ALTERNATIVE TO NASOPHARYNGEAL SWAB FOR DETECTION OF SARS-COV-2 USING RT-PCR

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Recommendation

This is to certify that **Mr. Salin Maharjan** has successfully completed his dissertation work entitled **"VALIDATION OF SALIVA BIOSPECIMEN: AN ALTERNATIVE TO NASOPHARYNGEAL SWAB FOR DETECTION OF SARS-COV-2 USING RT-PCR"** under my supervision.

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

_____

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Certificate of Evaluation

This is to certify that this thesis entitled "**VALIDATION OF SALIVA BIOSPECIMEN: AN ALTERNATIVE TO NASOPHARYNGEAL SWAB FOR DETECTION OF SARS-COV-2 USING RT-PCR"** presented to evaluation committee by **Mr. Salin Maharjan** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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Acknowledgement

At the end of this long journey of obtaining Master's Degree, I would like to take a moment to express my heartfelt gratitude to everyone who made this day come true.

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Salin Maharjan

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Glossary Acronyms

ACE2: Angiotensin Converting Enzyme 2

CDC: Centers for Disease Control and Prevention

cDNA: Complementary DNA

CoV: Coronavirus

COVID: Coronavirus disease of 2019

CTD: C-terminal domain

Ct: Cycle threshold

ERGIC: Endoplasmic reticulum-Golgi intermediate compartment

FDA: Food and Drug Administration

HE: Hemagglutinin esterase

IDSA: Infectious Diseases Society of America

ICTV: International Committee on Taxonomy of Viruses

kDa: Kilodalton

LAMP: Loop-mediated isothermal amplification

MERS-CoV: Middle East respiratory syndrome coronavirus

NPS: Nasopharyngeal swabs

NIAID: National Institute of Allergy and Infectious Diseases

NTC: No template control

NTD: N-terminal domain

PCR: Polymerase chain reaction

+ssRNA: Positive-sense single-stranded genomic RNA virus

PPE: Personal protective equipment

qRT-PCR: Real time reverse transcription polymerase chain reaction

RT-PCR: Reverse transcriptase-polymerase chain reaction

RdRp: RNA-dependent RNA polymerase

Rpm: Revolutions per minute

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

SEM: Standard error of mean

VTM: Viral transport mediums

WHO: World Health Organization

Abstract

Extensive and widespread testing is necessary to control the rapid spread of SARS-CoV-2 pandemic which has been rampant since its origin in 2019. The current gold standard for COVID testing, nasopharyngeal sampling (NPS) method presents various obstacles for a successful mass testing like need of highly trained personnel, transport media, personal protective equipment (PPE). Hence, we have tried to establish saliva sample as an alternative to NPS. In this study, patients visiting the KM biotech lab for PCR test were asked to provide saliva samples in addition to NPS sample. The saliva sample is then heat killed and directly processed for PCR amplification. The corresponding data from NPS and saliva samples are compared to gauge efficiency of saliva against NPS. We observed that the saliva is effective as NPS with sensitivity (80%) and specificity (91%) [$P<0.01$]. We found that 60% (110/180) samples tested positive in either or both the NPS and saliva test, while 21(11%) samples tested positive in NPS only and 7(4%) samples tested positive in saliva only. In conclusion, saliva assay demonstrated significant results against the NPS samples. Saliva assay could prove effective in controlling COVID pandemic when compared to painful and resource intensive NPS sample. This study is relevant, especially in case of countries like Nepal, where we are completely dependent on international market for transport mediums, PPE, swabs etc. Saliva assay could reduce our dependence significantly as it has substantially less resource requirements.

Keywords: SARS-CoV-2, COVID-19, Saliva assay, NPS, COVID testing, RT-PCR

Chapter 1

1. INTRODUCTION

1.1. Background

Coronavirus disease (COVID-19) has spread across the globe since its emergence in 2019 and has resulted in death of millions of people. This disease is caused by infection of virus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The International Committee on Taxonomy of Viruses (ICTV) named the virus according to its phylogenetic analysis. The virus is believed to originate from bats, which acquired mutations allowing it to cross the species infection barrier from bats to pangolins to humans. It was first reported in Wuhan, China (Liu et al., 2020). Although SARS-CoV-2 is the most devastating outbreak of coronavirus in 21st century, it is only the third pandemic caused by coronavirus. The first outbreak was caused during late 2002 by severe acute respiratory syndrome coronavirus (SARS-CoV), originating in Guangdong Province, China. Bats were the primary host for SARS-CoV as well. The second epidemic MERS-CoV (Middle East respiratory syndrome coronavirus) occurred in 2012 in Saudi Arabia. Infected camels acted as the host for MERS-CoV (Fadaka et al., 2020). Although SARS-CoV-2 is closely related to SARS-CoV and MERS-CoV, it is genetically distinct and different from them.

1.1. Classification

Viruses within the *Nidovirales* order comprises of four families: *Coronaviridae, Arteriviridae, Roniviridae* and *Mesoniviridae* families. *Coronaviridae* are the Nidovirales order's largest group, comprised of two subfamilies: *Coronavirinae* and *Torovirinae.* The *Coronavirinae* are further subdivided into four genera: Alpha (α), Beta (β), Gamma (γ) and Delta (δ)-CoVs based on phylogenetic clustering and pair-wise evolutionary distances in seven key domains of the replicase-transcriptase polyprotein (Wang et al., 2020).

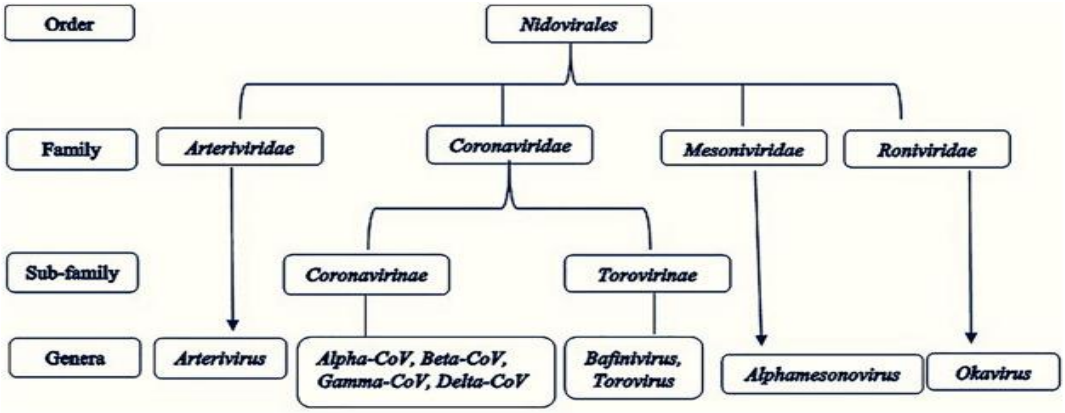


Figure 1: Taxonomy of Coronaviruses

Source: (Sofi, Hamid, & Bhat, 2020)

1.2. Structure

Coronaviruses(CoVs) are roughly spherical and enveloped in shape with size varying from 80 to 120 nm in diameter (Lai & Cavanagh, 1997). The club-shaped projections (spikes) emerging from the surface of the virion is the most prominent feature of CoVs. These spikes resemble a solar corona, where it gets its name from (Wang et al., 2020).

The four major structural proteins encoded by CoVs are: Spike (S), Membrane (M), Envelop (E), and Nucleocapsid (N).

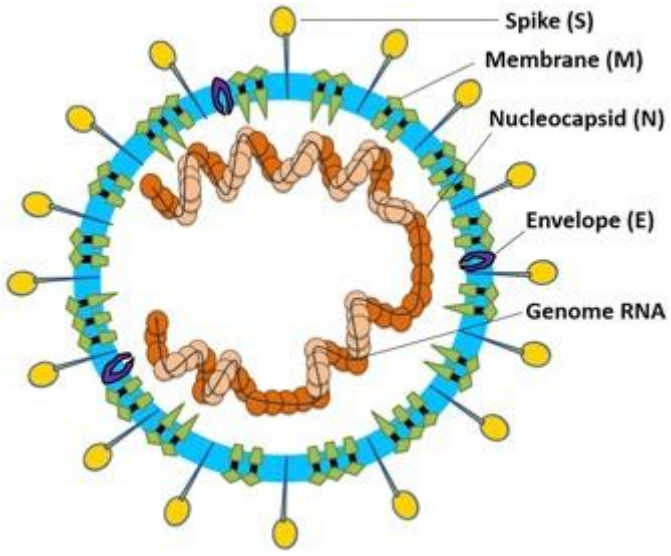


Figure 2: Structure of CoV virion

Source of picture: (Li. G, et al., 2020)

- **S glycoprotein**

The outermost component of the virion which is responsible for the attachment of virus to the cell is S glycoprotein (Lai & Cavanagh, 1997). Large, multifunctional class I viral transmembrane protein, S protein, is composed of two sub units- S1 and S2. The S1 subunit is responsible for receptor binding, while the second subunit, S2, facilitates fusion. S1 subunit can further be divided into two subdomains, the N-terminal domain (NTD) and C-terminal domain (CTD), and both act as receptor-binding domains with various hosts (Li. F et al., 2016). In some CoVs (specifically beta coronaviruses) hemagglutinin esterase (HE) are also present. They are shorter spike-like surface protein in addition to S glycoprotein that help in attachment and detachment of virus (Zeng et al, 2008).

- **M Protein**

It is the most abundant and main structural protein of envelop. It provides the overall shape to envelop. Consisting of three domains (a short N-terminal ectodomain, a triple-spanning transmembrane domain, and a C-terminal endodomain), M protein is a type III membrane protein. The M protein plays vital role during budding, envelope formation, assembly, and pathogenesis stages of the virus lifecycle (Neuman et al., 2011).

- **E Protein**

With the size of 8.4 to 12 kDa and composed of 76 to 109 amino acids, E proteins are the smallest major protein and highly variable in different species of CoVs (Masters, 2006). These integral proteins act as viroporin (ion channel) (Pervushin et al., 2009). Alteration of virulence of CoVs due to changes in morphology or tropism results in inactivation or absence of this protein (DeDiego et al., 2007).

- **N protein**

The helical nucleocapsid consist of the N protein whose size ranges from 43 to 50 kDa (Masters, 2006). Highly conserved and distinct domains found in N protein are namely, NTD, RNA-binding domain or a linker region (LKR), and a CTD. The viral genome is bound to the NTD end of N protein on 3’ end of the viral genome in highly divergent fashion, both in length and sequence (Dhama et al., 2020). N proteins are responsible for formation of

complex with viral genome. Also, during virion assembly, they facilitate M protein interaction and increases the transcription efficiency of virus (Chang et al., 2006).

1.3. Genome

Coronaviruses are positive-sense single stranded RNA viruses which are enveloped and no segmented. These viruses consist of largest known RNA genome ranging from 26 to 36 kilobases (Li. G et al., 2020). The viral genome possesses 5′ cap structure along with a 3′ poly(A) tail so that it can function as a messenger RNA(mRNA). The organization of the CoVs genome is 5′-leader-UTR-replicase-S (Spike)–E (Envelope)-M (Membrane)-N (Nucleocapsid)-3′UTR-poly (A) tail with accessory genes interspersed within the structural genes at the 3′ end of the genome(Figure 2) (Wang et al., 2020).

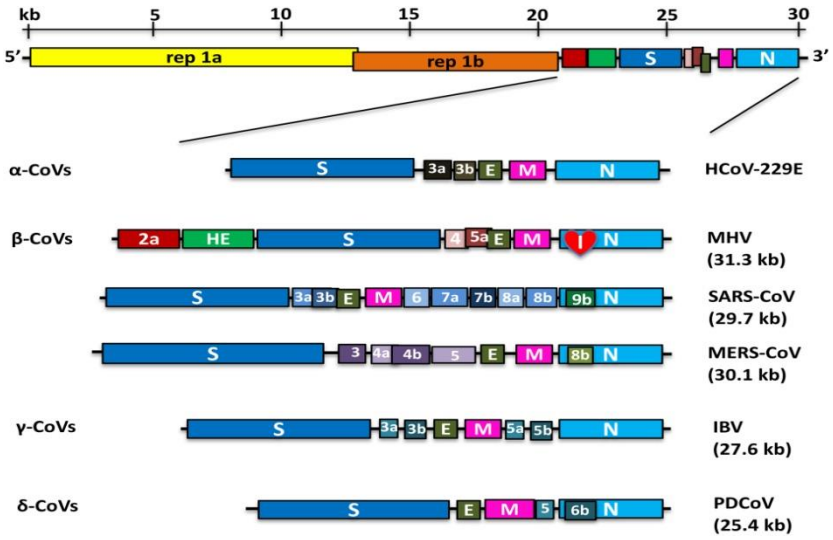


Figure 3: Genome Organization of Representative α, β, γ and δ-CoVs

Source of picture: (Wang et al., 2020)

1.4. Replication cycle

- Attachment and Entry

The interaction between S protein and its receptor initiates the initial attachment of virion with host. The receptor facilitating the binding of SARS-CoV to cells in humans is angiotensin-converting enzyme 2 (ACE2) (Li. C, et al., 2021). During adhesion, the catalytic domain N-terminal peptidase domain of ACE2 is recognized by the spike glycoprotein (Pizzato et al., 2022).

S protein attached to the receptor is cleaved activating it, by a protease from host cell. Of the two subunit included in S protein, S1 subunit mediates receptor binding and more conserved S2 subunit helps in fusion of virion and cell membrane by undergoing confrontational change (Wang et al., 2020).

- Replicase Protein Expression

Translation of the replicase gene from the viral genomic RNA is the next step in lifecycle of the virion. The virus particles are uncoated during entry into host cell and genome gains entry into the cytoplasm. 5′ methylated cap and a 3′ polyadenylated tail of CoV RNA genome allows it to act as a messenger RNA, which means host ribosomes can directly translate it. The viral genome is translated into 2 large overlapping polyproteins (pp1a and pp1ab) from ORF1a and ORF1b. 16 nonstructural proteins (nsp1 to nsp16) are formed from cleavage of polyprotein pp1ab. Nsps assemble to form suitable environment for RNA synthesis, formation of RNA-dependent RNA polymerase (nsp12), RNA helicase (nsp13), and exoribonuclease (nsp14) (Fehr & Perlman, 2015).

- Replication and Transcription

Assembly of multiple nsp into viral replicase complexes is followed by RNA synthesis. RNA-dependent RNA polymerase (RdRp), a main component of complex is directly involved in synthesis of RNA strand, both genomic and sub genomic RNAs. 3'co-terminal positive-sense sub genomic RNAs with full length viral genome form nested RNAs. Synthesis of positive sense RNA is done through intermediary negative sense strands as RdRp synthesizes negative sense RNA from genomic template (Fehr & Perlman, 2015).

- Assembly and Release

Once the replication is complete, viral structural proteins, S, E, and M are translated inside the endoplasmic reticulum. These proteins move along the secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). N protein encapsidated viral genome bud to form mature virion. Protein-protein interactions necessary in assembly of CoVs is directed by M protein (Fehr & Perlman, 2015).

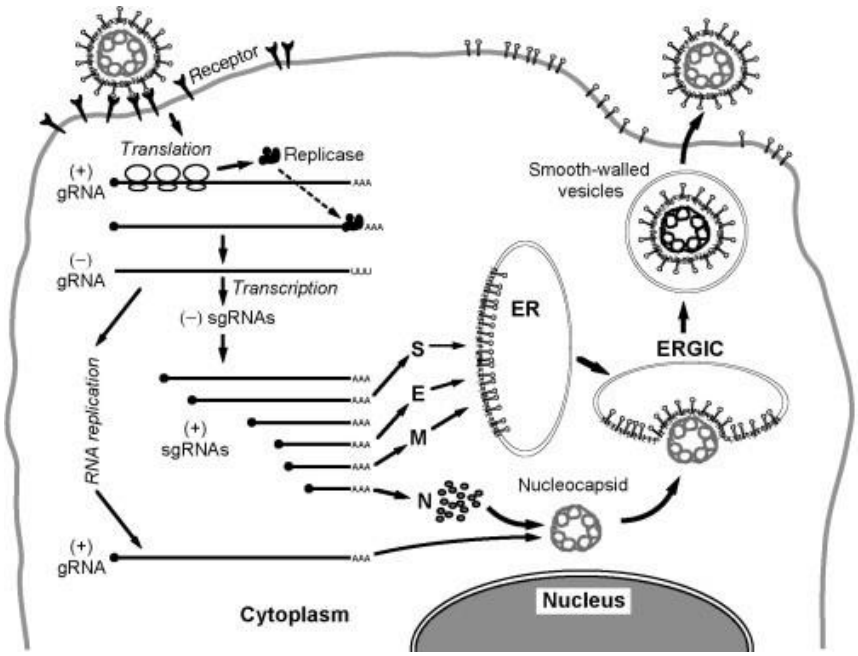


Figure 4: Life cycle of Coronavirus

Source of picture: (Masters, 2006)

1.5. Pathogenesis

We have not been able to determine the full picture of pathogenesis of CoV-19 yet (Sofi et al., 2020). Previously coronaviruses were expected to cause mild, self-limiting respiratory infections only. Scientists have isolated four coronaviruses that infect humans: α-coronaviruses (HCoV-229E and HCoV-NL63) and β-coronaviruses (HCoV-OC43 and HCoV-HKU1). The genetic variability of these viruses differs significantly. The low divergence of HCoV-229E explains why this variant fails to cross species barrier unlike HCoV-OC43, which can infect other species like mice (Sofi et al., 2020). The genetic sequence divergence of HCoV-229E from around the world is minimal, while HCoV-OC43 from same place but different time displays high variability (Wang et al., 2020).

The hypothesis of pathogenic stages of COVID has been generalized as: Viral invasion and replication, dysregulated immune response, multiple organ damage and recovery. Primarily alveolar epithelial cells in lungs are invaded by the virus. Presence of Angiotensin-converting enzyme 2 (ACE2) receptor in these organs is believed to be crucial in viral transmissibility and severity in humans (Li. C, et al.,2021). The viruses can infect ACE2 expressing immune cells like macrophages and dendrite cells as well. The global hospitalization data suggests

the incubation period of the virus to be 5.1 to 14 days (Sofi et al., 2020). The major symptoms of COVID are fever, cough, headaches, body pain, muscle aches. And since the main source of transmission is through direct contact and droplets (Ji et al., 2021),it is recommended to maintain minimum 6ft buffer and wear masks. Direct contact includes touching your eyes, mouth or nose with contaminated hands. Washing hands frequently with soap and using sanitizers is encourages to minimize the hazard risk.

1.6. Diagnosis

The symptoms of the SARS-CoV-2, fever, cough, etc, are very similar to the influenza symptoms. Correctly diagnosing the COVID infection is very important in limiting the spread of the virus and containing it. Currently, Reverse transcriptase-polymerase chain reaction (RT-PCR) assay is the gold standard for diagnosis of SARS-CoV-2. Nasal swab, aspirate, sputum, or blood samples can be obtained from the patients for PCR test to determine infection.

Various methods of SARS-CoV-2 diagnosis are;

1. Nucleic acid-based detection

   Modern sequencing technologies have made it possible to obtain whole genome sequences of organisms relatively easily and fast. After the emergence of SARS-CoV-2 virus, scientists were able study the virus and procure the whole genome in an effort to understand the virus and create potential diagnosis and treatment. SARS-CoV-2 consists of various proteins like ORF1a and ORF1b coding 16 nonstructural proteins such as RNA-dependent RNA polymerase, S (spike protein), E (envelop protein), M (membrane protein), and N (nucleocapsid protein). Such viral segments can be used to diagnose infection in patients (Wu, et al., 2022).

   a. Real time reverse transcription polymerase chain reaction (qRT-PCR)

      PCR testing has been the gold standard for the diagnosis of COVID-19. In this method, primers and probes are created from specific viral sequence, like gene segment coding for RdRp (RNA-dependent RNA polymerase), spike, nucleocapsid or enveloped proteins. The viral RNA is converted into cDNA by reverse

transcription process and then amplified. The viral load is determined by observing cycle threshold (Ct) values, lower the Ct value higher viral load (Sharma et al., 2021).

b. Isothermal detection

Although PCR testing is widely used for detection of CoV, it is time consuming, labor intensive and expensive. Isothermal detections improve upon these limitations of PCR testing and offer same sensitivity results at reduced cost and time.

Loop-mediated isothermal amplification (LAMP) is one of the variants of isothermal detection where 4-6 primers hybridize with various regions of target DNA. Strand displacing DNA polymerase insures fast amplification. In addition, reverse transcriptase enzyme can be incorporated to this method to detect RNA viruses. Reverse transcription LAMP (RT-LAMP) can be used to detect SARS-CoV under 30 min from saliva and nasopharyngeal samples of the patients (Jones et al., 2021).

Some examples of isothermal detection are; Loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), recombinase polymerase amplification (RPA) and rolling circle amplification (RCA) (Rabe A. & Constance, 2020).

2. Immunological methods

Any type of infection causes our body to display immunological response. Immunological assays detect these specific antibodies or antigens in our body to determine the extent of infection. Immunological method can be divided into two categories;

a. Serological tests: detection of serum antibody specific to SARS-CoV-2
b. Antigen detection: detection of specific antigens of SARS-CoV-2 based on specific antibody

Although serological tests are low cost and rapid, they present various obstacles. As these tests depend upon the concentration of antibodies and antigens present in blood, we cannot use these tests for early detection of infection. In addition, cross reactivity of antibodies can produce false results (Wu et al., 2022).

1.7. Current status of COVID-19

According to COVID-19 Weekly Epidemiological Update Edition 96 (published 15 June 2022), over 533 million confirmed cases with over 6.3 million deaths globally were reported (Weekly epidemiological update on COVID-19, 2022). Current active cases amount to 18,650,279 among which 18,613,994 (99.8%) are in mild Condition whereas 36,285 (0.2%) are serious or critical. Similarly of 523,722,820 closed cases 517,386,432 (99%) were recovered / discharged with 6,336,388 (1%) fatality (Worldometer, 2022).

In Nepal, 979297 total cases have been recorded as of 15 June 2022, of which 967248 (98.8%) have recovered. Case Fatality Rate of Nepal is at 1.2% with 11952 deaths (Ministry of Health and Population, 2022).

1.8. Problem statement

Wide spread testing to identify infected individuals and implementation of isolation to stop further spreading of the virus has been crucial strategy of most nations. The current gold standard for COVID testing, nasopharyngeal sampling process presents various obstacles. The sample collection process is very invasive and requires close contact between health workers and patients (Senok et al., 2020).The collection process is uncomfortable for the patients and can induce sneezing and cough which exposes the health workers too (Marta et al., 2021). In addition, highly trained personnel are required for the sampling process which is a major obstacle in mass screening for early detection of infection. Other resources requirements for sample collection like personal protective equipment (PPE), transport media, swabs exert very high financial burden and severely limit the scope of testing. In countries like Nepal, where we are heavily dependent on international market for supply of these equipment, the problem is even more severe.

1.9. Rational

Saliva assay is very attractive choice for detection of SARS-CoV-2 because of its cost effectiveness and ease of use. The saliva samples can be collected by patients themselves with guidance of health care workers which can be utilized in community setting for mass testing. Since saliva is not invasive process, individuals may be more receptive to testing for multiple times as required. This method will reduce the cost of testing as it reduces multiple requirements for testing as compared to nasopharyngeal sampling method. Currently various studies conducted have been with small sample size and in different settings, which has resulted in conflicting results (Senok et al., 2020). Hence, in this study we are trying to assess the accuracy of saliva sample in community setting compared to nasopharyngeal sample.

1.10. Hypothesis

- Null hypothesis (Ho): Saliva assay result is not as significant and accurate as nasopharyngeal sampling method.
- Alternate hypothesis (H1): Saliva assay result is as significant and accurate as nasopharyngeal sampling method.

1.11. Objectives

1.11.1. General Objective

- To validate saliva assay as an alternative method of SARS-CoV-2 detection for mass scale diagnosis.

1.11.2. Specific Objective

- Demographic analysis of the patients visited to KM TU-Biotech Corona Lab in the third wave of covid-19.
- To optimize the protocol for efficient sample processing and diagnosis
- Conduct the salivary assay for diagnosis of Covid-19 patients
- Compare the result with ongoing RT-PCR kits

Chapter 2

2. LITERATURE REVIEW

Since its first appearance on December 2019 in Wuhan, China, a novel corona virus, severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), causing corona virus disease 2019 (COVID-19) in humans have now developed into a pandemic (Rodriguez-Morales et al., 2020). According to World health organization (WHO), as of **1 April 2022**, there have been **486,761,597 confirmed cases** of COVID-19, including **6,142,735 deaths** (WHO coronavirus, 2022)**.**

2.1. SARS-CoV-2 genome and primer design

COVID-19 has taken heavy toll in world population and economy. To halt the infection from spreading further and to eradicate this pandemic reliable diagnostic tools and techniques are important. Real-time reverse transcription polymerase chain reaction (qRT-PCR) is the gold standard for detection of infection as it is the most specific and sensitive assay available to us. However, we need extensive knowledge about the genomic structure of the virus for this method to work. Due to this reason, there were no diagnostic kits available in the market at the start of pandemic. However, we have extensive knowledge of the virus now and many commercial kits are available for COVID-19 diagnosis.

Coronaviruses are enveloped, positive-sense single-stranded genomic RNA virus (+ssRNA). The full genome study of the SARS-CoV-2 has shown different regions of virus like ORF1ab or ORF8 regions, E gene, N gene, S gene, and RdRP genes (Figure 3). These genes can be targeted by primers and probes for detection of infection. But, the genomic structure of SARS-CoV-2 is very similar to SARS genome. Hence, in accordance with WHO guidelines, tests must be able to detect three different sets of gene for positive diagnosis- E gene, N gene and RdRP, ORF1ab. In these tests, one of the primers is programmed to detect a common gene from the beta-coronavirus group, while other primer is specific to SARS-CoV-2 (Kubina & Dziedzic, 2020). Detecting multiple genes in a single test helps in reducing the false negative results.

The primer and probe design used for the detection of specific gene varies greatly. While some studies like (Udugama et al., 2020) have found RdRP and E genes to show high sensitivity and poor sensitivity by N genes, other studies in contrast have found N gene to be the main component in detection of infection (Zhang. X et al., 2020). Hence, different kits can be found in the market which target different combination of genes for positive diagnosis. For example; "Real-time SARS-CoV-2 Assay" kit by Abbott relies on the amplification of RdRp and N genes while "cobas® SARS-CoV-2" kit by Roche targets ORF-1a and E-gene regions for CoV detection (Yüce et al., 2021). Different countries also have developed different primers and probes for the detection of COVID (Figure 5).

Institution	Gene target	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
U.S. CDC [33]	N gene	**N1:** GACCCCAAAATCAGCGAAAT	**N1:** TCTGGTTACTGCCAGTTGAATCTG	**N1:** FAM-ACCCCGCATTACGTTTG GTGGACC-BHQ1
		N2: TTACAAACATTGGCCGCAAA	**N2:** GCGCGACATTCCGAAGAA	**N2** FAM-ACAATTTGCCCCCAGC GCTTCAG-BHQ1
		N3: GGGAGCCTTGAATACACCAAAA	**N3:** TGTAGCACGATTGCAGCATTG	**N3:** FAM-AYCACATTGGCACCCGC AATCCTG-BHQ1
		RP-F RNAse: AGATTTGGACCTGCGAGCG	**RP-RRNAse:** GAGCGGCTGTCTCCACAAGT	**RP-P RNAse:** FAM-TTCTGACCTGAAGGCTC TGCGCG– BHQ-1
China CDC [165]	ORF1ab and N gene	**ORF1ab:** CCCTGTGGGTTTTACACTTAA	**ORF1ab:** ACGATTGTGCATCAGCTGA	**ORF1ab:** FAM-CCGTCTGCGGTATGTGGAAAG GTTATGG-BHQ1
		N: GGGGAACTTCTCCTGCTAGAAT	**N:** CAGACATTTTGCTCTCAAGCTG	**N:** FAM-TTGCTGCTGCTTGA CAGATT-TAMRA
Charité, Germany [41]	RdRp, E, N gene	**RdRp:** GTGARATGGTCATGTGTGGCGG	**RdRp:** CARATGTTAAASACACTATTAGCATA	**RdRp 1:** FAM-CAGGTGGAACCTCATC AGGAGATGC-BBQ
				RdRp 2: FAM-CCAGGTGGWACRTCATC MGGTGATGC-BBQ
		E: ACAGGTACGTTAATAGTTAATAGCGT	**E:** ATATTGCAGCAGTACGCACACA	**E:** FAM-ACACTAGCCATCCTTA CTGCGCTTCG-BBQ

Figure 5: Primer and probe design for SARS-CoV-2 detection

Source: (Udugama et al., 2020)

In the study conducted by (Vogels et al., 2020), where they compare sensitivity and efficiency of different SARS-COV-2 qRT-PCR assays, they found that under similar conditions the primer-probe sets from different nations performed satisfactorily except RdRp-SARSr (Charite), Germany, which had low specificity. Similarly,(Jung et al., 2020) in their study showed that the primers 2019-nCoV_N2 and N3 of the USA were the most sensitive for detection of N gene and the ORF1ab of China showed the best sensitivity for detection of

ORF1 gene. Hence, we could use primer-probe sets from different manufacturers in combination for better results.

2.2. Mechanism and optimization of assay conditions

Reverse transcription polymerase chain reaction (RT-PCR) is a derivative of PCR that was developed specifically for detection of RNA using reverse transcriptase enzyme. RT-PCR is generally combined with real time PCR (qPCR) to quantify the gene expression in sample.

The general procedure in RT-PCR consists of following steps (Yüce et al., 2021);

I. Synthesis of complementary DNA (cDNA) using reverse transcriptase enzyme following RNA isolation;

RT-PCR is specifically designed to detect RNA present in the sample, which are single stranded. But the working principle of PCR is based upon DNA, which is double stranded. Therefore, the RNA from the sample needs to be first converted into cDNA by the action of reverse transcriptase enzyme (Figure 6).

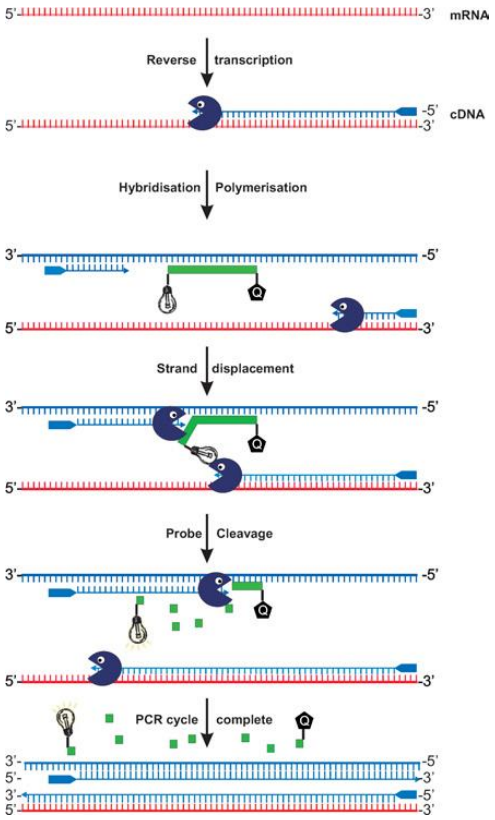


Figure 6: General steps in quantification of SARS-CoV-2 RNA in samples
Source: (Bustin & Mueller, 2005)

II. Amplification of cDNA by PCR and release of fluorescence;

After the template cDNA has been created, the template is exponentially multiplied by the PCR in multiple thermal cycles. In one cycle the template goes through various stages; denaturation of template, annealing of primers to the template, and elongation of the strand. These steps are temperature sensitive and PCR thermocycler controls the temperature to guide the amplification process. The denaturation of the template occurs at 95°C. At this temperature the two strands of cDNA separates creating two new template strands. The second step comprises of cooling the mixture to 55°C, at this temperature the primers can anneal to the template strand. In the third step, the temperature is increased to 72°C activating the polymerase enzyme. The polymerase enzyme adds the nucleotides to the end of primers elongating the strand. At the end of the cycle the temperature is increased again and the process repeats itself (Yalçinkaya & Koçoglu, 1992).

qRT-PCR [real-time RT (reverse transcription)-PCR] however uses fluorescent reporter molecules to detect the amplification of the target gene during each cycle. Probe based technique is used to detect COVID-19 infection from the samples, where specific fluorescent probes are used (Figure 6). During the polymerization of the strand, the hybridized probes are cleaved by the polymerase enzyme, releasing reporter dye and quencher dyes. The amplification of the target sequence is directly proportional to the fluorescence detected (Bustin & Mueller, 2005).

2.3. COVID vaccines

Vaccines have played major role in survival of human race against multiple pandemics before the current COVID-19 pandemic. The vaccines are designed so that they induce specific immune response in body. The active component (antigen) of vaccine is responsible for inducing this response, which could consist of different component from virus depending upon the vaccine type. Currently, most researched types of vaccines for SARS-CoV-2 include; RNA based vaccine, DNA vaccine, Protein subunit vaccine, and inactivated vaccine (Joshi et al., 2021).

Inactivated vaccine utilizes older technologies, where whole virus or a fragment of virus is used to illicit immune response. To create inactivated vaccine, viral cultures are created in lab, then heat or chemicals are used to inactivate them. Although these types of vaccines are relatively easy to create and elicit a strong immune response, these lack long lasting immune response. So, repeated doses and adjuvant maybe needed. Verocell, Coronavac, Covaxin are some of the inactivated vaccines approved for marketing. Although 2 doses of Verocell vaccine displayed strong response, its serum neutralizing activity against variants like delta, beta and omicron was found to be decreasing (Zhang Z, et al., 2022).

Protein subunit vaccine utilizes modern genetic engineering technologies to create viral antigen for effective immune response. Various COVID-19 proteins like S, E, N proteins have been studied to create immune response eliciting antigen. S1 and S2 domains of the S protein have been extensively explored for vaccine development. Targeting full length S protein or its subunits have shown to enhanced humoral and cellular immunity (Chung et al., 2021). Since these types of vaccines do not use whole virus, they are much safer than inactivated vaccines. But the difficulty in expression of S protein could affect the vaccine yield (Z. Zhang et al., 2022). NVX-CoV2373 by Novavax, a USA-based biotechnology company, SCB-2019 by Clover Biopharmaceuticals Inc, a China-based biotechnology company are some examples of protein subunit vaccine (Joshi et al., 2021).

RNA vaccine involves injecting individuals with S antigen encoding mRNA sequence, which once inside the body is translated by host cells to specific protein that induces immune response. Since this type of vaccine do not use whole viral components and produce very specific proteins, high-efficiency immune response is generated with minimum adverse effects. Moderna COVID-19 vaccine or mRNA-1273 created by joint venture of National Institute of Allergy and Infectious Diseases (NIAID) and Moderna Inc and Comirnaty or BNT162b2 vaccine from BioNTech and Pfizer are the two most prominent RNA vaccines available in market (Joshi et al., 2021).

DNA vaccine is designed such that a specific antigen coding strand of DNA is injected into the human body. DNA plasmids are used as vector to deliver it into the cell, where they can transcribe the immunogenic antigens. DNA vaccines have various advantages like stimulation of B and T cells, longer storage time compared to RNA vaccines and easier to

mass produce (Z. Zhang et al., 2022). INO-4800+electroporation by Inovio pharmaceuticals, nCoV vaccine by Zydus Cadila are some examples of the DNA vaccine (Joshi et al., 2021).

2.4. Sample types for COVID-19 diagnosis

Most governments require its citizens to obtain PCR test results when suspected of infection. Individuals, who test positive, are not allowed in any international flights and even medical care could be postponed in such instances due to risk of contaminating medical equipment. This highlights the importance of diagnosis for individuals in their day to day lives. Diagnosis also plays major role in a nation's policy making as well, in regards to how to better manage infection from spreading further and containing it.

Mass screening of large population is necessary for efficient containment effort of the pandemic. However, there are many constraints for mass screening. Some limitations in diagnosis based on different sample used are;

- Oropharyngeal/nasopharyngeal swabs (NPS):

  Nasopharyngeal swabs are generally performed to test respiratory infections; as such it has been the main form of sampling method for testing COVID-19. But this sampling method is not suitable for mass screening.

  The samples must be collected by trained personnel, so it is not as efficient as self-sampling methods. In addition, availability of materials like personal protective equipment (PPE), RNA extraction kits, and nasal/oral sterile swabs can severely limit the diagnosis process (Morales Angulo et al., 2020). This sampling method also puts the health workers in infection risk. The sample collectors need to be close contact with the patient, and tendency of nasal swab to discomfort some individuals resulting in sneeze or cough means exposure risk.

- Salivary samples

  Saliva sampling provides a noninvasive sampling alternative to NPS sampling method. This method reduces the cross infection risk to the healthcare workers and can even be use when PPE is hard to come by (Sun et al., 2021). Since this method can be self-

administered and relatively cheaper than NPS sampling, it can be utilized for mass screening process.

2.5. Comparison of Nasopharyngeal and Saliva samples

Diagnosis is the corner stone of containing any type of disease, especially so in cases of pandemics. Large population need to be tested constantly and relevant treatment should be provided accordingly. Nasopharyngeal sampling method, even though implemented for standard testing, has many drawbacks when it comes to mass testing. Hence scientists have been looking for a better alternative since the pandemic started (Butler-Laporte et al., 2021). Saliva samples have been considered as an alternative to NPS sampling but the studies have shown mixed results.

Ease of sampling

Saliva sampling is a far better choice in comparison to NPS when it comes to ease of sample procurement. Self-saliva sampling is pretty reliable, making it feasible for mass sampling procedure as well. During saliva sample collection process, patients are instructed to pool their saliva in mouth before collecting it in saliva collection tubes by passive drooling method. This method is noninvasive procedure and do not entail negative side effects like sneezing, cough, discomfort, pain, which can be felt during NPS sampling method. The aerosols created during sneeze and cough puts the health workers at direct contact and risk of infection as well (Marta et al., 2021). Hence, proper PPE needs to be available all the time.

In addition, we do not need to visit a sample collection center and queue up for our samples to be taken. Currently, we can order a saliva collection kit to our home and follow simple instructions to deposit our samples. This can then be mailed to the test laboratory to be processed. The results are made available online. Such devices have been FDA cleared for emergency use only for now.

Manpower required

Nasopharyngeal sampling process requires trained health workers during sampling process. NPS requires health workers to insert sterile swab inside the patient's nasal cavity, which

causes irritation and discomfort even when handled by trained professionals. Therefore, availability of trained personnel who are capable of sampling creates a bottle neck in COVID testing.

Also, RNA extraction process in the lab requires additional trained people to obtain results. The process even though simple is very long and is physically demanding. Although same is the process for sample processing is similar for saliva sample, it is much shorter and simpler in comparison to NPS samples.

Materials required

Nasopharyngeal swabs after extraction must be transported in Viral transport mediums (VTM) or Universal transport medium (UTM) which have been cleared by FDA for use. VTMs of different types can be found which in general contain buffer salt solution, amino acid source and antimicrobial agent. VTMs help to secure virus until further processing can be done. These mediums are not easy to come by, especially when the demand is very high, leading to shortages multiple times after COVID pandemic. This can act as a bottleneck in the process of mass COVID testing process (Smith et al., 2020). In addition, these are relatively very expensive than saliva collection tubes.

Specialized saliva collection tubes have been developed for effective and easy saliva extraction from patients. But simple standard sterile falcon tubes can also be used for saliva collection (Ember et al., 2022). Unlike saliva collection kits, these falcon tubes come in various sizes and using wide mouth falcon tube of 50 ml size can make the collection even easier. Also, obtaining falcon tubes is much easier compared to VTMs which can be produced by few companies only. This could be the key in eliminating the bottleneck created by lack of VTMs for mass COVID testing.

Test time

After the patients have provided their samples for COVID infection test in lab, the samples need to be processed to generate results. The RNA extraction is long and tedious process that takes long time before it can be amplified in PCR machine. Depending on the sample collection site and available health workers, it can take few hours to days (Barrell. A, 2020).

Since, PCR is done in bulk, test laboratories will wait till they have a full batch of samples. And the sample extraction is slow and painful process, hence it takes even longer.

Saliva samples, on the other hand, can be self-collected and do not take long relatively. That means people do not need to wait for a trained professional to take their sample. Samples can be collected in bulk and processed. They are easier to transport and their sample process time is much shorter than NPS samples as well. Therefore, saliva samples can provide much faster results.

Cost

The cost of COVID testing plays major role in the willingness of general public to be tested frequently for COVID infection. In this context saliva sample could significantly undermine NPS sampling. The ease of sampling process, ease of sample processing, low technical personnel requirement and other factors come together to significantly lower the cost of testing by transport media saliva. The cost of testing is calculated by considering the cost of associated materials like transport media, swabs, PPE, containers, personnel salary etc. In the study conducted in Canada, the use of saliva sample to NPS had estimated to save $636 105 per 100,000 samples (Bastos et al, 2021).Other studies have been conducted, where it has been estimated that, saliva samples could reduce the cost by 40% and personnel requirement by 20% (Butler-Laporte et al., 2021). Such margins are very significant considering its ramifications on the global pandemic.

Results comparison

Even though saliva sample seems to be better in every aspect mentioned till now, it does not matter if it cannot deliver the same quality result as NPS samples provide. And, it gets tricky here as multiple independent studies have produced varying degree of results; where some studies favor heavily towards saliva and some refute its viability as alternative to NPS.

Some results are not very promising regarding saliva sample. In the study (Torres et al., 2021), the sensitivity of saliva to NPS was found to be 46% only. The sensitivity of saliva samples dropped significantly when the Ct value of sample increased. At Ct value greater than 33, the sensitivity of saliva was 14.6 % only.

Although considered the gold standard, NPS samples are also prone to producing false results. Significance of such negative results can be valued by considering the clinical symptoms, which according to (Johnson et al., 2021) saliva samples retain high sensitivity on early stage and pre-symptomatic stages. In another study conducted by (Rao et al., 2021), out of 217 test subjects, 160 were tested positive. But the detection rate differed when comparing saliva samples and NPS samples. Saliva samples detected 149 positive samples (93.1%) while NPS was able to detect 84 (52.5%) positive samples out of 160 samples. But such results are possible only in controlled environments and the sensitivity of saliva samples decreases in community settings.

As demonstrated by (Sahajpal et al., 2021), the detection rate of COVID from saliva was lower than NPS samples while following protocol U. Under protocol U, samples were vortexed briefly and analyzed for COVID where as in the next phase under protocol SalivaAll, bead homogenizer was used to homogenize the sample before testing. This significantly increased the sensitivity of saliva sample than before. This shows saliva samples can be improved upon even further to increase their sensitivity and test reliability.

From this we can understand that not all test labs are same, not all protocols are perfect. There is going to be negative results no matter which sample we are using, but depending on the present context, where world is in need of alternative easier and cheaper COVID test, saliva sample seems viable. But we need to have a standard saliva testing protocol to ensure the reliability of the test.

Challenges of saliva as alternative

The benefits and advantages of saliva cannot be disregarded when it comes to diagnostics. But still it has not been adopted for standardized testing, even in present context where whole world is in search for alternative to expensive and invasive test i.e. NPS sample method. One of the main reasons for not adopting the saliva could be attributed to lack of data. More data is being generated with collective effort and interest in saliva as an alternative, but it is still not enough to warrant such large-scale change. As stated in Infectious Diseases Society of America (IDSA) guidelines “Saliva as the sole sample source for COVID-19 diagnosis cannot be recommended due to a paucity of studies” (Landry M, et al, 2020). The problem with low amount of data to extract information from is compounded

even further by the fact that, there isn't any standardized protocol for saliva. Even though high amount of data is being generated in current years, difference in sample processing procedure, handling, storage etc has made it challenging to correlate different studies.

Viscosity of the saliva could also be another problem that needs to be addressed. It could reduce the sensitivity of the test and also lead to pipetting errors which could be significant while working with low viral load samples for RT-PCR (Pijuan-Galito et al., 2021). Similarly, (Landry et al., 2020) report samples that were thick, stringy, and difficult to pipette, which required sputasol treatment. High amount of such samples from sick patients present a huge obstacle in terms of sample processing time and maintaining sterility of lab work space. Asides from the viscosity, the amount of saliva is also important factor to consider. In small children and certain diseased patients, who cannot generate enough saliva, this method is not applicable.

Chapter 3

3. MATERIALS AND METHODS

3.1. Sample collections, site and size

The samples were collected from TU Biotech Corona Lab, Kirtipur, Kathmandu, Nepal. From 10th Poush 2078 till 6th Magh 2078, patients visiting the lab for official COVID test were also asked to provide saliva sample in addition to NPS sample. The participants were made fully aware of the details of the study and a signed consent form was procured. All the participants visiting the lab for nasopharyngeal test were considered in the project for saliva assay. Both NPS and saliva samples were processed in same lab.

The sample size was calculated accounting for sensitivity and specificity of the test. The final sample size was determined to be 173 (193 accounting for 10% dropout). The sample size was calculated using the following formula;

The total COVID19 cases as of 20th February, 2022 was around 970000 according to MoHP website. Since the study population is infinitely large, 95% confidence interval and 6% margin of error was considered. The prevalence rate was calculated by dividing total no. of covid19 cases by total PCR tests done, which gave a sample size of 150. However, total of 184 samples were used for this study, out of which 154 were COVID positive and the rest were negative.

Sample size (n) = $Z^2_{\alpha/2} \times P \times (1\text{-}P)/d^2$

Where,

Z = Z Statistic for a level of significance

α = Level of significance

P = Expected proportion/ Prevalence rate

d = Absolute Precision

Prevalence Rate = $970000/5700000$

= 0.17

Sample size (n) = $1.96^2 \times 0.17\ (1\text{-}0.17)/0.06^2$

≈ 150

3.2. Nasopharyngeal Swab (NPS) sample;

3.2.1. Sample collection

For the collection of Nasopharyngeal samples CDC recommended instructions were followed (CDC, 2021);

Patients were asked to tilt their head back. Gently the swab was inserted into the nostril just touching the palate until resistance was felt, indicating contact with nasopharyx. At that moment, gentle rub and roll of the swab was performed. The swab was carefully removed and placed into Viral Transport Medium (VTM). The tubes were carefully labeled and stored in an icebox till further processing.

3.2.2. RNA extraction process

For RNA extraction, instruction provided by CWBIO was followed (Flex & Biomek, 2015);

A 1.5 ml centrifugal tube was taken and 200 µl sample, 200 µl lysis buffer, 300 µl isopropanol was added. Mixture was vortexed for 15 seconds, and kept at room temperature for 5 minutes with a constant temperature. The obtained solution was transferred to the adsorption column of collection tube and centrifuged at 12000 RPM (~ 13400 x g) for 1 minute (Gyrozen1536 centrifuge). The filtrate was discarded. And then the adsorption column was placed back into the collection tube. 500 µl washing buffer 1was added to the adsorption column, centrifuged at 12000 rpm for 1 minute and filtrate was discarded. Then the adsorption column was placed back into the collection tube. 500 µl washing buffer 2 was added to the adsorption column, centrifuged at 12000 rpm for 1 minute, and filtrate was discarded. Then the adsorption column was placed back into the collection tube and dry centrifuged at 12000 rpm for 2 minutes. Waste liquid in the collection tube was discarded. The column was left to dry at room temperature for 2 minutes. 40-100 µl RNase-Free water was added to the middle part of the adsorption column membrane, and kept for 2 minutes at room temperature then centrifuged for 1 minute at 12000 rpm. The samples were stored at -20°C until master mix was ready.

3.2.3. Uni-medica master mix preparation

The uni-medica master mix kit had two tubes, each containing RT-PCR reaction buffer and RT-PCR enzyme mix comprising 925µl and 75µlof components respectively. Besides, it also contained 100µl each positive and negative controls. 18.5µl of reaction buffer and 1.5µl of enzyme mix were added in an Eppendorf tube. This mixture was then short centrifuged and was used as our final master mix for amplification purpose.

3.2.4. Sample loading

To each 20µl of extracted RNA, negative control and positive control, 20µl of so prepared master mix was added into PCR stripes, resulting final volume of 40µl. The caps were tightened and were subjected to centrifugation at 6000rpm for 10 seconds according to the kit manual. Finally, the PCR stripes were placed into PCR machine.

3.2.5. Result analysis

For result analysis, the baseline was set at a region before the exponential amplification, where the fluorescent signals of all the samples are relatively stable. Similarly, the threshold was set right above the highest point of the negative control amplification curve to nullify the irregular noises interfering with the results.

3.3. Saliva sample;

IDT (2019-nCoV) Primer-probe kit was used in the detection of COVID-19 infection in saliva samples. This was done in collaboration with La Jolla Institute for Immunology, California, USA. The saliva assay kit and the optimized kit was developed and provided by Suzie Alarcon. The protocol mentioned in the paper (Alarcon et al., 2020) was followed.

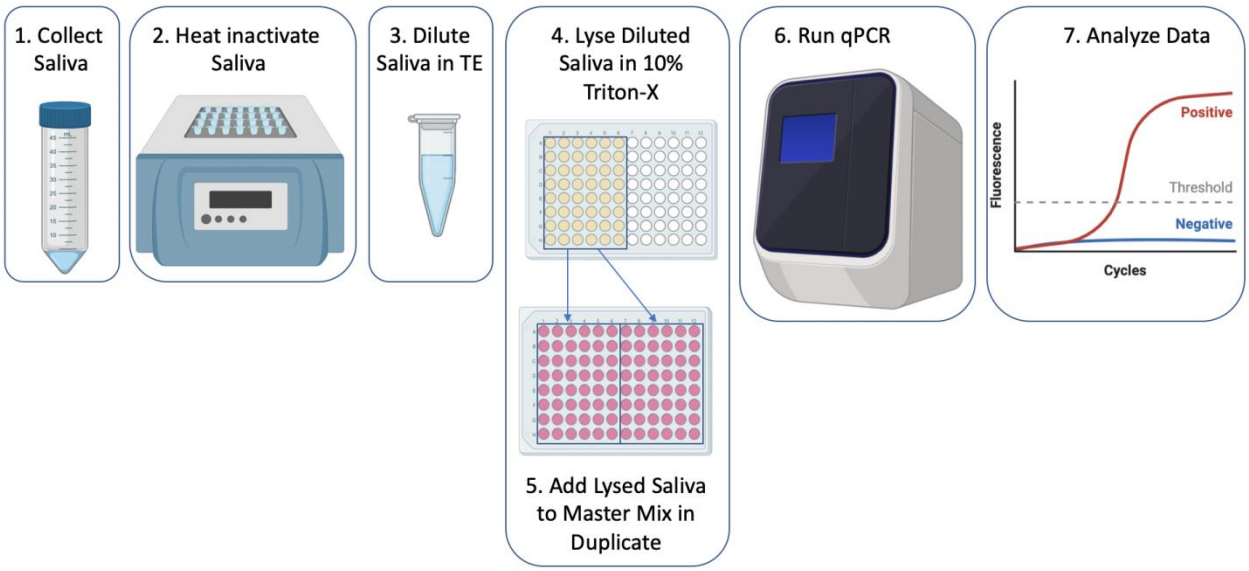


Figure 7: Graphic Overview of Protocol

Source of picture: (Alarcon et al., 2020)

3.3.1. Saliva collection protocol

Before the saliva collection process, patients were asked if they had eaten, chewed gum, brushed, used mouth wash, smoked within 1 hour (30 minutes minimum) before visiting the lab. If they performed any of the mentioned activities, they were not selected for the study. After the selection of patients, passive drooling technique was applied to collect saliva. Patients were instructed to allow the saliva to pool in their mouth until mouth felt full. Then, head was tipped forward and the saliva was allowed to drip into the collection tube without forcefully spitting into the tube (Tarsons 50ml graduated centrifuge tubes). Process was repeated till minimum volume (1-2mls was ideal, 50ul was minimum) of saliva was obtained. The tube was closed tightly. The outside of the collection tube was wiped with alcohol pads and placed inside biohazard transport bag. The alcohol pads were discarded.

3.3.2. Heat inactivation process

Working in a biosafety cabinet (Yakos65 SAFZONE Class II A2 Biosafety Cabinet), saliva samples were heat-inactivate using dry heat block (Taurus scientific). The obtained saliva samples were first sorted out and transferred into labeled eppendorf tubes. The saliva containing eppendorf tubes were placed into calibrated heat block, pre-heated to temperature (98°C x 5 minutes or 65°C x 30 minutes). In our experiment first option (98°C for 5min) was preferred. Then, 25% saliva dilutions in TE were made from the heat

inactivated saliva sample. 2ml eppendorf tubes were taken and labeled properly.750ul TE into each tube was dispensed. Then, 250ul of heat inactivated saliva was transferred into the tube containing 750ul TE. Caps were closed tightly. Stock/neat saliva could now be stored at -80C. Diluted saliva was used throughout the rest of the protocol.

3.3.3. For preparation of Master Mix

Primer/probe premixes (40X) and TaqPath (4X) were thawed on ice. Then a clean RNase, DNase free 2 ml Eppendorf tube was taken and master mix was created by mixing all the chemicals in (Table 1: Saliva assay master mix composition). For a 96-well plate, the following amounts were needed:

Table 1: Saliva assay master mix composition

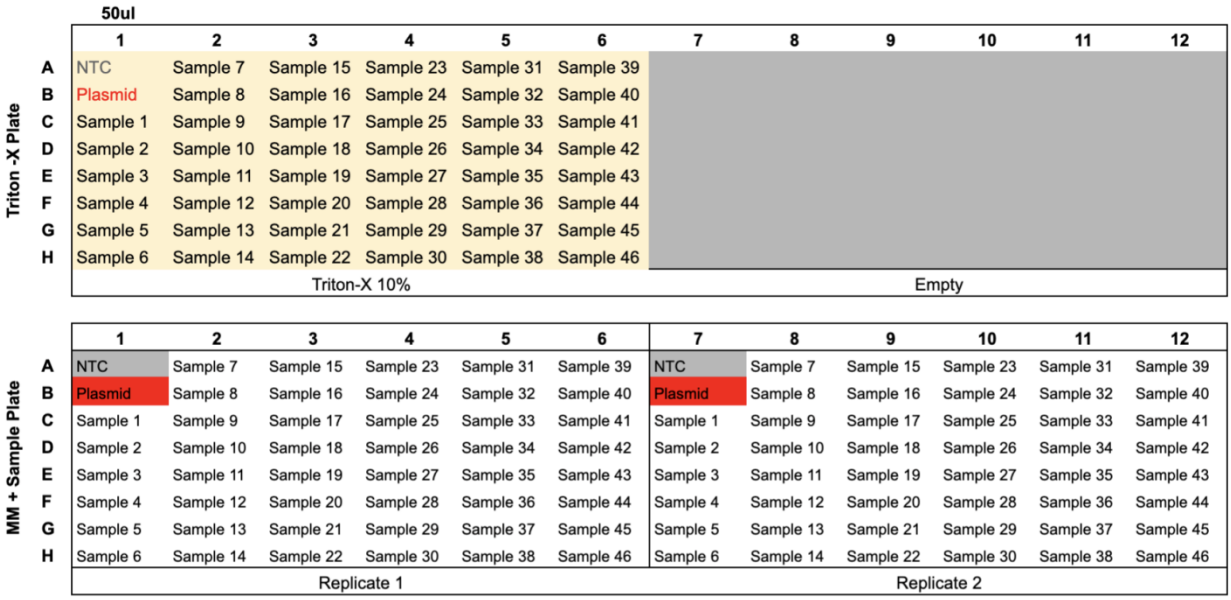
Master Mix N1, N2, RP				
# Samples	48			
Rxn Vol	20			
# of Replicates/Sample	2			
Overage	15%			
Component	ul x1	**ul batch**	[Stock] X	[Final] X
Premix N1	1.28	**140.8**	40	0.85
Premix N2	1.50	**165.6**	40	1
Premix RP	1.28	**140.8**	40	0.85
Saliva + TX	10	**1104.0**		
TaqPath	5	**552.0**		
H2O	0.95	**104.9**		
Total	**20.00**	**2208.0**		

Then, 10ul of master mix containing N1 premix, N2 premix, RP premix, TaqPath, and H2O was dispensed to each well of a 96-well OPTICAL PCR plate. Plate was sealed with foil and stored (Ideally for not more than an hour) at 4°C in the dark until ready to use.

3.3.4. For preparation of Lysis Plate

Tubes containing 25% saliva dilutions were centrifuged at 300g for 20 minutes (Gyrozen1536 centrifuge) at the beginning of this step. Once the centrifuge was running, 50ul of Triton-X was dispensed into half (48) the wells of a 96 well plate. One lysis well needed for each sample. After completion of 20 min centrifugation, tubes from centrifuge were removed and placed in rack or liquid handler. Carefully tubes were opened, taking care not to splash. 50ul of the upper aqueous phase of the diluted saliva samples was transferred

into a well containing 50ul of the Triton-X. As displayed in the example (Figure 8), NTC would go in A1; Plasmid in B1; Sample 1 in C1 etc of the Triton-X Plate. 50ul of NTC was transferred to one well of the Triton-X plate. 50ul of 200copy/ul Plasmid was transferred to one well of the Triton-X plate. With pipette set to 80ul, it was pipette mixed 6 times slowly taking care not to foam Triton-X. Then, plate was incubated for 5 minutes at room temperature to complete lysis reaction.



50ul

Triton -X Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39						
B	Plasmid	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40						
C	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41						
D	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42						
E	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43						
F	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44						
G	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45						
H	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46						

Triton-X 10% | Empty

MM + Sample Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
B	Plasmid	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Plasmid	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40
C	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41
D	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42
E	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43
F	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44
G	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45
H	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46

Replicate 1 | Replicate 2

Figure 8: PCR plate layout

After that, Master Mix plate prepared before this was removed from 4C storage and centrifuged at 2000RPM x 1 minute. Master Mix plates were constantly placed in ice pack while working on them. After incubation, using a P10 or P20 multichannel pipette (or 96 core head if available), 10ul of the lysed saliva/Triton mix from the Triton-X plate was pipette into 2 wells of the plate containing 10ul of Master Mix. Each lysis well was transferred to two wells in the final qPCR plate (MM+Sample Plate) as shown in lower portion of (Figure 8).

Each well of the plate now contained 10ul of Master Mix and 10ul of Lysed Saliva (Diluted Saliva + Triton Mixture). With pipette set to 15ul, each well was gently mixed 6 times. Plate was sealed with optical film suitable for real-time qPCR. Each well was visually inspected for expected volume. Then, plate was centrifuged at 2000 RPM x 1minute. The plate was visually inspected and sealed again after centrifugation.

Following cycling parameters was programmed in qRT-PCR cycler (Azure Cielo Real-time PCR);

Table 2: PCR cycle parameters

FDA CDC Instructions
Standard

Step	Deg C	Time	Purpose
1	25	2 min	UNG Incubation
2	50	15 min	RT Incubation
3	95	2 min	Enz Activation
4	95	5 sec	Amplification
5	56	30 sec	
7 Go to step 4 45 times total			

BioRad CFX

Step	Deg C	Time	Purpose
1	25	2 min	UNG Incubation
2	50	15 min	RT Incubation
3	95	2 min	Enz Activation
4	95	5 sec	Amplification
5	56	15 sec	
6	56	15 sec	Amplification /Read
7 Go to step 4 45 times total			

Dye channels were defined and targeted in software and applied to plate.

Table 3: PCR dye selected for each probe

Peak Channel (Quantstudio 3 and 5)	Color	Filter Wavelength (nm)[1]		Reporter Dye	Probe Name
		Excitation	Emission		
x1-m1	Blue	470 ± 15	520 ± 15	FAM	N1
x2-m2	Green	520 ± 10	558 ± 12	VIC	N2
x5-m5	Red	640 ± 10	682 ± 14	Cy5	RP

The program was saved and export location was designated. qPCR program was started. When qPCR program had been completed, the curves were inspected and adjusted thresholds for each target if necessary, according to software protocols to include amplification and disregarded noise such as background fluorescence. The generated data was exported according to software protocol.

Following reporting matrix was used to determine positive or negative result:

Positive Indicator Value Ct (or Cq) Value < 40

Negative Indicator Valu NaN or Ct (or Cq) Value > 40

Sample ID	N1	N2	RP	Plasmid	NTC	Test Type	Result	Next Step	Release Data/ Error Explanation ?
Sample A	+	+	+	+	-	Initial	Positive	Re-Test	No
Sample A (retest)	+	+	+	+	-	Confirmatory	Positive	Release Data	Yes
Sample B	+	-	+	+	-	Initial	Inconclusive	Re-Test	No
Sample B (retest scenario 1)	+	+	+	+	-	Confirmatory	Positive	Release Data	Yes
Sample B (retest scenario 2)	-	-	+	+	-	Confirmatory	Negative	Release Data	Yes
Sample C	-	+	+	+	-	Initial	Inconclusive	Re-Test	No
Sample C (retest scenario 1)	+	+	+	+	-	Confirmatory	Positive	Release Data	Yes
Sample C (retest scenario 2)	-	-	+	+	-	Confirmatory	Negative	Release Data	Yes
Sample D	-	-	+	+	-	Initial	Negative	Release Data	Yes
Sample E	-	-	-	+	-	Initial	Sample Fail	Re-collect sample	Yes
Sample F	any	any	any	If any batch control returns unexpected result, assay fail		Any	Assay Fail	Re-run plate	Yes
	Expected Batch Control Result			+	-				

3.3.5. Data Analysis

In case of NPS samples single test was done but undetermined results were retested for confirmation. All the saliva samples were tested in duplicates. Statistical data analysis, including calculations of mean, standard deviations, standard error of mean (SEM), etc were done with help of Microsoft Excel program. Graph plots and correlation analysis was done on Microsoft Excel program as well. The significance of all the results was analyzed by one tailed ANOVA test, paired T-test and Pearson's correlation coefficient. $P<0.05$ was considered significant.

Chapter 4

4. RESULT

4.1. Validation of the IDT saliva kit in Nepalese population

One hundred and ninety-five patients who visited the lab and fit the criteria (not eaten anything within 30 min of the test) were included in this study. 12 samples out of 195 were used to validate the procedure. Hence 183 samples were processed and compared to generate detailed data.

The kit was provided by La Jolla Institute for Immunology, California, USA along with optimized protocol. But to test if the kit and protocol was valid in Nepalese population, 12 samples (obtained from positive patients in isolation center) were tested if positive results could be obtained.

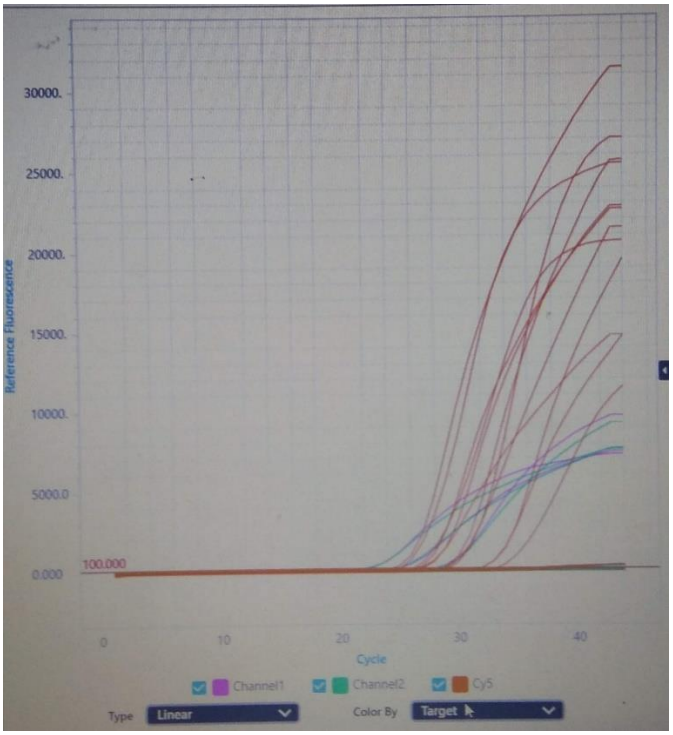


Figure 9: Amplification curve of validation samples

Out of the 13 samples collected and tested, 3 samples were tested positive by the saliva assay method. All of the participants were allegedly positive when the samples were obtained and had been in isolation center for varying number of days. None of the

participants were symptomatic at the time of sample collection. In Figure 9, we can see the amplification curve obtained after the completion of the PCR cycle.

Table 4: Ct values observed in validation samples

	Ct value		
Sample	**FAM (N1)**	**HEX (N2)**	**Cy5 (RP)**
SA05	42.509	41.799	25.047
SA09	28.073	28.014	28.523
SA12	21.999	22.107	34.743

In **Table 4** we can see the raw data obtained from the PCR machine for different probes assigned. Among the three samples which displayed Ct value, the sample SA05 had very high Ct value (greater than 40). Such result is not considered conclusive results and require retesting. However, in this validation stage, we were only trying to see if we could obtain gene amplification using IDT saliva assay kit in Nepalese population. Hence, retest for confirmation was not conducted and we proceeded for the next stage of the study.

4.2. Patient demographics and positive rate

The age of the patients ranged from 13-76 with the median age of 34. There were 114 males in the study and 69 female patients. The positive rate with respect to gender is showed in (Figure 10) and the distribution of patients according to their age is shown in (Table 5).

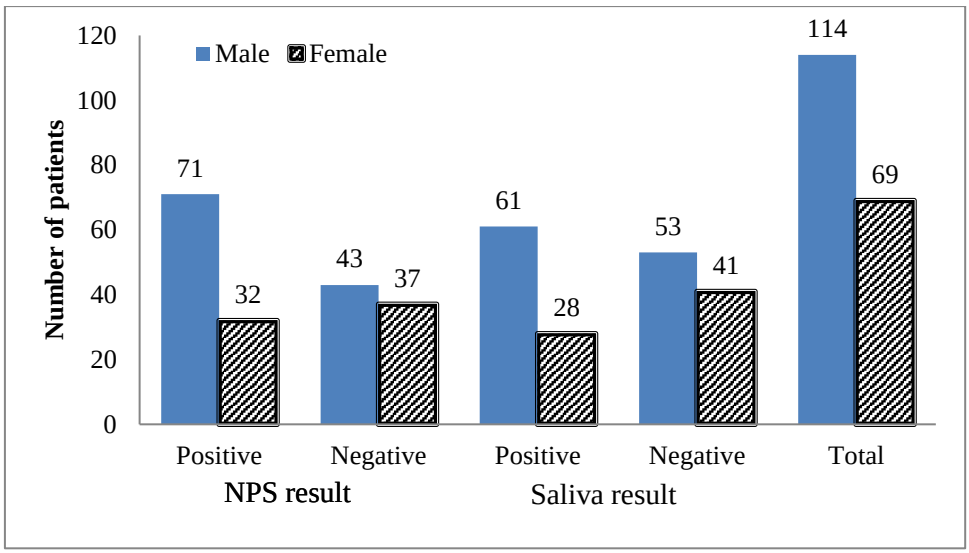


Figure 10: Positive rate differentiation according to gender

Out of 114 male and 69 female participants in the study, 62.28% of males and 46.38% of female tested positive by NPS sampling method whereas, 53.51% of males and 40.58% of females tested positive in Saliva assay. Hence, 37.72% males and 53.62% females tested negative in NPS. In saliva assay, 46.49% males and 59.42% females tested negative.

Table 5: Age demographic of patients

		NPS result		Saliva result		
MeSH terms	Age range	Positive	Negative	Positive	Negative	Total
Adolescent	13-18	6	2	3	5	8
Young Adult	19-24	11	12	11	12	23
Adult	19-44	74	50	67	57	124
Middle Aged	45-64	20	22	15	27	42
Aged	65-79	3	6	4	5	9
Total		103	80	89	94	183

The patients visiting the lab for COVID test were predominantly of age between 26-35. Out of 183 patients 37% of them were between 26-35 years of age. And the COVID positive rate was also highest among patients of this age group, 24 % of the patients in this age group were tested positive in NPS while 23% positive rate was observed from saliva assay. On the contrast, the positivity rate of patients between 16-25 and 36-45 was comparatively low. The low number of samples in age group below 15 years and above 65 years of age has limited the data interpretation in respective age bracket.

Considering the effect of age in different sampling method, it can be observed that the specificity of the saliva stayed consistently below that of NPS sampling in lower age groups but it gets better as the age of patients increases.

4.3. Clinical manifestation

The patients visiting the lab were mixture of symptomatic and asymptomatic condition. The major symptoms seen in the patients, as described by them were; fever, cough, sore throat etc. (Table 6)

Table 6: Symptoms and positive rate in patients

	NPS result		Saliva result		
Symptoms	Positive	Negative	Positive	Negative	Total (%)
Fever	58	9	45	22	67(36.61)
Cough	49	9	39	19	58(31.69)
Sore throat	18	7	17	8	25(13.66)
Muscle pain	35	4	28	11	39(21.31)
Asymptomatic	7	51	8	50	58(31.69)

The major symptoms included: fever (36.61%), cough (31.69%), sore throat (13.66%) and Muscle pain (21.31%), while 31.69% patients reported no symptoms. The onset of symptoms was in general 2-3 days prior to the COVID test.

The positivity rate of symptomatic patients was found to be very high in NPS method. However, the saliva assay displayed higher positive results in asymptomatic patients despite consistently displaying lower positivity rate in symptomatic cases.

4.4. Comparative diagnostic Tests on vaccinated population

The effectiveness of different vaccines in preventing COVID infection was studied by studying vaccines applied by patients before the CoV test (Table 7). Verocell vaccine was taken by the highest number of patients. 62% (107/173) of the vaccinated patients had taken Verocell, among whom 67 were tested positive in NPS assay and 57 were positive in saliva assay. Covishield vaccine displayed better protection against the COVID in comparison to Verocell but still 58% of the individuals with Covishield vaccine contracted the virus. By far the Pfizer vaccine seems the most effective vaccine in preventing infection. Only 2 out of 10 the patients who had previously taken Pfizer vaccine contracted the CoV infection.

Table 7: Comparison of effectiveness of vaccines

	NPS result		Saliva result		
Symptoms	Positive	Negative	Positive	Negative	total
Verocell	67	40	57	50	107
Covishield	19	14	16	17	33
Johnson	4	10	5	9	14
Pfizer	2	8	1	9	10
AstraZeneca	3	5	3	5	8
Moderna	1	2	0	3	3
Unvaccinated	6	4	6	4	10

4.5. Comparison of NPS and saliva results

Out of the 183 total samples, 60% (110/183) samples were tested positive for SARS-CoV-2 in either one of the tests (saliva or NPS) or in both tests. The sensitivity (80%) and specificity (91%) of the results obtained were significant with accuracy of 85% ($p<0.01$). Of the 110 positive samples, 82 (45%) samples tested positive in both saliva and NPS, while 21(11%) samples tested positive in NPS but negative in saliva and 7(4%) samples tested positive in saliva only (**Table 8**).

Table 8: Number of positive samples in distinct sampling methods

NPS sample	**Saliva sample**	**Positive patients (% of total positive samples)**
Total SARS-CoV-2 positive samples (Samples positive for at least one of sampling methods)		110 (100)
Positive	Positive	82 (45)
Positive	Negative	21 (11)
Negative	Positive	7 (4)
Negative	Negative	73

4.6. Comparison of cycle threshold (Ct) values

Here, the mean cycle threshold values of the different genes were measured to compare the performance of different sample types. In the study, we discovered that the Ct value from saliva samples is consistently greater than that of NPS samples (Table 9).

Table 9: Comparison of RT-PCR Ct values between sampling methods

Sampling method	**E gene**		**N gene**		**RdRp**	
	Ct mean [95%CI]	SEM	Ct mean [95%CI]	SEM	Ct mean [95%CI]	SEM
NPS	26.93 [25.73-28.13]	0.61	21.52 [20.1-22.94]	0.71	24.47 [23.32-25.62]	0.578
Saliva	30.39 [29.24-31.54]	0.579	28.57 [27.41-29.73]	0.582	29.66 [29.06-30.26]	0.30

4.7. Correlation between Ct values of different genes

After the general study of mean Ct values, all the individual data points were plotted and corresponding gene data was studied to obtain better understanding of the data. Since 2 different master mixes were used during PCR analysis for saliva and NPS samples, the genes amplified were correlated according to the reporter dye assigned to them i.e. VIC was used to report N gene in NPS sample and N2 gene in saliva samples. Hence N gene from NPS and N2 from saliva were studied for correlation with each other. Similarly, E gene from NPS and N1 gene from saliva were compared. Internal control genes, namely, ORF lab gene for NPS and Rp for saliva were studied.

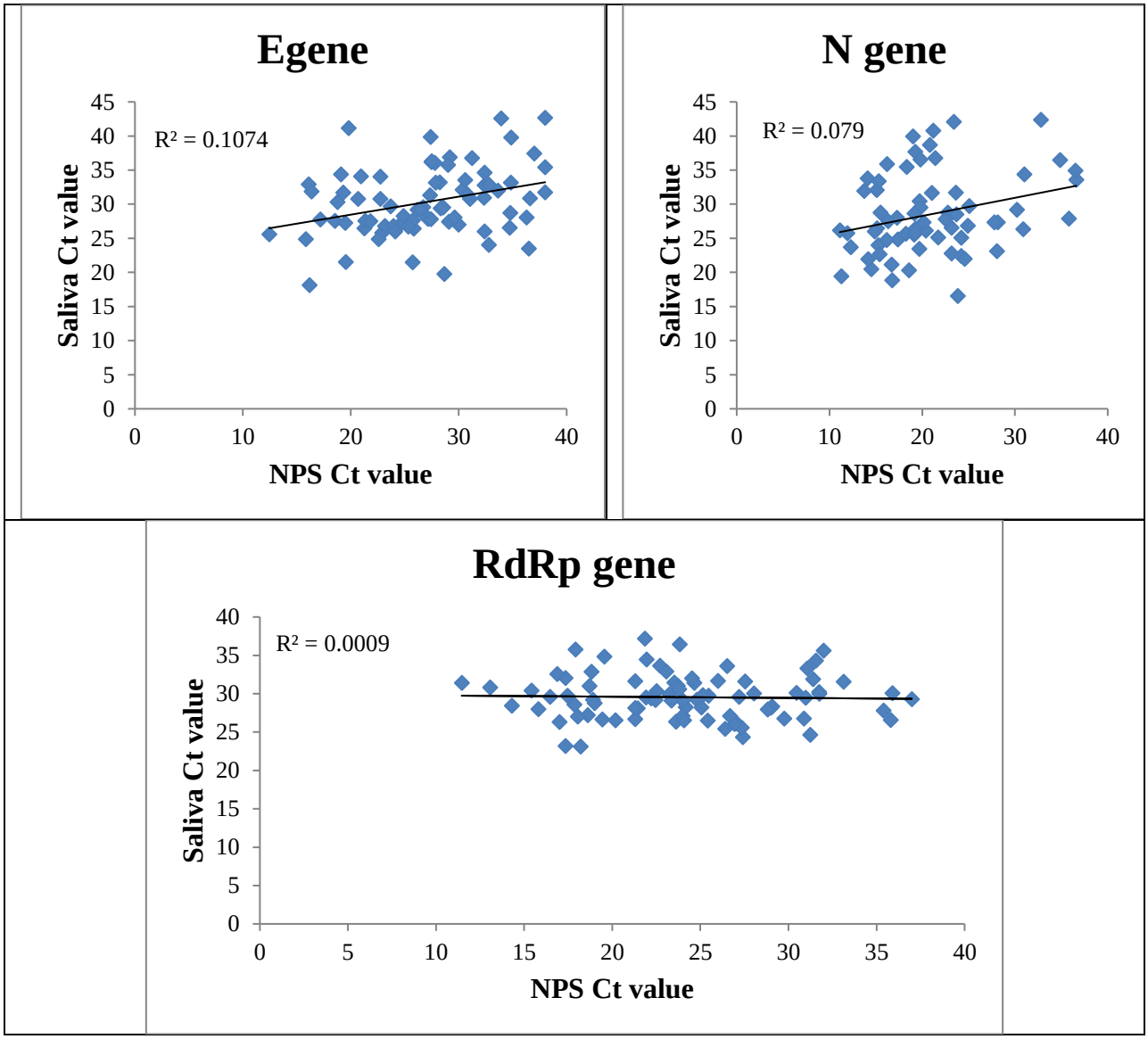


Figure 11: Graph plot of Ct values from NPS against saliva sample

The graph plot of the corresponding Ct values is shown in (Figure 11). Positive correlation was observed for all three amplified-target, Egene-N1 gene ($r= 0.33$, $p<0.01$), N gene-N2 gene ($r= 0.28$, $p<0.01$) and ORF lab- Rp ($r= 0.12$, $p<0.01$).

Chapter 5

5. DISCUSSION

Scientists have been looking into the saliva samples as diagnostic tool for very long now, given their noninvasive and easy to procure characteristic. Especially in case of large scale and repeated testing in high risk population (Landry, M., 2020). A comparable result between saliva sample with current gold standard, NPS sampling has been shown in this study.

The result obtained from the comparative study of NPS sampling method and saliva assay can be interpreted from multiple angles. From the demographics perspective, in our study, the COVID detection rate in children under 15 seems to fall off in saliva samples when compared to NPS sample. It could be attributed to low number of samples, but other factors like low viral load in the saliva and low saliva generation itself could be the cause for it. This finding is in concordance with the study conducted by Ana Laura et al., 2021, where they test the sensitivity of saliva for COVID in children specifically. They found that COVID results in children showed lower positive cases than in adults. Citing the low sensitivity of the results they have concluded that the saliva test cannot, as of yet replace the NPS sampling method.

However, our study showed saliva sample is particularly good at detecting infection in asymptomatic patients. But NPS samples tested positive for symptomatic patients significantly higher than saliva samples. Such results have been reported by multiple independent studies as well. Positive test result from saliva despite negative NPS result could mean that mild or subclinical infections detection by saliva could be viable (Wyllie et al., 2020). In another study, the infection detection rate of the saliva in asymptomatic patients was significantly higher than in NPS (Teo et al., 2021). However other studies had completely opposite results. (Alkhateeb et al., 2021) found the sensitivity of saliva to be too low in saliva. Such early detection capability is especially important in cases of pandemics. Isolating infected individuals from population as soon as possible can significantly help in reducing secondary infections.

Kit used and the sample collection technique of saliva can also affect the detection rate of COVID. In our study IDT master mix was used to diagnose various gene amplification. In recent times, many different saliva assay master mixes are available in market. Since there are no standard guidelines to follow, different studies are using different master mixes to varying degree of success. Some of the studies which produced similar results to our study used different master mix for amplification. (Vogels et al., 2021) showed high positive agreement between saliva and NPS tests using SalivaDirect test kit. (Tapia et al., 2021) were successful in showing positive concordance results against NPS test using Allplex^{TM} 2019-nCoV Assay Kit in their study. Apart from the kit used, we have not addressed any of the other factors, like; early morning samples, saliva gargle methods, drinking water before sampling etc, while sampling in our study. The only restriction placed for sample collection was that, patients who had eaten anything within 30 min were not considered. As demonstrated by Goldfarb et al., 2021 in their study where self-collected gargle samples performed extremely well against NPS samples collected by health care workers.

Another aspect of the COVID infection we tried to look into in our study was the effectiveness of vaccines taken. For this we simply correlated the data of vaccine used by patients and the PCR test results. We observed that 63% (67/107) of the patients who had previously taken Verocell vaccine contracted COVID in NPS samples. While this number was much lower in saliva assay 53% (57/107), still shows the low effectivity of vaccine in preventing infection. According to WHO, the Verocell vaccine provides 79% efficacy against SARS-CoV-2 infection after 2 dosages(World Health Organization, 2022). Pfizer vaccine was the most successful vaccine in our study in preventing COVID infection with 25% (2/8) positive rate in NPS and 10% (1/10) positive rate in saliva assay. In a study where efficacy of different vaccines was compared, Pfizer vaccine showed 95% efficacy in preventing symptomatic COVID 19 (Fiolet et al, 2022). Although our study generated similar results to these studies, small number of patients with Pfizer vaccine limits our interpretation.

The results obtained in our study were very significant with accuracy of 85%. The sensitivity of the test was 80%, which is in concordance with various previous similar studies(Torres et al., 2021)(Uddin et al., 2021). While some independent studies showed concordance of 100% (Azzi et al., 2020), (Yoon et al., 2020), our results are still competitive and relevant. These results could still be improved upon. Since no standard protocol has been established

yet, many researchers are working on their own to better the efficiency and outcome of saliva assay. As shown by (Sahajpal et al., 2021) and (Wyllie et al., 2020), the sensitivity of the saliva samples are higher when the samples are obtained early in the morning. Others in the meanwhile have managed to improve the saliva assay by use of homogenizer beads (Sahajpal et al., 2021). These studies show that there is a possibility for saliva samples to be used as an alternative to the current NPS sampling method.

Based on the sensitivity of saliva assay we have tried to establish saliva as a valid alternative to NPS sampling method. The saliva assay technique can still be improved upon and a standard protocol needs to be established, so that researchers have a basic guideline to further their research. Because currently the interest in saliva has risen in scientific community, but lack of standard has resulted in dilution of research quality. The conflicting results seen now days against saliva can be attributed to lack of standard as well.

Chapter 6

CONCLUSION

This research aimed to validate the saliva biospecimen as an alternative to the nasopharyngeal sample swab for SARS-CoV-2 detection using PCR. On the basis of the comparative analysis of the data generated by NPS samples and saliva assay in this study, it can be concluded that saliva sample can indeed be applied for detection of SARS-CoV-2 in cost effective and efficient manner. The positive correlation of NPS and saliva sample data along with high sensitivity and specificity further validates this claim. The better result of saliva assay against NPS for asymptomatic patients could indicate its usefulness in detecting COVID before manifestation of symptoms in patients. This finding is especially relevant in current world situation, where we are in need of easy to use, cheap and reliable alternative diagnostic tool. By adopting the saliva assay as an alternative, poor nations like Nepal could significantly reduce our dependence on advanced nations for various materials, from VTMs to sterile swabs.

Limitation of study

- The optimal sampling time period (after showing of first symptoms or exposure) could vary for saliva and NPS samples. This factor was not considered in the experiment.
- Saliva obtained early in the morning have been shown to produce better results. The sampling time of the day was not considered in this experiment.
- Different master mix kits were used to process NPS and saliva samples. This could result in divergent results.

Future Recommendations

- Qualitative study of saliva against NPS could provide better understanding.
- Better optimized techniques could reduce the performance gap between saliva and NPS samples.
- Homogenization of saliva samples could provide better results.

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Appendix

- Consent form:

Detection of SARS-CoV-2 using qRT-PCR in saliva obtained from asymptomatic or mild COVID-19 patients

सम्बन्धि अध्ययनमा सहभागी मञ्जुरीनामा फाराम

विश्व महामारीको रुपमा फैलिरहेको COVID-19 ,कोरोना भाइरसको नयाँ प्रजाति SARS–COV–2 ले हुने भाईरल संक्रमण हो। यो श्वास प्रश्वास , खोक्दा, हाच्छ्युँ गर्दा निस्कने थुक, खकार तथा सिगाँनका कणहरुका माध्यमबाट सर्दछ। माथि उल्लेखित शिर्षक Detection of SARS-CoV-2 using qRT-PCR in saliva obtained from asymptomatic or mild COVID-19 patients
मा नेपालमा हाल कोरोना भाईरस संक्रमणको प्रभाव र प्रकृति बुझ्नका लागि गरिएको एउटा अनुसन्धान हो । तपाईंलाई यस अनुसन्धानमा सहभागी गराउनुको मुख्य उद्धेश्य तपाईमा देखिएको स्वास्थ्य समस्या कोरोना संक्रमणले गर्दा हो वा होईन, हो भने यसले तपाईलाई कस्तो प्रभाव पारिरहेको छ, तपाईंमा यसको संक्रमणकाल कति रहेको छ अध्ययन गर्नु हो ।

तपाई यस अनुसन्धानको स्वयंसेवकको रुपमा आफ्नो मुख बाट तरल पदार्थ(Saliva) दिनुपर्नेछ । तपाइबाट लिएको जैविकपदार्थलाई Kirtipur Municipality-TU Biotech Corona Laboratoryमा विभिन्न साधन प्रयोग गरि अध्ययन गरिन्छ र प्रश्नपत्र (Questionnaire) प्रयोग गरेर पनि तथ्याङ्क निकालिन्छ । तपाईबाट लिइएको जैविकपदार्थ र यसबाट आएको तथ्याङ्कप्रयोग गरि कुनै किसिमको व्यापारिकरण गरिने छैन ।

तपाईबाट लिइएको जैविकनमुना तथा तथ्याङ्क अनन्त समयसम्म भण्डार गरेर राखिनेछ र भविष्यमा चाहीएको खण्डमा प्रयोग गर्न सकिनेछ ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनमा हुने परिक्षणमा तपाइको शरिरमा नविन कोरोना भाइरस भए नभएको विषयमा RT-PCR प्रविधि प्रयोग गरि गरिन्छ र यसले रोगको पहिचान गरी संक्रमण कम गर्न मद्दत गर्न सक्छ । तपाईले Saliva दिदा केही जोखिम हुने छैन ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुने छैन ।

यो रोग नेपालमा मात्र नभएर संसारभर नै महामारीको रुपमा फैलिरहेको छ । यसले बेलाबेलामा आफ्नो स्वरुप फेरीरहने र नयां नयां भेरीयन्टको रुपमा फैलिने गरेको पनि पाइएको छ । त्यसैले तपाईको शरीरमा भईसको मात्रा कति छ र उक्त भाईरस निस्कृय हुन कति समय लाग्यो भनी अध्ययन गर्नाले नेपालमा यो रोगको अवस्था थाहा पाउन तपाइको सहभागिले ठूलो भूमिका खेल्ने छ ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैल आफ्नो इच्छाले बिना जरिवाना ,बिना डरत्रास, अनुसन्धानकर्ता समक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

सहभागीको हस्ताक्षर :

(अथवा सहभागीको हकमा अनुमती प्रदान गर्ने व्यक्तिको हस्ताक्षर)

सहभागिको नाम :

ठेगाना :

सम्पर्क नं :

अनुसन्धानकर्ताको नाम :

अनुसन्धानकर्ताको सम्पर्क नं :

अनुसन्धानकर्ताको हस्ताक्षर :

मिति :

- Institutional research approval letter

Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu

Tribhuvan University

Institute of Science and Technology

Kirtipur, Kathmandu, Nepal

Institutional Review Committee

IRC/IoST Chairperson

Assoc. Prof. Dr. Surendra Gautam
Asst. Dean-Academics, IoST

IRC/IoST Members

Prof. Dr Anjana Singh

Prof. Dr Krishna D Manandhar

Prof. Dr Sangeeta Rajbhandary

Prof. Dr Shankar P Khanal

Prof. Dr Kumar Sapkota

Prof .Dr Prakash Ghimire

Prof. Dr Chhatra M Sharma

Assoc. Prof. Dr Megha R Banjara

Member Secretary

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Head, Central Department of Microbiology

IRC/IOST Secretariat

Central Department of Microbiology

Phone: 4331869

Ref. No.: 971/078/078 Date: 12 April, 2022

PI: **Prof. Dr. Krishna Das Manandhar**
M.Sc student: **Salin Maharjan**
Central Department of Biotechnology,
Tribhuvan University (TU),
Kirtipur, Kathmandu

Ref.: IRC Ethical Approval of research proposal entitled **"Validation of saliva sampling as an alternative to nasopharyngeal swab for detection of SARS-CoV-2 using RT-PCR"**

Dear **Prof. Dr. Manandhar**,

It is our pleasure to inform you that the above mentioned proposal submitted on 15 March, 2022 (Regd. No **IRCIOST-22-0027**), following independent expert review and discussion in the IRC/IoST meeting held on **11 April, 2022** has been approved for implementation [start date 12 April, 2022 and end date 11 October, 2022], maintaining ethical principles, set by the Nepal Health Research Council.

The investigators have to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure including deviation of the protocol, data management and budget need to be submitted in detail with justification for seeking prior approval to implement the proposed change including extension of the date, in the protocol.

Further, the researchers are also directed to follow the national ethical guidelines published by Nepal Health Research Council during the implementation of research. You are required to submit the final report to the IRC within a month of completion of the research, as planned in the approved proposal.

If you have any questions, please contact the Institutional Review Committee of Institute of Science and Technology, Tribhuvan University.

Thanking you,

.....
Assoc. Prof. Dr. Komal Raj Rijal
Member Secretary
Institutional Review Committee
Institute of Science and Technology
Tribhuvan University

- Primers and probes used for Saliva assay;

Manufacturer: Integrated DNA technologies (IDT)

S.N	Primers/Probes	Catalogue no.	Lot no.
1.	nCOV_N1 Forward Primer	10006830	0000591228
2.	nCOV_N1 Reverse primer	10006831	0000604126
3.	nCOV_N1 probe	10006832	0000591233
4.	nCOV_N2 Forward Primer	10006833	0000586232
5.	nCOV_N2 Reverse Primer	10006834	0000586233
6.	nCOV_N2 Probe	10007049	0000604708
7.	RNase P Forward Primer	10006836	0000583665
8.	RNase P Reverse Primer	10006837	0000583167
9.	RNase P Probe	10007062	0000593832
10.	nCOV_Positive Control	10006621	0000568956