VALIDATION OF RAPID ANTIGEN TEST AS DIAGNOSTIC TOOL FOR DETECTION OF SARS-CoV-2 AS COMPARED WITH REAL TIME RT-PCR

M.Sc. Thesis

2022

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For the Partial Fulfillment of the Requirement of
M.Sc. Degree in Biotechnology

Suman Gautam

Exam Roll No.: BT-521/074

TU Registration No. : 4-2-103-94-2012

VALIDATION OF RAPID ANTIGEN TEST AS DIAGNOSTIC TOOL FOR DETECTION OF SARS-CoV-2 AS COMPARED WITH REAL-TIME RT-PCR

M.Sc. Thesis

2022

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by:

Suman Gautam

Exam Roll No.: BT-521/074

TU Registration No.: 4-2-103-94-2012

Supervisor:

Prof. Dr. Krishna Das Manandhar

Central Department of Biotechnology

Tribhuvan University

ACKNOWLEDGEMENT

A journey of the dissertation that started before COVID-19 seemed never ending has finally come to an end after 2.5 years. This pandemic was frustrating as well as great opportunity for me. Writing this thesis has been fascinating and extremely rewarding. On this overwhelming moment I would like to express my deepest gratitude to all the people who made this actually possible and contributed to the final result in many different ways.

To commence with, I express my sincere and deepest gratitude to my supervisor **Prof. Dr. Krishna Das Manandhar**, Head of Department for the continuous support through my dissertation, for his patience, motivation and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor and mentor for my thesis.

I would like to express my sincere gratitude to Dr. Dipendra Pandey, Medical Superintendent, Trishuli Hospital Nuwakot and Sagar Ghimire, Senior public health officer, Head of DHO Nuwakot. It was his support and permission that made my work in Trishuli Hospital Covid-19 Nuwakot Laboratory possible without shortage of Chemicals and Kits even in the pandemic.

My in-depth sincere thanks goes to **Prasamsha Nepal** and **Samarpan Shrestha Rajbhandari,** and entire team of Trishuli Hospital Nuwakot without them I would not have been able to collect and process the samples.

I gratefully acknowledge **DHO Nuwakot, CDO Nuwakot, Ministry of Industry, Commerce and Supplies, National public health laboratory, Province public health laboratory Bagmati, Bagmati Province Government Nepal** for supply of Chemicals and Kits for the diagnosis of SARS-CoV-2 in the patients of Nuwakot, Rasuwa and Dhadhing District.

My deepest gratitude to Central Department of Biotechnology, Tribhuvan University for providing me the platform for this research and all respected faculty members of Central Department of Biotechnology **Dr. Ganga Kharel, Dr. Rajani Malla, Dr. Gauri Shankar Manandhar, Dr. Pramod Aryal, Dr. Suresh Subedi, Dr. Jarina Joshi, Dr. Smita Shrestha, Mrs. Alina Shree Sapkota, Mrs. Preeti Regmi, Ms. Pragati Pradhan,** and all the staffs of this department who have helped me to finish my research.

I am obliged to my lab partners **Gyaneshwor Tharu**, **Binita Rijal, Rajani kumara Gurung, Shyam sundar Ojha, Laxmi Bhatta, Prasamsha Nepal, Samarpan Shrestha Rajbhandari** for extending their unstinted support, timely motivation, sympathetic attitude and unfailing help during the course of entire study. I sincerely admire the contribution of **Roji Raut** along with **Rashmi Thapa** and everyone who has been with me throughout this research. I sincerely express my gratitude from the core of my heart to all the patients who gave their samples and consented to participate in this research.

Finally, and most importantly, none of this could have been possible without the love and patience of my family. I would like to dedicate this work and express my heartfelt gratitude to my family: my mother, my father and my brother for their constant encouragement, prayers and unequivocal support.

Suman Gautam

GLOSSARYACRONYMS

AEC2:	Angiotensin Converting Enzyme 2
Ag:	Antigen
ARDS:	Acute respiratory Distress Syndrome
CDC:	Center for Disease Control
CFR:	Crude Fatality Rate
COVID-19:	Corona virus Disease-2019
CT:	Cycle Threshold
CTSB:	Cathepsin B
CT-scan:	Computed Tomography Scan
CTSL:	Catheosin L
E protein:	Envelope protein
ELISA:	Enzyme-linked immunosorbent Assay
FiO2:	Fraction of Inspired Oxygen
hAEC2:	Human Angiotensin Converting Enzyme 2
Kb:	Kilobases
LAMP:	Loop Mediated Isothermal Amplification
M protein:	Membrane protein
MERS:	Middle East respiratory Syndrome
MHC I:	Major Histocompatibility Complex I
MHC II:	Major Histocompatibility Complex II
Mm of Hg:	millimeter of Mercury
MOHP:	Ministry of Health and Population Nepal
N protein:	Nucleocapsidprotein
NAAT:	Nucleic Acid Amplification Test

nCoV-19:	Novel corona virus-2019
NHPL:	National Public Health Laboratory
NPS:	Nasopharyngeal Swab
NSPS:	Non-structural proteins
ORFs:	Open Reading Frames
PaO2:	Partial pressure of Oxygen
POCT:	Point of care testing
PPE:	Personal protective Equipment
qRT-PCR:	Quantitative Reverse Transcription Polymerase chain reaction
RAT:	Rapid Antigen test
RBD:	Receptor Binding Domain
RNA:	Ribonucleic Acid
rtRT-PCR:	Real Time Reverse Transcription Polymerase Chain Reaction
S protein:	Spike protein
SARS-CoV-2:	Severe Acute Respiratory Syndrome Coronavirus 2
SpO2:	Oxygen saturation
TMPRSS2:	Transmembrane serine protease 2
UTRs:	Untranslated Regions
VTM:	Virus Transport Medium
WHO:	World Health Organization

Table of Contents

ACKNOWLEDGEMENT i
GLOSSARYACRONYMS ii
List of Tables vi
List of Figures vii
ABSTRACT 1
Chapter I 2
1.0 INTRODUCTION 2
1.1 Background of COVID-19 infection 2
1.2 Global burden of COVID-19 3
1.3 History of COVID-19 in Nepal 3
1.4 Taxonomy of COVID-19 4
1.4.1 Virion morphology 5
1.4.2 Genomic organization 5
1.5 Detection Protocol 6
1.6 Rationale of the Study 8
1.7 Research Hypothesis 8
1.8 Objective 9
CHAPTER-II 10
2.0 LITERATURE REVIEW 10
2.1 Review of literature related to corona viruses 10
2.1.1 History of Corona viruses 10
2.1.2 Evolution of SARS-CoV-2 10
2.1.3 Corona viruses: Structure, genome, and classification in general 11
2.2 Review of literature related to SARS-CoV 2 11
2.2.1 Origin and Spread of SARS-CoV 2 11
2.2.1.2 SARS-CoV-2: Origin, spread and current statistics in Nepal 12
2.3 Proteins of SARS-CoV-2 14
2.3.1 Structural Proteins of SARS-CoV-2 14
2.4 Pathogenicity 15
2.5 Transmissibility 16
2.5 Clinical features 17
2.5.1 Clinical manifestations of SARS-CoV 2 17
2.6 Laboratory Detection 22

2.6.1 Real time PCR 23
Chapter III 25
3.0 Methods and Methodology 25
3.1 Study Design 25
3.2 Study Site 25
3.3 Study Population and sample size 25
3.4 Duration of the Study 26
3.5 Inclusion and Exclusion Criteria 26
3.6 Ethical Consideration 27
3.7 Sample collection, transport and storage 27
3.8 Rapid SARS-CoV-2 antigen detection assay 27
3.9 Reverse transcription Real time PCR 28
3.10 Sequencing and Sequence analysis 30
3.11 Analysis of Data 30
CHAPTER-IV 33
4.0 RESULTS 33
4.1 Incidence 33
4.2 Real time RT-PCR and SARS-CoV-2 antigen assay 33
4.3 Diagnostic Sensitivity and Specificity of RAT compare to rtRT-PCR 35
4.4 CT value distribution of rtRT-PCR target genes 38
4.5 CT value distribution of rtRT-PCR target genes in comparison to RAT positive and negative result 39
4.6 Sequence analysis and Phylogenetic Analysis 40
Chapter V 45
Discussion 45
Limitation of Study 49
CHAPTER-VI 50
Conclusion 50
Recommendation 50
Chapter VII 51
Reference 51
APPENDIX 57

List of Tables

Table 4.1 Age and Gender wise distribution of Antigen rapid test 34

Table 4.2: Gender wise distribution of cases.....34

Table 4.3: Age and Gender wise distribution of rtRT-PCR test.....35

Table 4.4: contingency table showing rtRT-PCR vs RAT result. 36

Table 4.5: Diagnostic performance of SD Biosensor RAT 37

Table 4.6: Diagnosis performance of Panbio RAT.....37

Table 4.7: overall diagnosis performance of RAT 38

List of Figures

Figure 1.1: Classification of RNA group virus and origin of SARS-CoV-2 4

Figure 1.2: Representation of SARS-CoV-2 5

Figure 1.3: SARS-CoV-2 structure with genome 6

Figure 4.1: CT value distribution of RT-PCR in all the positive cases..... 38

Figure 4.2: CT value distribution of RtRT-PCR target genes with comparison to RAT positive and negative. 39

Figure 4.3: Genetic distance determination of S-53..... 41

Figure 4.4: Genetic distance determination of S-54..... 42

Figure 4.5: Genetic distance determination of S-55..... 43

Figure 4.6: Genetic distance determination of S-77..... 44

ABSTRACT

Introduction: Testing of samples from suspected SARS-COV-2 individuals with rtRT-PCR result leads to delayed in detection of infection. Early recognition of SARS-CoV-2 infection is crucial for both the improvement of turnaround time and limiting the spread of the virus in the Community. Huge number of people affected in pandemic, it is important to use a test that give faster results and can be used on large number of sample. Rapid Antigen Test fulfills both the criteria. **AIM:** The aim of our study was to evaluate the diagnostic performance of two antigen rapid diagnostic kits (RAT) to diagnose SARS-CoV-2 infection in comparison to rtRT-PCR. So, we evaluated two commercially available antigen kit. **Methods:** A laboratory based descriptive Cross-sectional study was performed in Trishuli Hospital Nuwakot from January 2021 to December 2021. **Result:** Out of 1295, 472 tested positive in RAT test while 715 tested positive for SARS-CoV-2 RNA genome via rtRT-PCR. Specificity was 98.2% for Panbio and 96.9% for SD Biosensor. Sensitivity for sample with CT ≤ 20 was 93.5% and 94.2% and for sample with CT ≤ 25 was 93.7% and 93.5% and for CT ≤ 30 was 82.9% and 81.4% with $p < 0.001$ in case of Panbio and SD Biosensor respectively. The overall diagnostic performance of RAT showed 64.2% sensitivity and 97.8% specificity. Agreement with PCR was excellent for high viral load sample CT<20: Panbio, kappa=0.921; SD Biosensor, Kappa= 0.912 and CT value <25: Panbio, Kappa=0.926; SD Biosensor, Kappa=0.906. Significant difference (p value <0.0001) was observed between RAT+ and RAT- results when compared to CT value obtained from the rtRT-PCR. The diagnostic accuracy for RAT was 79.2% with kappa=0.59 showing moderate agreement and empirical benchmark was set with Youden's Index 0.597 to be administered as diagnostic purposed. **Conclusion:** The RAT performed well as a POCT test for early diagnosis of COVID-19 in primary healthcare centers. More crucially finding from our study is that RT-PCR proven SARS-CoV-2 infection and negative by RAT are not likely to be infectious.

Keywords: SARS-CoV-2, rtRT-PCR, RAT, Sensitivity, Specificity

Chapter I

1.0 INTRODUCTION

1.1 Background of COVID-19 infection

Coronavirus disease 2019 (COVID-19) is defined as the Contagious infection or disease condition caused by a novel corona virus (nCoV-19) recently known as Severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) which has no previously known history of infection to the mankind. The first official record of a case of COVID-19 was from Wuhan, China by the WHO in 31st December 2019 however, according to professionals the cases were detected as early as 17th November 2019. Afterwards, it has spreads all over the world rapidly and subsequently leading to pandemic after affecting 7 continents, which was declared as outbreak of Public Health Emergency of International Concern by WHO on 30th January, 2020 and global pandemic in 11th March, 2020 (Adil et. al. 2021). Having wide range of clinical manifestation from asymptomatic/mild symptoms to life threatening condition of hospitalization in incentive care unit with involvement of multiple system i.e. respiratory tract, gastrointestinal, hepatic along with neurological leading to death. The first case of infection was probably from the Zoonotic agent (from animal to human) has emerged from the Huanan seafood market, in Wuhan state of Hubei province in china where livestock animals are also traded (Sahin et. al. 2020). The principal mode by which human to human transmission takes place is through contact within 6 feet by respiratory droplets carrying SARS-CoV-2 whereas secondary mode of transmission includes close contact (e.g. Shaking hands, touching contaminated surface), airborne transmission and virus release during coughing, sneezing, talking, spitting (Cennimo DJ, 2022). Onset of symptoms may develop from 2 days to 14 days from exposure to the virus. Infection may be asymptomatic i.e. no symptoms, mild to moderate i.e. symptoms such as fever, cough, aches, lethargy but no difficulty in breathing at rest; severe i.e. symptoms with shortness of breath or difficulty in breath and raised in respiratory rate suggestive of pneumonia; or critical i.e. need of external respiratory support due to severe acute respiratory syndrome or acute respiratory distress syndrome (ARDS) (Dinnes et. al. 2020).

1.2 Global burden of COVID-19

The unanticipated outbreak of COVID-19 infection by coronavirus was first record reported from Wuhan, China in December 2019. WHO declared COVID-19 as Public Health Emergency of International Concern on 30 Jan 2020 and a global pandemic on 11th March 2020 (Zheng, 2020). In response, various mitigate measures (Islam,2020 & Gupta et. al. 2020) and eradication strategies (Lu et. al. 2021) were adopted worldwide aiming to reduce huge damage and reach nil cases. However, due to lack of preparedness and implementation of response plans as effectively hence resulted in the rapid spread of SARS-CoV-2 globally within the one month of first case conformation by WHO (The Lancet, 2020 & Basnet et. al. 2021). As of 5thJune 2022, 535171130 COVID-19 positive cases were reported with 506011648 recovered, 22839537 active cases and 6319945 death cases. This data showed 1.18 % death rate and 98.82% recovery rate in the World (Worldometer, 2022).

1.3 History of COVID-19 in Nepal

The Ministry of Health and Population of Nepal confirmed the first case of COVID-19 in 23rd January 2020 and second case after around 2 months in March. As of one year later from the reporting of first case 22nd January 2021, 268,948 COVID-19 positive cases were reported, with 263,546 recovered and 1,986 death cases (MOHP Nepal 2021). This data showed nearly 0.74% death and about 98% recovery rate in Nepal. The case fatality rate (CFR) was 0.5% up to 30th March in Nepal (Panthee et. al. 2020). Till now 5th June 2022, 979199 COVID-19 positive cases, 967137 recovered and 11952 death cases are reported, with 1.22% death and 98.77% recovery rate. The CFR was increased gradually to 1.2% by 5th June 2022(MOHP Nepal, 2022). In total, 5715414 qRT-PCR tests were performed in Nepal, indicating about 17.13% current prevalence of COVID-19 among the qRT-PCR tested population as compared with 2.5% as of March 31st, 2020 (Worldometer Nepal Population 2021). As of reviewing, the prevalence of COVID-19 among the qRT-PCR tested population is higher than the neighboring countries, China (0.14%) and India (5.06%) (Worldometer, 2022).

1.4 Taxonomy of COVID-19

Severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) causative agent of coronavirus disease 2019 (COVID-19) belongs to Coronaviridae family, Betacoronavirus genera. They are categorized into four important genera viz Alpha, Beta, Gamma and Delta coronavirus. The classification along with origin of SARS-CoV-2 is portrayed in figure 1.

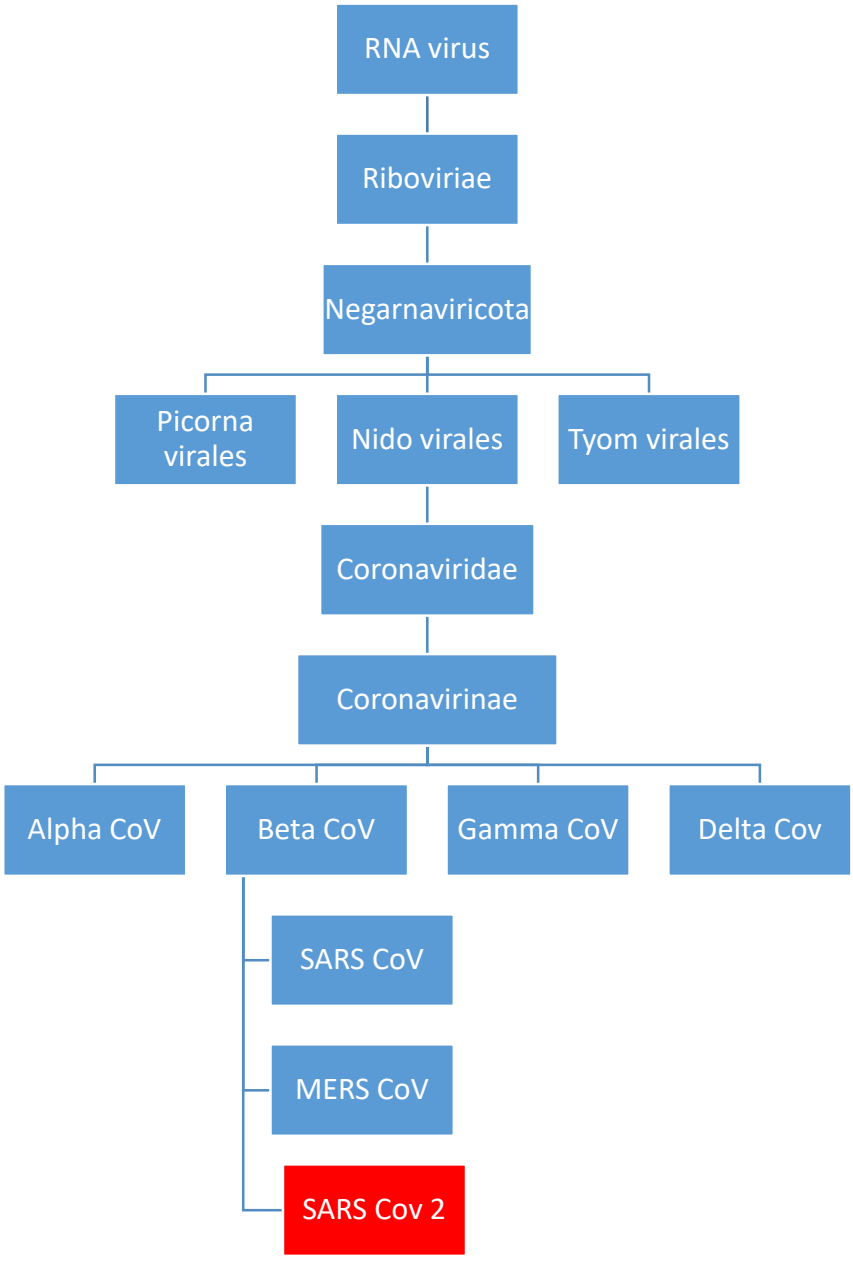


Figure 1.1: Classification of RNA group virus and origin of SARS-CoV-2 (Pal et. al. 2020). CoV: coronavirus; SARS CoV 2: severe acute respiratory syndrome coronavirus 2; MERS CoV, Middle East respiratory syndrome coronavirus; RNA, ribonucleic acid

1.4.1 Virion morphology

The Betacoronavirus SARS-CoV-2, an enveloped virus having a large non–segmented positive sense single stranded RNA packaged within a helical caspid (encapsidated) by Nucleoprotein(N). The viral lipid enveloped incorporate three structural transmembrane proteins: Spike (S) protein, Envelope (E) protein and Membrane (M) protein, which are involved on binding with host receptor (through spike glycoprotein), mediate membrane fusion during virus entry in host cell and budding process respectively. SARS-CoV-2 virions, roughly spherical/ ellipsoidal shape with variable diameter of about 100 nm with prominent S trimers protrude from lipid bilayer.

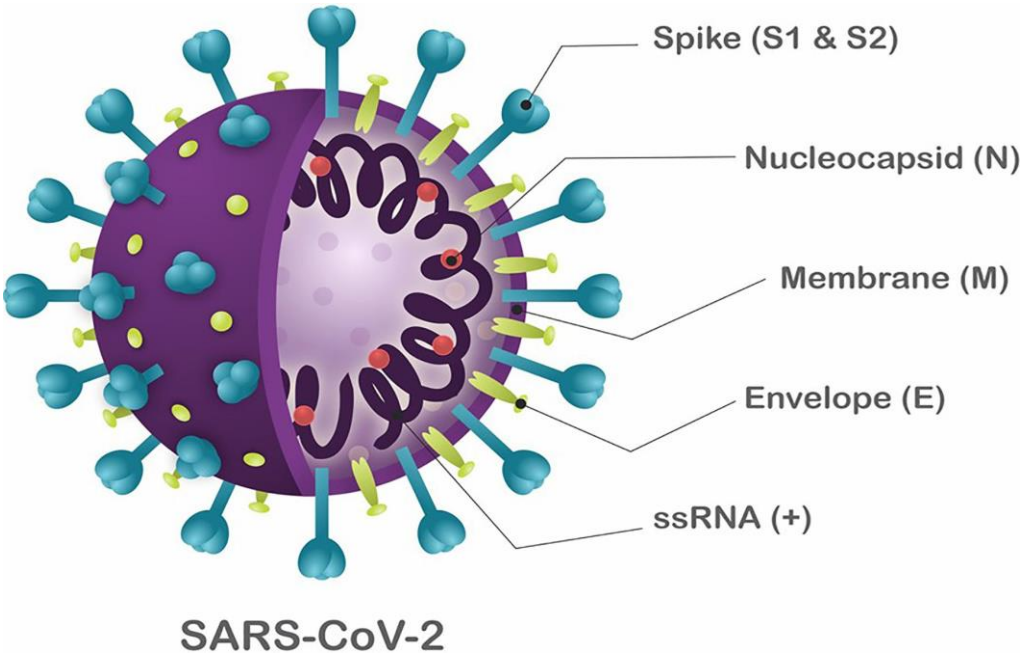


Figure 1.2: Representation of SARS-CoV-2 (Nuthalapti, 2021)

1.4.2 Genomic organization

The SARS-CoV-2 genome consists of a single stranded positive sense RNA molecule in range of 26 to 32 kb, which comprise 6-11 open reading frame (ORFs) encoding 9680amino acid polyprotein. The first ORF (ORF 1a/b) comprises two-third (67%) of the viral genome translate two poly-protein, pp1a and pp1ab, and encodes for 16 non-structural protein (nsp) while the remaining ORFs encodes for accessory and Structural proteins. The remaining one-third part of

viral genome encodes 4 essential structural proteins: Spike(S) surface glycoprotein, Envelope (E) protein, Membrane (M) protein and Nucleo-capsid (N) protein and also several accessory proteins that interfere in host defense mechanism (Guo et. al., 2020) The SARS-CoV-2 ORF is flanked at its 5' terminus by an Untranslated regions(UTR) of about 265 nucleotides and a longer UTR of 358 nucleotides at its 3' terminus.

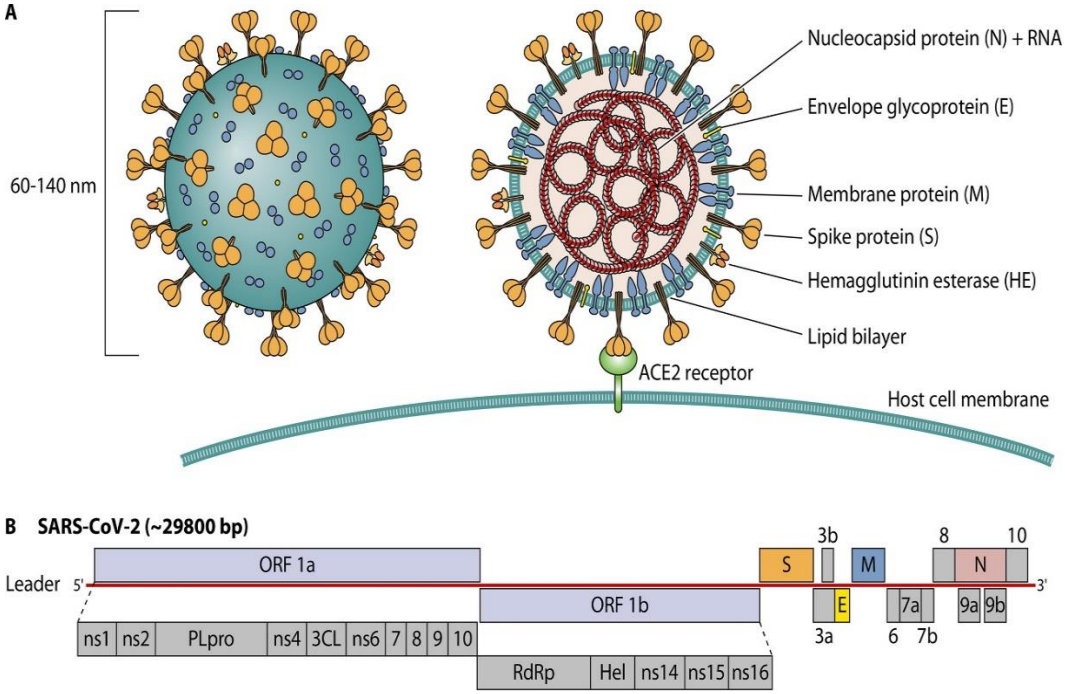


Figure 1.3: SARS-CoV-2 structure with genome (Sefaibadi et. al. 2021)

1.5 Detection Protocol

The SARS-CoV-2 genome publication in January 2020 (Wu et.al. 2020) led to the rapid development of real-time reverse transcription polymerase chain reaction (rtRT-PCR) tests for the diagnosis of COVID-19 while avoiding cross-reactions to other known coronaviruses. Among early versions, one was developed in China that targeted the ORF1ab and N genes of viral RNA (Chinese national institute,2020) while another version was developed in Germany that targeted the RdRp, E, and N genes (Corman et. al. 2020). The ORF1ab/RdRp, E, N, and S genes are the targets most frequently used for SARS-CoV-2 detection by RT-PCR. Real-time RT-PCR tests were developed and implemented thereafter by many laboratories around the world (Chan et.al. 2020, Pang et.al. 2020, Reusken et. al.

2020) even as COVID-19 became a global pandemic that continued to spread rapidly. A listing of tests and protocols is maintained online by the World Health Organization (WHO). The rapid development and deployment of RT-PCR tests has been essential for the ability to measure and control the spread of SARS-CoV-2. However, the urgency of the pandemic has meant that the diagnostic tests were deployed without first being supported by clinical studies to measure the diagnostic error rates (Pfefferle et. al. 2020).

Early and rapid confirmation during the acute phase of illness is important as well as crucial step for patient management, epidemiological monitoring and lowering fatality rates. For laboratorial diagnosis of SARS-CoV-2, several techniques have been developed; these include virus isolation, Nucleic acid amplification tests (Real time reverse transcription PCR, LAMP, Gene-Xpert), immunoassays (ELISA, RDTs), and biochemical tests using nucleotide probes or biosensor. Virus isolation is expensive, requires experienced staff, sophisticated equipment's and time-consuming needs more than 7 days to complete the assay. Gene-Xpert also required sophisticated equipment and trained personal. Most widespread method to detect SARS-CoV-2 is Molecular amplification techniques to detect RNA (RT-PCR, qRT-PCR), are gradually being accepted as a gold standard, have a high specificity and sensitivity. However, these techniques require expensive and sophisticated equipment with experienced staff, high turnaround time making them unpractical for laboratories with limited resources and in case of emergency.

In an outbreak, community screening of suspected cases needs to be done to establish the presence of COVID-19 transmission in the community. Therefore, rapid accurate and confirmatory detection of SARS-CoV-2 using simple method is an urgent need for effective clinical management and control SARS-CoV-2 pandemic. To eliminate the complex and time consuming protocol along with the sophisticated equipment and experienced staff the use of Rapid Antigen Test has emerged as a promising diagnostic tool for larger number of suspected cases, tracing contacts and preventing widespread community transmission of the virus.

1.6 Rationale of the Study

The pandemic due to SARS-CoV-2 has become one of the major public health issue affecting the global economy and life of the people. Due to the lack of early diagnosis, isolation of infected patients, effective contacts tracing the virus is spreading rapidly. Nowadays, reverse transcriptase real time polymerase chain reaction (rtRT-PCR) is consider as the reference method for the diagnosis of COVID-19. However, rtRT-PCR required expensive equipment, expertise to perform test, take long turnaround time and required sophisticated specialized laboratory to perform the test. Therefore, there is a need of rapid, inexpensive and simple test procedure as an alternative of rtRT-PCR for early diagnosis and isolation of the infected patients. Although being different alternative, Rapid antigen test can be the potential alternative for rtRT-PCR in case of rapid diagnosis, inexpensive and simple procedure.

This is especially important because early recognition of suspected patients allows timely initiation of appropriate Infection Prevention and Control measures. The use of validated Rapid antigen kit also helps in decreasing the burden on laboratories, in addition to early detection, case isolation and prompt management and treatment strategy.

So to validate the diagnostics performance of Rapid antigen test (RAT) in comparison to rtRT-PCR as a potential alternative the study is carried out.

1.7 Research Hypothesis

Null Hypothesis(Ho):

Ho = The Rapid Antigen Test can't be used as diagnostic tool for the early detection of SARS-CoV-2 in field in place of rtRT-PCR

Alternative Hypothesis (H1):

H1 = The Rapid Antigen Test can be used as diagnostic tool for the early detection of SARS-CoV-2 in field in place of rtRT-PCR

1.8 Objective

1.8.1General Objectives

- To check the performance of SARS-CoV-2 Rapid antigen test as a diagnostic tool for early diagnosis of COVID-19 in comparison to real time reverse transcription PCR.
- Molecular characterization of SARS-CoV-2 virus isolated in clinical sample.

1.8.2Specific Objectives

- Detection of SARS-CoV-2 virus by one step Real time reverse-transcription PCR in all suspected cases.
- Detection of SARS-COV-2 virus by using SARS-CoV-2 Rapid antigen test in all suspected cases.
- Comparison between rtRT-PCR test and RAT result in all suspected cases.
- Determination of Sensitivity, Specificity, Positive Predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio, accuracy of RAT compared to rtRT-PCR.
- Molecular characterization and phylogenetic analysis of SARS-CoV-2 virus isolated from clinical sample.

CHAPTER-II

2.0 LITERATURE REVIEW

Literature review is an important part in research process which helps in acquaintance with relevant research literature and state current knowledge for research. The main purpose of this chapter is to review the available literature from past publications, journals, survey reports, documents, published books and previous research studies. Literature was reviewed throughout the program during problem selection, proposal writing, data collection and interpretation and report writing.

2.1 Review of literature related to corona viruses

2.1.1 History of Corona viruses

In 1965, Tyrell and Bynoe isolated a human coronavirus was for the first time from the nasal secretions of a male child with a common cold (Singhal, 2020). The morphological similarity of the isolated virus with solar corona (crown like) as observed by the electron microscope gave those viruses the name 'corona virus'. Such appearance is mainly due to the presence of spike- glycoprotein which radiates from the viral surface (Esakandari et. al., 2020).

2.1.2 Evolution of SARS-CoV-2

The identification of the origin, native host(s) and evolution pathway of the virus that causes an outbreak of a pandemic is very critical. The molecular mechanism of its cross-species spread can be understood and proper control measures to prevent it from further spreading can be implemented by having a good knowledge of the evolution pathways of that pandemic causing virus (Xu et. al., 2020). The seven conserved replicas domains in ORF1ab has 94.6% sequence identity in amino acids between SARS-CoV-2 and SARS-CoV reflects the close similarity between these two viruses despite the fact that some of the six major ORFs of SARS-CoV-2 genes share less than 80% identity in nucleotide acids to SARS-CoV (Zhou et. al., 2020).

2.1.3 Corona viruses: Structure, genome, and classification in general

Corona viruses are large roughly spherical with unique surface projections (Goldsmith et. al., 2004) with variable sizes, generally averaging a diameter of 120 nm, with extreme sizes known from 50 nm to 200 nm and an average total molecular weight of 40,000 kilo Daltons (Masters, 2006). The corona viruses are protected by lipid bilayer envelop, membrane proteins and nucleocapsid when outside the host cell (Neuman et. al., 2011).

The membrane (M), envelope (E) and spike (S) structural proteins are anchored in the lipid bilayer which comprises the viral envelop (Lai and Cavannagh, 1997). The E and M protein are the structural proteins that combined with the lipid bilayer shape the viral envelope and maintain its size whereas S proteins are needed for interaction with the host cells (Fehr and Pearlman, 2015).

Corona viruses are enveloped viruses with a positive sense SS-RNA genome and a nucleocapsid of helical symmetry with genome sizes ranging from approximately 26 to 32kilobases and are one of the largest among RNA viruses (Woo et. al., 2010). The genome organization for a coronavirus is 5'-leader-UTR-replicase (ORF1ab)-spike (S)-envelope (E)-membrane (M)-nucleocapsid (N)-3'UTR-poly (A) tail. The open reading frames 1a and 1b, which occupy the first two-thirds of the genome, encode the replicase polyprotein (pp1ab) which self cleaves to form 16 non-structural proteins (nsp1–nsp16) (Fehr and Pearlman, 2015). The latter reading frames encode for four major structural proteins namely spike, envelope, membrane and nucleocapsid. In between these reading frames, the reading frames coding for the accessory proteins are dispersed and the role and number of these coded accessory proteins varies accordingly with the specific corona virus (Snijder et. al., 2003).

2.2 Review of literature related to SARS-CoV 2

2.2.1 Origin and Spread of SARS-CoV 2

2.2.1.1 A global scenario

Wuhan, capital city of Hubei province and a major transportation hub of China started presenting to local hospitals with severe pneumonia among adults of

unknown cause in December 2019 (Singhal et al, 2020). Many of the initial cases had a common exposure to the Huanan wholesale seafood market where trading of the live animals was also done (Wang et al, 2020). The activation of the surveillance system (put into place after the SARS outbreak) took place and respiratory samples of patients were sent to reference laboratories for etiological investigations of the then suspected pneumonia cases (Wang et. al.,2020). This outbreak was notified to the World Health Organization on December 31st 2019 by China and on 1st January the closing of sea food market in Huanan took place (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports/.).

The origin of the virus was traced back to Huanan sea food market when environmental samples from the Huanan sea food market also tested positive for the virus, implying that virus originated from there(https://www.xinhuanet.com/2020-01/27/c_1125504355.htm). The fact that human-to-human transmission was occurring was suggested when the number of cases infected with the virus started increasing exponentially, some of which did not have exposure to the Huanan sea food market (Huang et. al., 2020). The first fatal case was reported on 11th Jan 2020and the massive migration of Chinese during the Chinese New Year fueled the epidemic to spread on alarming rates.

Local human-to-human transmission was occurring in the countries other than China and this assumption was supported by the fact that Cases of COVID-19 in these countries started being reported in those with no history of travel to China (Camilla et. al., 2020).

2.2.1.2 SARS-CoV-2: Origin, spread and current statistics in Nepal

On Jan 13, 2020, a 32-year-old man, a Nepalese student at Wuhan University of Technology, Wuhan, China, with no history of co-morbidities, returned to Nepal. The patient visited the outpatient department of Sukraraj Tropical and Infectious Disease Hospital, Kathmandu, with a cough. He had become ill on Jan 3, 6 days before he flew to Nepal with his claims that he had no exposure to the so-called

wet market in Wuhan. Upon real-time RT-PCR assays at the WHO laboratory in Hong Kong the throat swabs obtained from the patient tested positive for COVID-19 (Bastola et. al., 2020).

On 16 February, 175 Nepalese nationals were flown into Nepal from China after exit screening and the repatriated citizen's transportation was managed under quarantine from Tribhuvan International airport to quarantine site at Kharipati, Bhaktapur district, east of Kathmandu where these individuals were placed under 24-hrs surveillanceSample of all 175 persons, sampled on 16 February, were reported negative on 19 February. A dedicated ambulance service was available to one of three hospitals—STIDH, Patan hospital or APF hospital in case of a positive test or in case of onset of the symptoms amongst these individuals(://heoc.mohp.gov.np/update-on-novel-corona-virus-2019_ncov/).

On May 16, 2020 Nepal reported the first death from COVID-19 on a female who had recently given birth on May 8 (https://www.aa.com.tr/en/asia-pacific/nepal-registers-its-first-death-from-COVID-19/1843635). Despite the lockdown measures applied on different occasions, the spread of corona virus in the different parts of countries is still rising and continues to mount pressure on the government authorities for proper control and surveillance of COVID-19.

Nepal stands on the 40th position on the worldwide scenario with total number of infections suggested by the statistics of 252474 reported cases with 1765 deaths and a population of 241392 detected cases have already recovered as of December 19, 2020(https://www.worldometers.info/coronavirus/).Considering the possibility of travel of infected cases within the internal borders of the country, high vigilance coupled with a strong response plan is required to address the current risk of COVID-19. In this regard, a directive from the Government of Nepal is still required for educating the public to respond to the outbreak through necessary precautions and inform travelers about possible risk despite the current efforts of the government which does not support the confinement of the spread of disease in today's context (Shrestha et. al., 2020)). There is necessity to identify and contain suspected cases from the site of origin. COVID-19 and past outbreak scenarios should be a learning experience for Nepal

not only on emergency management but also towards developing a strong surveillance system and taking preventive actions for similar events as we cannot deny the fact that these types of serious complications won't emerge in the future.

2.3 Proteins of SARS-CoV-2

The 29.3 kilobases of 2019 Coronavirus gene consits of 1273 amino acids with spike glycoprotein (S), envelope protein (E), membrane protein (M) , nucelocapsid protein (N), receptor binding motif (RTF), M pro, NSP7, NSP13. Some of the membrane proteins that are present in SARS-CoV-2 includes RNA polymerase, 3-chemotrypsin-like protease, papain-like protease, helicase , and accessory proteins.

2.3.1 Structural Proteins of SARS-CoV-2

2.3.1.1 Spike Protein

The spike protein of SARA-CoV-2 contains about 1273 amino acids and it has 141778 kDa molecular weight (Chan et. al., 2020). It has 50-100 timers of spikes where each spike protein consists of an ectodomain element, transmembrane element, and short C fragment. There are two ectodomains : (a) S1 – facilitates the receptor binding and (b) S2- stem that forms a timer. These protruding projections from the surface are responsible for binding and fusing with the membrane (Anderson et. al.,1983). S protein plays vital role in viral entry into the host cell as it has high affinity to the angiotensin-converting enzyme 2 (ACE2) receptors which are expressed in the pneumocytes in human. The presence of glutamine 493, asparagine 501, leucine 455, phenylalanine 486 and serine 494 amino acids in spike protein helps in binding with ACE2 receptors. Along with these amino acids, receptor binding domain (RBD) present in S1 subunit recognizes and binds to human ACE2.

2.3.1.2 Nucleocapsid Protein

The N protein in SARS-CoV-2 known as the nucleocapsid protein is responsible for the interaction with cellular processes and fusion of virus (Huanget. al., 2004). N protein consists of a N terminal domain (NTD) and C terminal domain (CTD) and a serine rich linker (SR) present between them . Repication transcription

complexes (RTC) belonging to N protein plays key role in viral genome synthesis (Gerber et. al., 2019). It is found to inhibit the type I Interferon (IFN) which decreases the immune responses in the host cell. The increase amount of N protein increases the viral RNA synthesis once it comes in contact with Heterogenous nuclear ribonucleoprotein (hnRNPA1) (Luo, Chen et al. 2005). The presence of Arginine at 94 position and Tyrosine at 122 are reported to be essential to bind to SARS-CoV RNA (McBride et. al., 2014). Host samples express the N protein during early stages of infection. N protein is involved in various activities that are essential for the proliferation and functioning of virus.

2.3.1.3 Envelope Protein

Envelope Protein (E) has 76-109 amino acids with 8-12 kDa in size (Fung and Liu, 2018). It is integral membrane protein that is composed of NTD, hydrophobic domain and C terminal chain. E protein are responsible for the production of viroporins that are vital for viral assembly, release of virus, mediate pathogenicity and cause cytotoxicity (Ye and Hogue 2007).

2.3.1.4 Membrane Protein

The membrane protien (M) encompasses the highest percentage among all proteins in the virus (Alsaadi and Jones, 2019). M protein is found to exist in two forms - long and compact form. Spike protein is present above these two forms. M protein plays key role in translation and produces the virions after interacting with E protein. M protein inhibits the Nuclear Factor Kappa B activation by interacting with I Kappa B Kinase that enhances the viral pathogenicity. Among the terminals of M protein, C termianl blocks the interaction of 3-phosphoinositide-dependent protein kinase 1 (PDK1) with Protein Kinase B (PKB) that ultimately leads to the apoptosis (Tsoi, Li et al. 2014). Along with this, M protein is involved in the activation of β interferons (IFN-β) (Wang and Liu, 2016). M protein is most responsible for increasing the inflammatory responses in the host and the formation of ribonucleoprotiens.

2.4 Pathogenicity

The same human cell receptor Angiotensin Converting enzyme 2 (ACE-2) is employed by SARS-CoV-2 and SARS for their entry into to the host cell whereas

MERS-CoV uses dipeptidyl peptidase 4 (DPP4) to enter host cells (Wan et. al., 2020). The works of Wan et al suggests SARS-CoV-2 binds ACE2 more efficiently than the 2003 strain of SARS-CoV, although less efficiently than the 2002 strain. Further, they have predicted that a single nucleotide mutation on the RBD of SARS-CoV-2, if it occurs, could further increase its pathogenicity.

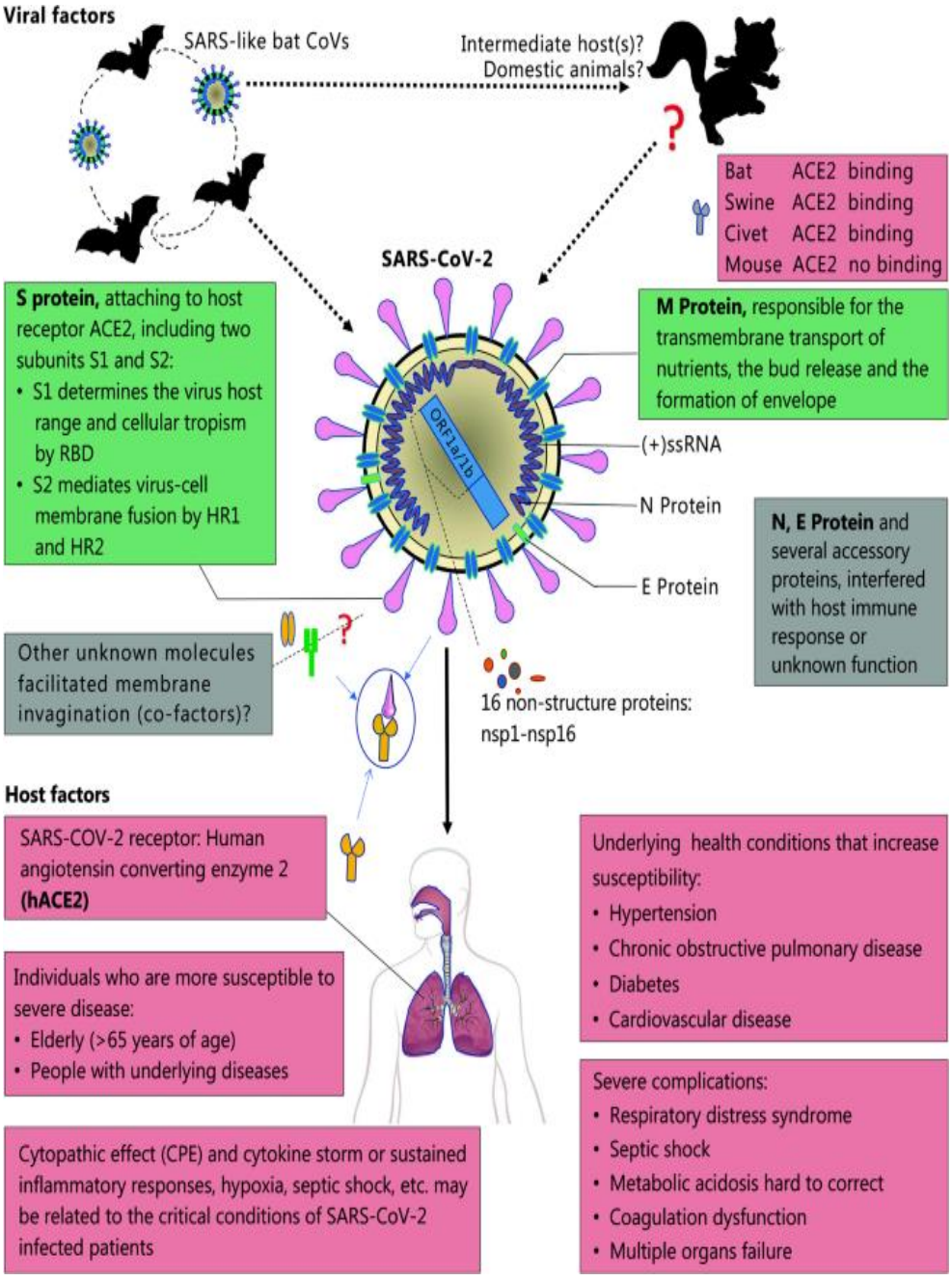


Figure 2.1: Pathogenicity of SARS-CoV-2 (Guo et .al., 2020)

2.5 Transmissibility

The reproductive number (R_0) reflecting the transmissibility of virus was found in the range of 2 and 2.5 for SARS-CoV-2 which is higher than that for SARS (1.7–

1.9) and MERS (<1), as estimated by the World Health Organization, which in turn favors the higher pandemic potential possessed by SARS-CoV-2(Chen et. al., 2020; Luiet. al., 2020 and Wu et. al., 2020). There might be certain fluctuations in the values of R_0 since the estimation of R_0 depends on the estimation method used, and the current estimate can be biased by insufficient data and the short onset times of the diseases, as stated by Liu and colleagues. The fatality rate for novel corona virus is estimated to be 2.3% (Chen, 2020) which is a lower value in comparison to SARS (9.5%) and significantly lower when compared to MERS (34.4%) (Munster et al, 2020).

2.5 Clinical features

The onset of dyspnea within 5 days, ARDS within 8 days in 30% of cases, and the need for invasive mechanical ventilation and extracorporeal membrane oxygenation (ECMO) in 17% and 4% of cases, respectively are the major indications relating to the unfavorable clinical course in case of patients diagnosed with COVID-19 (Wang et. al., 2020). The clinical course of MERS seems to be characterized by a more frequent development of ARDS and the need for invasive life support; especially amongst smokers and elderly patients whereas the earlier mentioned findings of clinical manifestations for COVID-19 are in conjunction with SARS percentages (Azhar et. al., 2019). A direct renal cytopathic effect induced by the virus involving DDP4 receptors facilitating the entry of virus, which are largely represented in tubules and glomeruli accounts, for the acute kidney injury in MERS (Cha et. al., 2015) which seldom occurs in SARS and SARS-CoV-2.

2.5.1 Clinical manifestations of SARS-CoV 2

2.5.1.1 Signs and symptoms

Regarding the four comprehensive studies on the epidemiological and clinical characteristics of SARS-CoV-2 infected patients the most common signs and symptoms of patients are fever and cough (Chen et. al., 2020, Guan et. al.,2020 Huang et. al., 2020 and Wang et. al.,2020). Fatigue was another symptom amongst the patients as complained by 96% of patients (n=138) in one study by

Huang and colleagues but was less outstanding (18%, n=44) in another report by Wang and colleagues.

A combinational analysis of the common recorded signs or symptoms of the reported cases found that cough was observed in around 68% of the SARS-CoV-2 infected patients; the number of patients with fever is relatively more (90%) compared to cough (Zheng, 2020). In addition, shortness of breath or dyspnea, muscle ache, headache, chest pain, diarrhea, hemoptysis, sputum production, rhinorrhea, nausea and vomiting, sore throat, confusion, and anorexia were also observed in a proportion of the patients (Guan et. al., 2020, Huang et. al., 2020 and Wang et. al.,2020).

Pneumonia is a typical characteristic of the SARS-CoV-2 infected patient, now termed as Coronavirus Disease 2019 (COVID-19), demonstrated on diagnosis methods like computer tomographic (CT) scan or chest X –ray (Zhong et. al.,2020 and Chan et. al.,2020). The quick development of acute respiratory failure and other serious complications was accompanied by the acute respiratory infection symptoms displayed by the patient in the early stages of the disease (https://www.who.int/publications-detail/clinical-management-of-severe-acute-respiratory-infection-when-novel-coronavirus-(ncov)-infection-is-suspected). All of the first three patients reported by the China Novel Coronavirus Investigating and Research Team developed severe pneumonia and two among these three patients showed a common feature of fever and cough (Zhu et. al., 2020). 75% within 99 patients demonstrated bilateral pneumonia and the remaining 25% unilateral pneumonia in the chest X-ray and CT imaging as shown in a study (Chen et. al., 2020). Overall, 14% of the patients showed multiple mottling and ground-glass opacity as presented on the same study. One of the early cases of coronavirus infection in the United States detected pneumonia on the tenth day of infection with basilar streaky opacities in both lungs as shown by chest radiography (Holshue et.al.,2020).

2.5.1.2 Modes of transmission

The wide spread of SARS-CoV-2 infection within a short period time was boosted by the fact that SARS-CoV-2 Spike (S) protein had 10- to 20-fold higher affinity to

human angiotensin-converting enzyme 2 (ACE2) receptor than that of SARS-CoV based on the Cryo-EM structure analysis of S proteins, implying the highly contagious and more infectious nature of SARS-CoV-2 infection than initially suspected (Wrapp et. al.,2020). The entry of SARS-CoV-2 into host cells is facilitated upon the recognition and binding of S protein to ACE2 receptor of the host cells, similar to SARS (Zhou et al, 2020). The high affinity of S protein to ACE2 receptor as shown in studies likely contributes to the quick spreading of virus (Wrapp et. al., 2020). The human organs with high ACE2 expression level, such as lung alveolar epithelial cells and enterocytes of the small intestine, are potentially the target of SARS-CoV-2 as indicated by the fact that ACE2 receptor are the human receptors for the virus (Zou et. al., 2020).

Current knowledge for SARS-CoV-2 transmission is largely based on the knowledge for the transmission regarding the similar coronaviruses, particularly SARS-CoV and MERS-CoV, where droplets, contact and fomites are responsible for human-to-human transmission (Zheng et. al.,2020). Although the well-established route of viral infection so far, is believed to be through respiratory droplets and contact with COVID-19 affected individuals so far (Signorelli et. al., 2020). The possible modes of transmission are discussed below under the following sub-headings.

2.5.1.2.1 Saliva

The abundance of Angiotensin converting enzyme 2 (ACE2) in the epithelial cells of the oral mucosa with the fact that high expression of this receptor cells is seen on the tongue (95.86%) than in the buccal and gingival tissues outlines the role of saliva in the possible spread of the SARS-CoV-2 infection (Xu et al, 2020). The role of saliva can be of both friend (diagnostic tool for viral detection) and of foe (a more common mode of transmission via aerosols and salivary droplets) (Han et al, 2020).

2.5.1.2.2 Surface contact

Infected surfaces may still help to transmit the virus despite the control of person-to-person transmission of SARS-CoV-2. The indirect contact with the surfaces or objects contaminated by the infected person may cause the spread of

this zoonotic virus, as this virus displays the ability of staying for longer durations on various surfaces making it contaminated for hours and days. Also, the large viral droplets expired by the infected person may also get deposited on the surface in their close proximity. The viability of SARS-CoV-2 on plastic and stainless steel for a maximum of 72 hours, and eventually a decline in the virus stability and titer is displayed in a study (van Dormaelan et al, 2020). Another study shows that on inanimate surfaces, another coronavirus strain (HCoV-229E) is capable to survive for up to nine days (Carraturo et. al., 2020).

2.5.1.2.3 Aerosols and Airborne

The possible route of spread of virus maybe aerosols and airborne transmission helped by coughing, sneezing, or even talking (Mukhra et. al.,2020). The droplets and aerosols produced by infected individuals on the regular basis may play an important role in the transmission of the virus (Klompas et. al., 2020) and the smaller size of the droplet nuclei particles of <5µm diameter are considered to be the carriers as these can persist in the air for longer durations and can be transported to distances greater than one meter (Moraswka et. al., 2020). The infectious virus can remain in the aerosols for approximately 3 hours with a gradual reduction from 103.5 to 102.7 TCID50 per liter of air under controlled conditions (Moraswka et. al., 2020). A viral transmission rate of approximately 41.3% through respired air by infected patients in a nosocomial environment suggested an urgent need of consideration of the airborne passage of transmission (Wang et. al., 2020). The possibility of air borne route of transmission of viral particles except the indoor confined environment was also suggested by WHO on a update released on 29th June 2020(https://www.who.int/news-room/commentaries/detail/transmission-of-sars-cov-2-implications-for-infection-prevention-precautions).

2.5.1.2.4 Faeco-Oral transmission

The gastrointestinal manifestations in COVID-19 patients outline the probability of shedding viral RNA or infectious virus in the stool and fecal matters of the infected individual (Mukhra et. al., 2020). The fecal-oral route as a probable route of transmission is fueled by the fact where presence of viral RNA in feces, on toilet seat and washbasin sink samples is reported (Young et. al., 2020). The

capability of the virus to survive in fecal samples for up to 4 days outlines the possibility of the fecal-oral route of transmission of SARS-CoV-2(Amirian, 2020). Likewise, other studies have reported presence of viral RNA in stools even after negative test results of the respiratory samples (Xu et. al., 2020 and Chen et. al., 2020). However, the fecal-oral route of transmission is still debatable due to the lack of strong evidence for possibility of viral replication in fecal swabs.

2.5.1.2.5 Other possible modes of transmission

The sexual route of transmission for SARS-CoV-2 virus (Patri et. al., 2020) surfaced following the reports of fecal-oral transmission (Peng et. al., 2020). Both direct or indirect exposure of oral-anal contact within few days even after recovery, with the shedding the viral RNA still seen on some cases in feces, such sexual behaviors could result in alternative ways of transmission of the virus (Patri et. al., 2020). The vertical transmission of SARS-CoV-2 virus from mother to fetus or neonate is still debatable. The shedding of the RNA particles on the milk of lactating mothers could hint on the maternal to neonate transmission but this need to be validated through further studies (Grob et. al., 2020).

Conjunctival secretions might be another possible mode of viral transmission; as conjunctival mucosa and respiratory tract are connected by the nasolacrimal duct and share the same ACE-II receptor cell of SARS-CoV-2 (Sun et.al., 2020). Lu et al. have raised the possibility of transmission through ocular surface. More research is suggested to develop a detailed understanding of the transmission mechanism through tear secretions and ocular surfaces.

Another major concern regarding the transmission of COVID-19 is via asymptomatic carriers who are responsible for transmitting the virus during the incubation period (Ye and Hogue, 2020). There have been reports of asymptomatic carriers of SARS-CoV-2, a case in Wuhan, where a 10 years old boy behaved as an asymptomatic carrier of virus, showing lung infiltrates on CT scans (Chan et. al., 2020). These findings underline the role of asymptomatic carriers as one of the probable sources of SARS-CoV-2 infection posing a great threat to increase the infection and further calling out for an immediate evaluation of transmission dynamics of the epidemic.

2.6 Laboratory Detection

Everyone who has symptoms revealing of COVID-19 and the people with high risk of exposure to SARS-CoV-2 should be tested to rule out the infection. Testing may be

a. Diagnostic testing: Testing intended to rule out or identify the current or recent infection in individuals with sign and symptoms consistent of COVID-19 or suspected exposure to SARS-CoV-2.
b. Screening testing: To identify the asymptomatic or no suspected exposure to SARS-CoV-2.

Such testing should employ either a nucleic acid amplification test (NAAT) or an antigen test to detect SARS-CoV-2. Testing may also be used for screening, determining the length of a patient's isolation period, and other non-diagnostic purposes. (CDC 2022)

A number of diagnostic tests for SARS-CoV-2 infection (e.g., NAATs, antigen tests) have received Emergency Use Authorizations (EUAs) from the Food and Drug Administration (FDA), but no diagnostic test has been approved by the FDA. Diagnostic tests have been authorized for use by trained personnel in several settings, including lab facilities. They can also be used in point-of-care settings, where the test is performed by trained personnel at or near the place where the specimen was collected. Point-of-care settings include physician offices, pharmacies, long-term care facilities, and school clinics. Antigen tests can be self-administered, and most can be used in point-of-care settings, allowing results to be available within minutes. Some NAATs can also be self-administered at home or in other non-health care locations and shipped to a laboratory for testing.

Although nasopharyngeal specimens remain the recommended samples for SARS-CoV-2 diagnostic testing, nasal (anterior nares or mid-turbinate) or oropharyngeal swabs are acceptable alternatives. Lower respiratory tract samples have a higher yield than upper respiratory tract samples, but they are often not obtained because of concerns about aerosolization of the virus during sample collection procedures. Some of the tests that have received EUAs can also be performed on saliva specimens, but the quality of saliva specimens can

be highly variable. Studies are currently evaluating the use of other sample types, including stool samples.

2.6.1 Real time PCR

Real time is the most reliable method of detection and measurement of products generated during PCR which is equivalent to the amount of template present at start of PCR process. TaqMan probe is a fluorogenic non extendable probe which has a fluorescent reporter dye attached to its 5' end and a quencher dye at its 3' end. The fluorogenic probe anneals downstream from the primer site which is cleaved by the 5' nuclease activity of Taq polymerase enzyme during extension phase when the target sequence is present. When the target sequence is not present, the probe is intact, fluorescence resonance energy transfer occurs which leads to the absorbance of the fluorescence emission of the reporter dye by the quenching dye. When Taq polymerase cleaves probe during PCR the separation of reporter and quencher dyes takes place which increases fluorescence from the reporter dye.

After the cleavage, the probe is removed from the target strand, which allows primer extension to continue to end of template strand which does not interfere with exponential accumulation of PCR product. The additional reporter dye molecule is cleaved in each consecutive cycle which in turn leads to increase in fluorescence intensity that is proportional to amount of amplicon produced.

TaqMan probe: A forward primer, a reverse primer and a probe is used. The assay is dependent on 5' to 3' nuclease activity of Taq polymerase. The probe has Fluorescent reporter and quencher dye covalently bonded at the 5' and 3' end respectively. The intactness of probe leads to occurrence of FRET which is due to proximity of the reporter and quencher dye as a result of which fluorescence emission does not occur. When target sequence is present during PCR the annealing of probe to target takes place and Taq polymerase cleaves probe allowing an increase in fluorescence emission. The intensity of fluorescence is increased in proportion to amount of amplicon produced (Aryal et. al., 2005).

On the basis of fluorescence emission data, the computer software constructs amplification plots. Baseline describe about the PCR cycles in which the reporter

fluorescence signal accumulates but is below the limit of detection by the machine.

- RN: It is the difference between fluorescence emission of product at each time point and fluorescence emission of baseline.

Threshold: It is arbitrary value chosen by computer being based on variability of baseline. The threshold cycle for a simple is a fluorescent signal which is detected above the threshold.

Cycle Threshold (CT): CT is defined as the fractional number of PCR cycle at which reporter fluorescence is greater than the threshold (minimum detection level). The essential component in production of accurate and reproducible data is based on CT which is a basic principle of Real time PCR. The lower CT value indicates greater amount of target nucleic acid present in the sample.

During the early cycles of PCR exponential amplification of the target sequence takes place which is the time when CT value occurs. If more template is present at beginning of the reaction, a fewer number of cycle can lead to a point at which fluorescent signal is recorded statistically significance above the background. (Higuchi et. al. 1993)

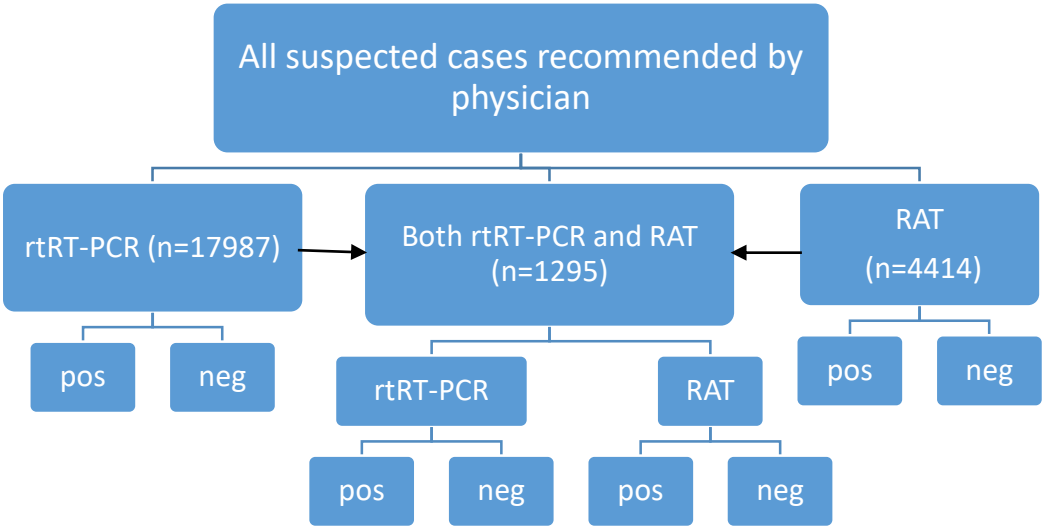
Chapter III

3.0 Methods and Methodology

3.1 Study Design

This was a descriptive, cross-sectional study. Quantitative study was carried out to access SARS-CoV-2 infection in central region of Nepal along with the diagnostic performance of rapid antigen kit vs rtRT-PCR at Trishuli Hospital Nuwakot.

The research design of entire study is presented below:



3.2 Study Site

Study site was the Trishuli Hospital Nuwakot, Nepal. The study was conducted in Molecular department, Department of Pathology, Trishuli Hospital Nuwakot.

3.3 Study Population and sample size

3.3.1 Study population

Nasopharyngeal swab(NPS) specimens was collected from all the patients visiting Trishuli Hospital Nuwakot and respective local bodies of Nuwakot, Dhading and Rasuwa district suspected of having COVID-19 infection and processed for Real time RT-PCR. For Antigen test all the NPS samples were collected in Trishuli hospital COVID-19 RT-PCR laboratory.

3.3.2 Sample size

The required Sample size was calculated as $n= [z^2P(1-P)]/e^2$

Where z=1.96 in 95% Confidence interval(CI)

P=proportion= 0.5

e= margin of error=0.03

$n= [1.96^2 \times 0.5(1-0.5)]/0.03^2$

n=1067.111

n ≈ 1068

The minimum of 1068 samples were required for the study. As per the convenience and availability we have included 1295 sample in our study.

3.4 Duration of the Study

The samples were collected from January 2021 to December 2021 after recommendation of Clinician/ Physicians.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion Criteria

1. All the patients clinically suspected for COVID-19 infection

   Symptoms of COVID-19 infection

   a. Fever or chills
   b. Cough
   c. Shortness of breath or difficulty breathing
   d. Fatigue
   e. Malaise
   f. Muscle or body aches
   g. Headache
   h. Loss of taste or smell
   i. Sore throat
   j. Congestion or runny nose
   k. Nausea or vomiting
   l. Diarrhea
   m. Sputum production
   n. Loss of appetite
   o. Respiratory distress

2. People giving consent and willing to participate.

3.5.2 Exclusion Criteria

1. Those patients not giving consent.
2. Patient not willing to participate in the study.

3.6 Ethical Consideration

For the collection and processing of sample, verbal consent was taken from patient parties and ethical clearance was obtained from the Trishuli Hospital Nuwakot. The respondent were patients themselves or the family members and mother/father of the children under 5 years of age. Test was performed by direct involvement of the researcher himself.

3.7 Sample collection, transport and storage

Nasopharyngeal swab sample was collected in well labeled Virus Transport Medium (VTM) from patients suspected with COVID-19 infection using Personal Protective Equipment (PPE) and the swab stick was broken from the specified region and the VTM was sealed properly and store in 2-8^{o}C until test performed within a day or during transport (with 3 layer packing) otherwise delayed in processing of sample, the VTM was stored in -20^{o}C until tested.

3.8 Rapid SARS-CoV-2 antigen detection assay

3.8.1 Standard Q COVID-19 Ag kit (SD Biosensor®, Chuncheongbuk-do, Republic of Korea)

Standard Q COVID-19 Ag test (SD Biosensor) is a rapid chromatographic immunoassay for the qualitative detection of SARS-CoV-2 nucleo-capsid (N) protein antigen in human nasopharyngeal specimens. This rapid antigen test device has two pre-coated lines on the nitrocellulose membrane on result window: control (C) and test (T) lines which are not visible before applying any specimen. The control (C) region is coated with mouse monoclonal anti-chicken Igγ antibody; the test (T) region is coated with mouse monoclonal anti-SARS-CoV-2 antibody against SARS-CoV-2 N antigen. Detectors for SARS-CoV-2 N antigen presented in the specimen are mouse monoclonal anti-SARS-CoV-2 antibody conjugated with color particles. The antigen–antibody color particle

complex migrates via capillary force and is captured by the mouse monoclonal anti-SARS-CoV-2 antibody coated on the test (T) region. The colored test (T) line's intensity depends on the amount of SARS-CoV-2 N antigen presented in the sample. The test result was read in 15–30 min. For positive COVID-19 antigen result, two colored lines of control (C) and test (T) lines were presented.

3.8.2 Panbio COVID-19 Ag Rapid Test Device **(**Abbott Panbio™ Orlaweg, Germany**)**

PanbioCOVID-19 Ag Rapid Test Device (Panbio) is a rapid chromatographic immunoassay for the qualitative detection of SARS-CoV-2 Nucleo-capsid protein antigen in human nasopharyngeal specimens. This rapid antigen test device has two pre-coated lines on the nitrocellulose membrane strip on result window: control (C) and test (T) lines which are not visible before applying any specimen. The control (C) region is coated with mouse monoclonal anti-chicken Igɣ antibody; the test (T) region is coated with immobilized anti-SARS-CoV-2 antibody. Detectors for SARS-CoV-2, human IgG specific to SARS-CoV-2 Ag gold conjugate and chicken IgY gold conjugate with the specimen. The antigen–antibody color particle complex migrates via capillary force and is captured by the anti-SARS-CoV-2 antibody coated on the test (T) region. The colored test (T) line's intensity depends on the amount of SARS-CoV-2 presented in the sample. The test result was read in 15–20 min. For positive COVID-19 antigen result, two colored lines of control (C) and test (T) lines were presented.

3.9 Reverse transcription Real time PCR

3.9.1 RNA Extraction

RNA of SARS-CoV-2 virus was extracted using CWBIO Viral DNA/RNA kit and the extracted RNA was soon stored in the freezer to prevent further degradation of viral RNA for further molecular research.

First of all, the reagent was prepared as the manufacturer instruction and appropriate amount of isopropanol and 100% ethanol were added to Wash buffer 1 and wash buffer 2 respectively.

200 µL of swab sample after vortexing was pipette out in 1.5mL micro-centrifuge tube and 200 µL of lysis buffer was added along with 300µL isopropanol. The solution was mixed by pulse vortexing for 5-10 seconds and incubated at room temperature for 5-10 minutes and vortex for 10 minutes with constant temperature mixer of 1200 rpm. Then 700µL of the solution was transfer to the adsorption column in a 2 ml Collection tube and centrifuge at 12000 rpm (~ 13400 g) for 1 minute. The filtrate liquid was discarded and the adsorption column was kept back into the collection tube. The adsorption column was carefully opened and 500µL washing buffer (Wash buffer 1) was added and centrifuge at 12000rpm for 1 minute. Then the filtrate liquid was discarded and the adsorption column was kept back in collection tube and the process repeated by wash buffer 2. Then dry spin of adsorption column was done without adding any liquid by centrifuge at 12000rpm for 2 minutes. Then the adsorption column was transfer into the new 1.5 ml micro-centrifuge tube and left at room temperature for 2 minute to dry. The adsorption column cap was open carefully after drying and 40-100µLRNAase free water was added to the middle part of the adsorption column membrane. The cap was closed and the incubated at room temperature for 2 minute. Finally, the tube was centrifuged at 12000rpm for 1 minute and the RNA collected in the micro-centrifuge tube was stored at -80^{o} C to prevent degradation.

3.9.2 rtRT-PCR

One step Real time reverse transcription PCR was performed as the instructions in SARS-CoV-2 Fluorescent PCR kit, Maccura. The kit includes the multiplexed real time RT-PCR test for the quantitative detection of SARS-CoV-2 RNA. The kit contains three primer/probe sets specific to different genomic region of SARS-CoV-2 such as ORF1 ab, N gene and E gene along with primer and probes for the internal control. The one step Real time RT-PCR was performed along with positive, negative and internal controls pre-extracted as sample during the sample RNA extraction process.

3.10 Sequencing and Sequence analysis

The NPS sample collected from patients which were positive for rtRT-PCR were send to CSIR-Institute of Genomics and Integrative, Delhi through NPHL for whole genome sequencing and the sequence was submitted to GISAID.

3.10.1 Phylogenic tree construction

The phylogenic tree was constructed by aligning sequence in AudacityInstant alogorithim and test maximum Likelihood Tree. The 11.7 Million sequence available in Gene Bank from different parts of world were searched for related genomes, obtained from blast result were included in the multiple sequence alignment and subsequent construction of the phylogenetic tree.

3.11 Analysis of Data

The data obtained were entered process and filter in Excel. The graph was prepared using Excel, Graph pad prism 8. The mean value was compared using Mann-Whitney U test. Sensitivity, specificity, PPV, NPV, Likelihood ratio, Accuracy were calculated using 2x2 contingency table. The Cohens Kappa value, P value and Youden's index were calculated for validation of the protocol.

2x2 contingency table	rtRT-PCR		Total
	Positive	Negative	
Antigen Positive	a True positive	b False positive	a+b
Antigen Negative	c False Negative	d True Negative	c+d
Total	a+c	b+d	a+b+c+d

Calculation Formula

1. Sensitivity: It is the ability of the test to correctly identify patients with a disease.

   Sensitivity: [a/(a+c)] x 100

2. Specificity: It is the ability of the test to correctly identify patients without the disease.

   Specificity: [d/(b+d)] x 100

3. Positive predictive value (PPV): It is the ratio of patients truly diagnosed as positive to all those who had positive test results. It is also known a Precision. It is proportion of cases giving positive test result that are truly positive.

   PPV: [a/(a+b)] x 100

4. Negative predictive value (NPV): It is the ratio of subjects truly diagnosed as negative to all those who had negative test results (including patients who were incorrectly diagnosed as healthy). It is proportion of cases giving negative test result that are already healthy.

   NPV: [d/(c+d)] x 100

5. Likelihood ratio: Likelihood ratios (LRs) represent another statistical tool to understand diagnostic tests. LRs allow providers to determine how much the utilization of a particular test will alter the probability. A positive likelihood ratio, or PLR, is the "probability that a positive test would be expected in a patient divided by the probability that a positive test would be expected in a patient without a disease." In other words, a PLR is the true positivity rate divided by the false positivity rate. A negative likelihood ratio or NLR is "the probability of a patient testing negative that has a disease divided by the probability of a patient testing negative who does not have a disease." Unlike predictive values, and similar to sensitivity and specificity, likelihood ratios are not impacted by disease prevalence. The formulas for the likelihood ratios are below.

   Positive Likelihood Ratio=Sensitivity/(1-Specificity)

   Negative Likelihood Ratio= (1- Sensitivity)/Specificity

6. Accuracy: Accuracy is an absence of bias.

   Accuracy: (a+d)/ (a+b+c+d)

7. Cohen's kappa: kappa value is use to ascess the degree of agreement of the test done by multiple appraisers when the appraiser evaluates the same sample. Cohen's Kappa is popular for measuring assessment agreement between 2 raters.

   For Cohen Kappa (K) = [pr(a)-pr(e)] / [1-pr(e)]

Pr(a)= (a+d) / 10

Pr(e)= (a% x c%) + (b% x d%)

a%= (a+c)/ a+b+c+d

b%= (b+d) /a+b+c+d

c%= (a+b) / a+b+c+d

d%= (c+d)/ a+b+c+d

Kappa value interpretation:

<0 :	No agreement
0- 0.20:	Slight agreement
0.21 - 0.40:	Fair agreement
0.41- 0.60:	Moderate agreement
0.61- 0.80:	Substantial agreement
0.81-1.0:	Perfect agreement

8. Youden index (J): Youden's J statistic (also called Youden's index) is a single statistic that captures the performance of a dichotomous diagnostic test. The cut-off point for having an acceptable Youden index is **50%**. Any value below 50% denotes an overall lack of the diagnostic test to detect either disease or health. If the Youden index is not over 50%, then the test does not meet empirical benchmarks for being administered for diagnostic purposes. The Youden index is a measure of a diagnostic test's ability to balance sensitivity (detecting disease) and specificity (detecting health or no disease).

   Youden index: Sensitivity+Specificity-1

CHAPTER-IV

4.0 RESULTS

The chapter deals with the analysis and interpretation of findings of the study. The descriptive study was conducted from January 2021 to December 2021 in the Trishuli Hospital Nuwakot. The collected data were analyzed and presented in the different figures and tables.

4.1 Incidence

4.1.1 Real time reverse transcription polymerase chain reaction (rtRT-PCR)

A total of 17987 nasopharyngeal swab (NPS) samples were collected from suspected COVID-19 cases from 1st January 2021 to 31st December 2021 for Real Time Reverse Transcription PCR (rtRT-PCR). Of the sample tested for detection of COVID-19, 7956 samples (44.23%) were positive by rtRT-PCR while 10031 samples (55.77%) were negative for SARS-CoV-2 RNA. Of which 63.79% (n=11474) were male and 36.21% (n=6513) were female with an average age of 36.6 years (ranging from 4 months to 101 years).

4.1.2 Rapid Antigen test (RAT)

Among 4414 suspected COVID-19 cases, NPS sample was collected and processed immediately according to manufactures instruction for Rapid antigen test (RAT) during study period. Out of 4414 samples, 951 (21.5%) were found to be positive for anti-SARS-CoV-2 antibody. Of these 4414 suspected cases, 1295 recommended by clinician or physician were tested parallel and had a concomitant rtRT-PCR test result.

4.2 Real time RT-PCR and SARS-CoV-2 antigen assay

Among 1295, 36.45% (n=472) were RAT positive and 823 were RAT negative with an average age of 40.4 years (ranging from 6 months to 99 years) and Standard deviation of 18.6 years. In the study population, highest number of suspected cases 319 (24.63%) belonged to the 20-30 years' age group followed by 30-40 years' age group (n=298) whereas lowest number 7 (0.54%) to above 90 years' age group. Among a total 472 anti-SARS-CoV-2 antibodies positive cases, higher positive rate was seen in the 30-40 years' age group followed by 20-30 years' age

group 26.69% and 23.52% respectively. In contrast, there was a very low positivity rate 0.42% and 0.63% in the age group more than 90 years and less than 10 years respectively. Gender wise, among total suspected cases 53.67% (695/1295) were males and 46.33% (600/1295) were females. Positive case among male was observed to be 36.96% which constituted 19.84% of total cases and 35.83% were females which comprised 16.60% of total cases. This gender bias, a male to female ratio of 1.16:1 was present in our study. (Table 4.1 and 4.2).

Table 4.1 Age and Gender wise distribution of Antigen rapid test

Class	Male			Female			Ag neg	Ag pos	Total
	Neg	Pos	Total	Neg	Pos	Total			
0-10	11	1	12	7	2	9	18	3	**21**
10-20	38	11	49	26	8	34	64	19	**83**
20-30	95	45	140	113	66	179	208	111	**319**
30-40	101	66	167	71	60	131	172	126	**298**
40-50	73	53	126	41	28	69	114	81	**195**
50-60	45	39	84	35	21	56	80	60	**140**
60-70	35	20	55	43	14	57	78	34	**112**
70-80	28	13	41	37	10	47	65	23	**88**
80-90	9	8	17	10	5	15	19	13	**32**
90-100	3	1	4	2	1	3	5	2	**7**
Total	**438**	**257**	**695**	**385**	**215**	**600**	**823**	**472**	**1295**

Table 4.2: Gender wise distribution of cases

Sex	Total no. of sample	No. of positive sample (%)	% of positive cases in total (n=1295)	Male: Female among positive cases
Antigen				
Male	695	257 (36.96%)	19.84	1.16:1
Female	600	215 (35.83%)	16.60	
Total	1295	472 (36.45%)		
PCR				
Male	695	378 (54.39%)	29.19	1.12:1
Female	600	337 (56.17%)	26.02	
Total	1295	715 (55.21%)		

Out of 1295, 715 (55.21%) were rtRT-PCR positive and 580 (44.79%) have negative result. Among a total 715 rtRT-PCR positive cases, higher positive rate was seen in the 30-40 years' age group followed by 20-30 years' age group 25.17% and 22.94% respectively whereas there was a very low positivity rate 0.42% and 0.98% in the age group more than 90 years and less than 10 years respectively. Gender wise, Positive case among male suspects was observed to be 54.39% which constituted 29.19% of total cases and 56.17% were females which comprised 26.02% of total cases. (Table 4.3 and 4.2) Out of 1295, 715 were rtRT-PCR positive and 472 had RAT positive i.e 66.01% (472/715) analytical sensitivity of RAT compare to rtRT-PCR.

Table 4.3: Age and Gender wise distribution of rtRT-PCR test

Class	Male			Female			PCR Neg	PCR pos	Total
	Neg	Pos	Total	Neg	Pos	Total			
0-10	7	5	12	7	2	9	14	7	**21**
10-20	30	19	49	18	16	34	48	35	**83**
20-30	71	69	140	84	95	179	155	164	**319**
30-40	73	94	167	45	86	131	118	180	**298**
40-50	52	74	126	24	45	69	76	119	**195**
50-60	32	52	84	22	34	56	54	86	**140**
60-70	24	31	55	26	31	57	50	62	**112**
70-80	19	22	41	28	19	47	47	41	**88**
80-90	6	11	17	8	7	15	14	18	**32**
90-100	3	1	4	1	2	3	4	3	**7**
Total	**317**	**378**	**695**	**263**	**337**	**600**	**580**	**715**	**1295**

4.3 Diagnostic Sensitivity and Specificity of RAT compare to rtRT-PCR

We evaluated the performance characteristics of two SARS-CoV-2 antigen detection kit (Standard Q COVID-19 Ag test and PanbioCOVID-19 Ag rapid test Device). The sample used in the study was mainly composed of symptomatic participants and rests of participants were considered as higher COVID-19 risk participants with mild symptoms and close contact. When analyzing the results, we categorized the data on the basis of quantitative Ct-Value in 4 accumulative categories (<20, <25, <30 and <35). For SD Biosensor, sensitivity ranged from

94.2%, 93.5%, 81.4% and 64.9% in CT value <20, <25, <30 and <35 respectively. Whereas for Panbio, sensitivity range from 92.9%, 93.9%, 83.8% and 63.8%. Gradual decline in the test performance was observed as the CT value increased whereas the Specificity of the kits was found to be 96.9% and 98.3% for SD Biosensor and Panbio respectively. The overall sensitivity and specificity of both the RAT compared to rtRT-PCR is 93.5%, 93.7%, 82.9% and 64.2% in CT value <20, <25, <30 and <35 respectively whereas sensitivity is 97.8% (Table 4.5, 4.6 & 4.7). In 44.23% calculated period prevalence in our study, Negative predictive values (NPV) were 95.5%, 94.9%, 86.8% and 77.7% for SD Biosensor and 94.6%, 95.3%, 88.4% and 77.4% for Panbio and Positive predictive value (PPV) was 96%, 96%, 95.4% and 94.3% of SD biosensor and 97.7%, 97.8%, 97.5% and 96.7% of Panbio for CT<20, CT<25, CT<30 and Ct<35 respectively. The overall NPV was 95.0%, 95.1%, 87.8% and 77.8% and PPV was 97.1%, 97.1%, 96.8% and 96.8% for CT value <20, <25, <30 and <35 respectively.

Table 4.4: contingency table showing rtRT-PCR vs RAT result.

SARS-CoV-2	**rtRT-PCR**							
	CT < 20		**CT < 25**		**CT < 30**		**CT < 35**	
	+	**-**	**+**	**-**	**+**	**-**	**+**	**-**
SD Biosensor								
Antigen +	130	7	172	7	179	7	179	7
Antigen -	8	216	12	216	41	216	97	216
Panbio								
Antigen +	170	6	261	6	280	6	280	6
Antigen -	13	351	17	351	54	351	159	351
Overall								
Antigen +	300	13	433	13	459	13	459	13
Antigen -	21	567	29	567	95	567	256	567

Table 4.5: Diagnostic performance of SD Biosensor RAT

SD Biosensor	**CT < 20**	**CT < 25**	**CT < 30**	**CT < 35**
	% (95% CI)	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**
Sensitivity	94.2 (89.0-97.0)	93.5 (88.9-96.2)	81.4 (75.7-86.0)	64.9 (59.1-70.2)
Specificiy	96.9 (93.7-98.5)			
PLR	30.39	30.17	26.26	20.93
NLR	0.06	0.07	0.19	0.36
PPV	96 (91.8-98.1)	96 (91.8-98.1)	95.4 (90.5-97.8)	94.3 (88.2-97.2)
NPV	95.5 (91.5-97.6)	94.9 (91.4-97.0)	86.6 (82.9-89.9)	77.7 (74.3-80.6)
Accuracy	95.8 (93.3-97.0)	95.3 (92.8-97.0)	89.2 (85.9-91.7)	79.2 (75.4-82.2)
Kappa value	0.91 (0.868-0.956)	0.91 (0.864-0.947)	0.78 (0.726-0.840)	0.59 (0.529-0.659)
SE of Kappa	0.022	0.021	0.029	0.033
p-value	<0.0001	<0.0001	<0.0001	<0.0001
Youden index	0.917	0.918	0.809	0.596

* P value was calculated using Fisher's exact test.

Table 4.6: Diagnostic performance of Panbio RAT

Panbio	**CT < 20**	**CT < 25**	**CT < 30**	**CT < 35**
	% (95% CI)	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**
Sensitivity	92.9 (88.2-95.8)	93.9 (90.4-96.1)	83.8 (79.5-87.4)	63.8 (59.2-68.1)
Specificiy	98.2 (96.4-99.2)			
PLR	51.61	52.17	46.56	35.44
NLR	0.07	0.06	0.16	0.37
PPV	97.7 (95.1-99.0)	97.8 (94.6-97.6)	97.5 (94.6-98.9)	96.7 (92.9-98.5)
NPV	94.6 (91.2-96.8)	95.3 (92.7-97.0)	88.4 (85.6-90.8)	77.4 (79.9-79.7)
Accuracy	96.5 (94.6-97.7)	96.4 (94.6-97.6)	91.3 89.0-93.2)	79.3 (76.3-81.9)
Kappa value	0.92 0.886-0.956	0.93 0.896-0.956	0.83 0.784-0.867	0.6 0.546-0.648
SE of Kappa	0.018	0.015	0.021	0.026
p-value	<0.0001	<0.0001	<0.0001	<0.0001
Youden index	0.912	0.905	0.783	0.594

* P value was calculated using Fisher's exact test.

Table 4.7: Overall diagnostic performance of RAT

Overall	CT < 20	CT < 25	CT < 30	CT < 35
	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)
Sensitivity	93.5 (90.2-95.7)	93.7 (91.1-95.6)	82.9 (79.5-85.8)	64.2 (60.6-67.6)
Specificiy	97.8(96.2-98.7)			
PLR	42.5	42.59	37.68	29.18
NLR	0.07	0.06	0.17	0.37
PPV	97.1 (95.0-98.3)	97.1 (95.0-98.3)	96.8 (94.3-98.1)	95.9 (92.7-97.0)
NPV	95 (92.5-96.7)	95.1 (93.2-96.6)	87.8 (85.5-89.8)	77.5 (75.5-79.3)
Accuracy	96.2 (94.8-97.3)	96 (94.6-97.0)	90.5 (88.6-92.1)	79.2 (76.9-81.3)
Kappa value	0.92 0.890-0.945	0.92 (0.894-0.942)	0.81 (0.775-0.843)	0.6 (0.556-0.636)
SE of Kappa	0.014	0.012	0.017	0.02
p-value	<0.0001	<0.0001	<0.0001	<0.0001
Youden index	0.921	0.902	0.825	0.597

* P value was calculated using Fisher's exact test.

4.4 CT value distribution of rtRT-PCR target genes

RT-PCR CT values of each target gene were studied independently to further demonstrate the performance RT-PCR test results. For the ORF 1ab gene the mean CT value is 20.14, for N gene mean CT value was 21.03 and for E gene was 21.63. The difference between the 3 genes were statistically significant (P value <0.05) (figure 4.1).

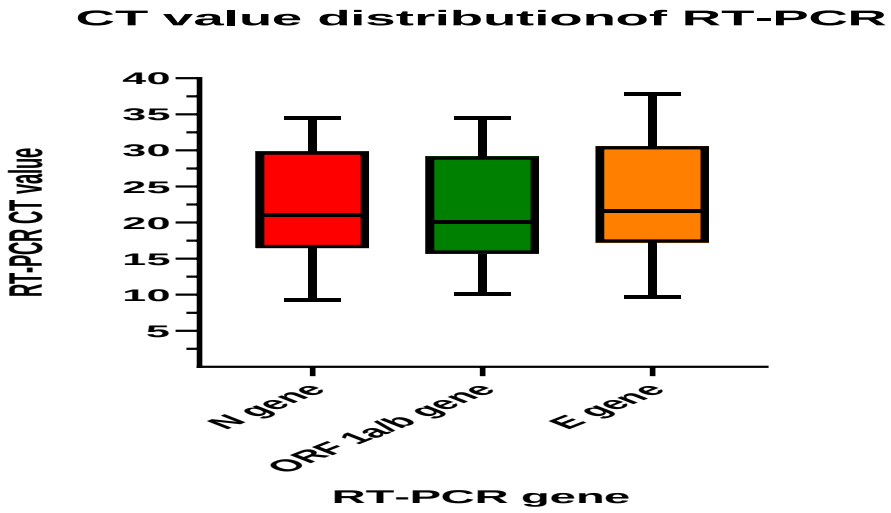


Figure 4.1: CT value distribution of RT-PCR in all the positive cases. The middle horizontal line inside the box denotes the Mean. The lower and upper Whiskers represent minimum and maximum CT value distribution. * P value < 0.05 was calculated using Mann-Whitney U test.

4.5 CT value distribution of rtRT-PCR target genes in comparison to RAT positive and negative result

CT values of each target gene were studied independently to further demonstrate the performance of antigen test results. For ORF 1ab gene, in the RAT positive had median CT value of 17.0, while in RAT negative had a median value of 30.45. For N gene, RAT positive had median CT value of 17.89 and the RAT negative had median CT value of 30.85. For E gene, the cases detected by RAT had median CT value of 18.71 and cases missed by RAT had a median CT value of 31.69. Figure 4.2 demonstrate the box plot graph of ORF 1ab gene, N gene and E gene with CT value in RAT positive and negative test results. The statistical significant difference between the two group that is RT-PCR +/ RAT + and RT-PCR +/ RAT – for individual genes with p value <0.0001 was observed.

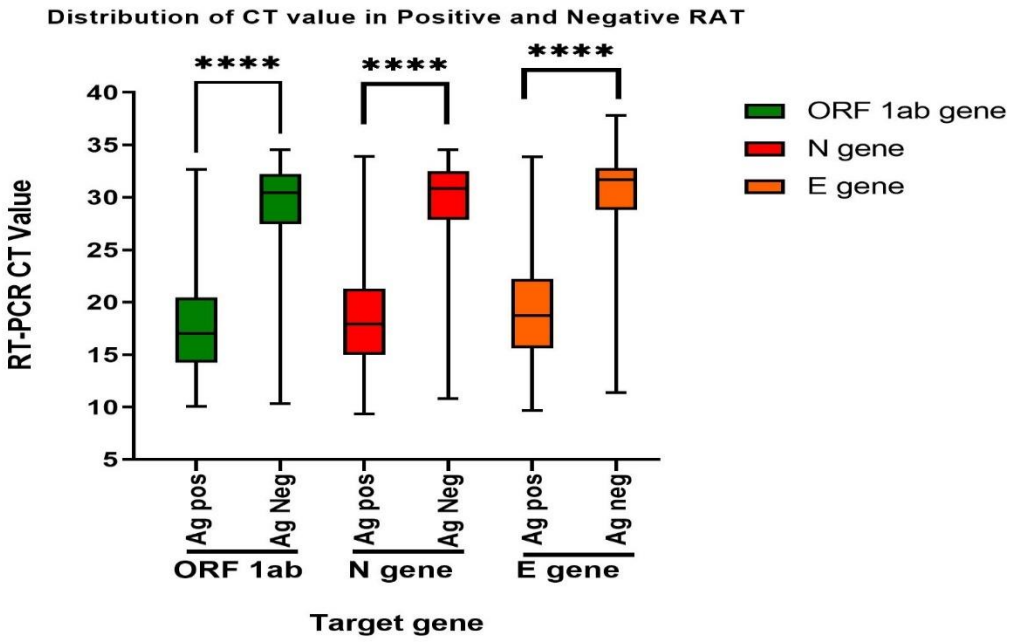


Figure4.2: CT value distribution of rtRT-PCR target genes with comparison to RAT positive and negative. The middle horizontal line inside the box denotes the Mean. The lower and upper Whiskers represent minimum and maximum CT value distribution. **** represents p value < 0.0001 was calculated using Mann-Whitney U test.

4.6 Sequence analysis and Phylogenetic Analysis

The 4 sample were send for whole genome Sequencing to CSIR-Institute of Genomics and Integrative, Delhi through NPHL and was submitted to GISAID and Accession Id was assign EPI_ISL_2674082, EPI_ISL_2674083, EPI_ISL_2674084 and EPI_ISL_2674106. Then blast result was obtained from GISAID. The maximum likelihood phylogenetic tree constructed by aligning the sequence of tested sample related samples and reference sequence from GISAID.

Sequence 1: hCoV19/Nepal/S53/2021|EPI_ISL_2674082|20210528

There were 100 related genomes found (at a distance of 4 or less from the uploaded sequence). The minimum quality of the matches was 0.9. Amongst the related genomes, the most frequent country was USA (30% of genomes), the most frequent lineage was B.1.617.2 (31% of genomes), and 80.2% of the related genomes were from samples collected between 2021-05-19 and 2021-12-08.

Virus name:	hCoV-19/Nepal/S-53/2021
Accession ID:	EPI_ISL_2674082
Type:	Betacoronavirus
Clade:	GK
Pango Lineage:	Unassigned (Pango 4.1.1 PLEARN-v1.11) - last assigned as AY.1 (Pango v.4.1 PANGO-v1.11), Delta (B.1.617.2-like) +K417N (Scorpio)
AA Substitutions:	Spike D614G, Spike D950B, Spike K417N, Spike L452R, Spike P681R, Spike T19R, Spike T478K, Spike W258L, M I82T, N D63G, N G215C, N R41W, N R203M, NS3 S26L, NS3 T151I, NS7a T120I, NS7b T40I, NSP3 A488S, NSP3 P1228L, NSP3 P1469S, NSP4 S34F, NSP4 T492I, NSP4 V167L, NSP6 T77A, NSP12 G671S, NSP12 P323L, NSP12 T141I, NSP13 P77L, NSP14 A394V
Variant:	VOC Delta GK (B.1.617.2+AY.*) first detected in India

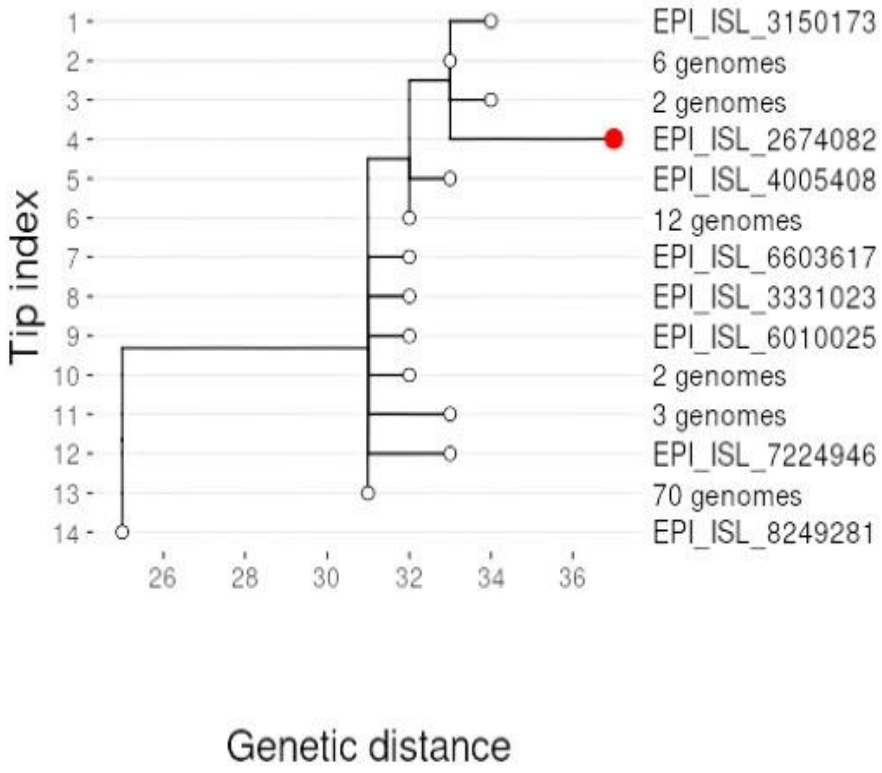


Figure 4.3: Genetic distance determination of S-53

Sequence 2: hCoV19/Nepal/S54/2021|EPI_ISL_2674083|20210529

There were 133 related genomes found (at a distance of 6 or less from the uploaded sequence). The minimum quality of the matches was 0.901. Amongst the related genomes, the most frequent country was USA (46.6% of genomes), the most frequent lineage was AY.75 (82% of genomes), and 82% of the related genomes were from samples collected between 2021-04-20 and 2021-08-04.

Virus name:	hCoV-19/Nepal/S-54/2021
Accession ID:	EPI_ISL_2674083
Type:	Betacoronavirus
Clade:	GK
Pango Lineage:	AY.75 (Pango v.4.1 PLEARN-v1.11), Delta (B.1.617.2-like) (Scorpio)
AA Substitutions:	Spike A222V, Spike D614G, Spike D950B, Spike L452R, Spike N1187I, Spike P681R, Spike T19R, Spike T478K, M I82T, N D63G, N R203M, NS3 S26L, NS7a C23F, NS7a T120I, NS7a V82A, NSP3 P822L, NSP4 A446V, NSP6 T181I, NSP6 V149A, NSP12 G671S, NSP12 P323L, NSP13 P77L, NSP14 N176S
Variant:	VOC Delta GK (B.1.617.2+AY.*) first detected in India

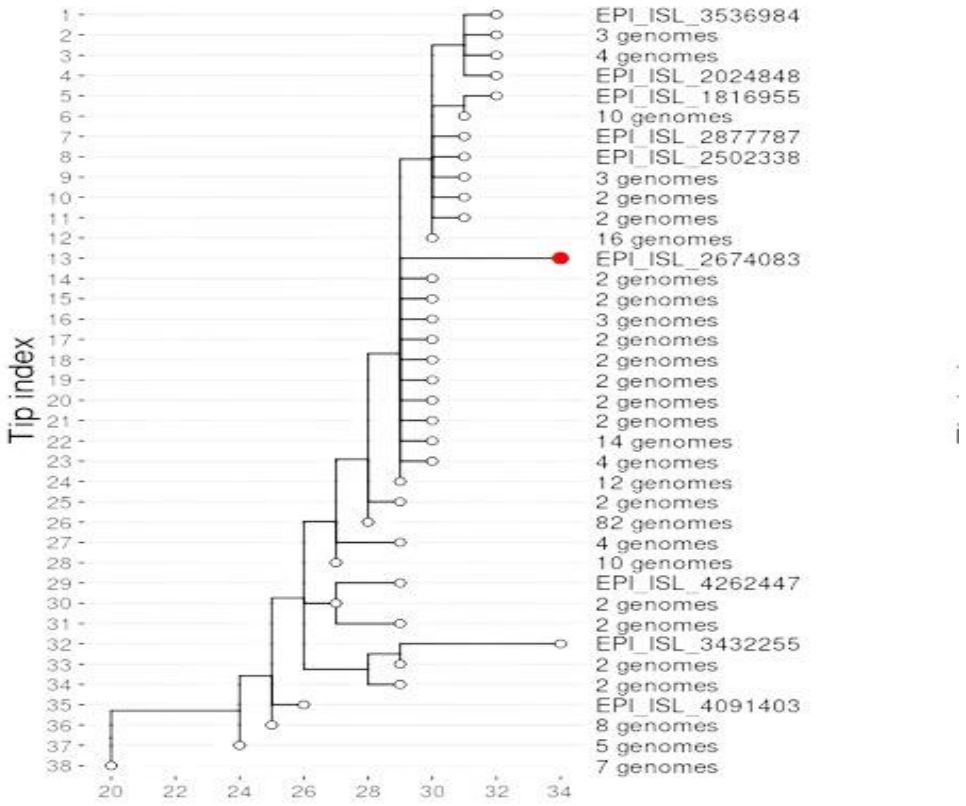


Figure 4.4: Genetic distance determination of S-54

Sequence 3: hCoV19/Nepal/S55/2021|EPI_ISL_2674084|20210601

There were 137 related genomes found (at a distance of 3 or less from the uploaded sequence). The minimum quality of the matches was 0.902. Amongst the related genomes, the most frequent country was USA (40.9% of genomes), the most frequent lineage was AY.75 (70.1% of genomes), and 81.1% of the related genomes were from samples collected between 2021-04-21and 2021-08-13.

Virus name:	hCoV-19/Nepal/S-55/2021
Accession ID:	EPI_ISL_2674084
Type:	Betacoronavirus
Clade:	GK
Pango Lineage:	Unassigned (Pango 4.1.1 PLEARN-v1.11) - last assigned as AY.75 (Pango v.4.1 PANGO-v1.11), Delta (B.1.617.2-like) (Scorpio)
AA Substitutions:	Spike A222V, Spike D614G, Spike D950B, Spike G142D, Spike L452R, Spike P681R, Spike T19R, Spike T478K, M I82T, N D63G, N R203M, NS3 S26L, NS7a V82A, NSP3 P822L, NSP4 A446V, NSP6 T181I, NSP6 V149A, NSP12 G671S, NSP12 P323L, NSP13 P77L
Variant:	VOC Delta GK (B.1.617.2+AY.*) first detected in India

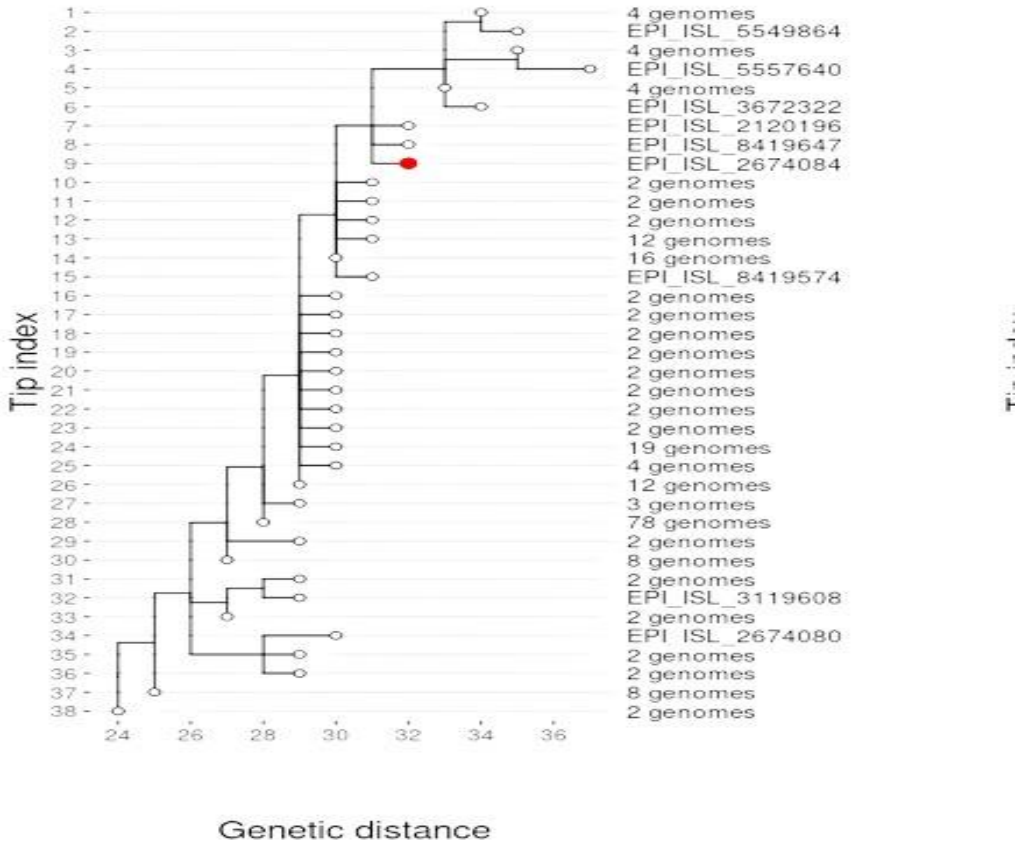


Figure 4.5: Genetic distance determination of S-55

Sequence 4: hCoV19/Nepal/S77/2021|EPI_ISL_2674106|20210530

There were 100 related genomes found (at a distance of 3 or less from the uploaded sequence). The minimum quality of the matches was 0.911. Amongst the related genomes, the most frequent country was USA (74% of genomes), the most frequent lineage was AY.44 (49% of genomes), and 80.9% of the related genomes were from samples collected between 2021-07-30 and 2021-11-27.

Virus name:	hCoV-19/Nepal/S-77/2021
Accession ID:	EPI_ISL_2674106
Type:	Betacoronavirus
Clade:	GK
Pango Lineage:	AY.112 (Pango v.4.1 PLEARN-v1.11), Delta (B.1.617.2-like) (Scorpio)
AA Substitutions:	Spike D614G, Spike D950B, Spike L452R, Spike P681R, Spike T19R, Spike T95I, Spike T478K, Spike V1104L, M I82T, N D63G, N G215C, N R203M, NS3 G172C, NS3 S26L, NS7a T120I, NS7a V82A, NS7b T40I, NSP1 D33G, NSP3 A488S, NSP3 P1228L, NSP3 P1469S, NSP4 T492I, NSP4 V167L, NSP6 T77A, NSP12 G671S, NSP12 P323L, NSP13 P77L, NSP14 A394V, NSP16 P236S
Variant:	VOC Delta GK (B.1.617.2+AY.*) first detected in India

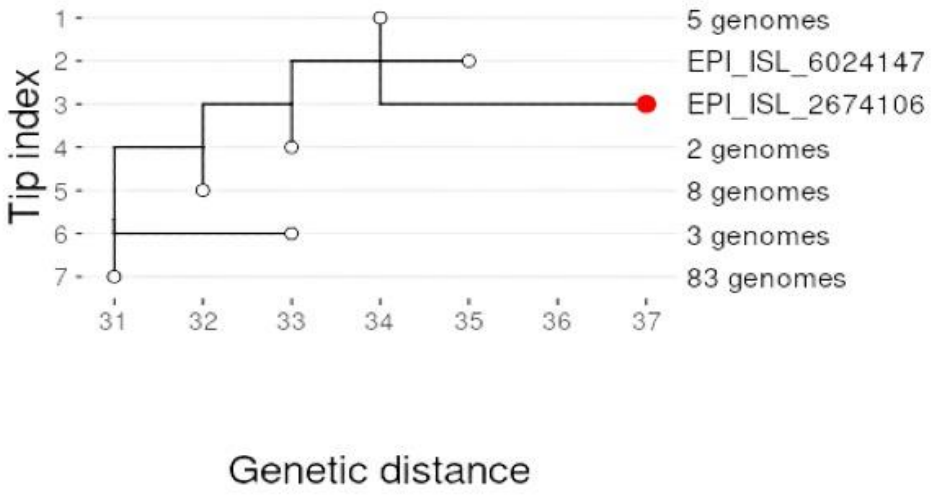


Figure 4.6: Genetic distance determination of S-77

Chapter V

Discussion

The ongoing pandemic of COVID-19 with viral diversity is continually challenging the substantial need of rapid testing kits for the detection of infectiousness as new SARS-CoV-2 strain emerges and spread out globally. An early diagnosis of COVID-19 will not only prevent the potential outbreak of new strain but also help in isolating and control the transmission because social distancing, home quarantine and lockdown cannot be the long term remedy. So, to prevent the outbreak as a presumptive public health measure, regular basis testing or massive screening of symptomatic, doubtful as well as asymptomatic should be done. For the detection of new variant lineages, it is a crucial and desperate to validate the rapid diagnostic techniques by regular evaluation to challenge the assay to detect infection in different health care setting and populations for optimizing the test protocol in every condition.

In context of 3rd world countries like Nepal, with one of the lowest GDPs in world and weak health care system the surveillance and diagnosis of SARS-CoV-2 is a serious public health challenge. As RT-PCR is the "gold standard" for the detection of SARS-CoV-2 but it is required sophisticated equipment, experienced staff, costly facilities, complex and time consuming protocol hours' of turnaround time (due to high sample load) making them unpractical for laboratories with limited resources, mass screening, contract tracing, disease surveillance and in case of emergency. To eliminate the complex and time consuming protocol along with the sophisticated equipment and experienced staff, Rapid Antigen test can be the potential candidate with easy procedure giving result within 30 minutes and easy result interpretation.

As of 5th June 2022, 979199 (17.13%) was positive for rtRT-PCR out of 5715414 test performed in Nepal. Of total 1200971 RAT performed, 140261 (11.68%) turned out to be positive (MOHP Nepal) In comparison to MOHP Nepal data, we have slightly higher positive rate in RAT (21.5%) and higher positive result for rtRT-PCR (44.23%) supported by Ghimire et.al (41.7%). This higher positivity rate in comparison to national data is expected due to the symptomatic population,

disease patients and close contact person visiting hospital for diagnosis of infection rather than for screening.

Most of the study from different part of world illustrated higher SARS-CoV-2 infection in males compare to female, this gender bias is also present in our study with 52.9% and 47.13% in rtRT-PCR whereas 54.45% and 45.55% in RAT for male and female respectively (He et. al.2020, Scohy et. al.,2020, & Mandal et. al., 2022). Our study illustrated the highest prevalence rate on age group 30-40 years followed by age group 20-30 years which is in correspondence with the result obtained by Undurraga et al, 2020. In contrast, similar study from Nepal reported the higher prevalence rate at 20-30 years followed by 30-40 years (Mandal et al.,2022 and Sharma et al).

Comparison of RAT with rtRT-PCR, there is a major difference in analytical sensitivity of the assay. The sensitivity of antigen test ranges from 30% to 40% lower than the RT-PCR depending on the sample from the symptomatic or asymptomatic subjects (Cochrane library 2021). The similar result was obtained from our study with 66.01% analytical sensitivity. This lower analytical sensitivity has advantage of unlikely detecting the leftover viral RNA after the active infection who are on recovery state (Mina et al 2020) and reduces the probability of unnecessary isolation and Quarantine of suspect along with testing load. (Pilarowski et al 2021, Ford et al 2021).

Overall sensitivity of our test was 93.5%, 93.7%, 82.9% for CT value <20, <25 and <30 and specificity of 97.8% with almost perfect agreement between both tests (k= 0.917, k= 0.918 and k= 809 respectively and p value <0.0001) which fulfilled the WHO criteria of ≥80% sensitivity and specificity of ≥ 97% on comparison to PCR indicating the reliability for the diagnosis of SARS-CoV-2. In contract, CT value <35 has the sensitivity of 64.2% which indicating the RAT are unable to detect the SARS-CoV-2 antigen having low viral load and low CT value (Mandal et. al 2022 &Chaimayo et.al.2020, Aoki et.al. 2021, Mak et.al. 2020) which also have the moderate agreement with the rtRT-PCR (k=0.596).

The clinical sensitivity reported by the manufacturer for SD Biosensor was 84.97% and clinical specificity of 98.94%. In our study we found much high

sensitivity for CT <20 (94.2%) and CT <25 (93.5%) with almost perfect agreement (k=0.912 & k=0.906) and almost similar value for CT <30 (81.4%) with substantial agreement (k=0.783). In case of Panbio, manufacturer reported the sensitivity of 91.4% and specificity of 99.8% which was correspondence with our data of sensitivity CT value <20 (92.9%) and <25 (93.9%) with almost perfect agreement Cohen's kappa value (k=0.921 & k=0.926) with rtRT-PCR and specificity of 98.3% for all 4 class of CT value. Whereas in CT <30 sensitivity was slightly low (83.8%) and dramatically very low (63.8%) in CT value <35. The results obtained in our study are comparable with studies based on rapid antigen test across the world. From previously published data, we found that test sensitivity was directly related to the viral load showing up to 100% result in sample with high viral load (CT value <25) and gradually decreases as the viral load decreases (CT value 25-<30, 30- <35 and >35) from 95% to 22.2 % respectively (Nalamushi et al, Zhang et al, Kruttgen et. al., Lefever et. al) despite the used of different genetic target in RT-PCR and automated result reading used for rapid antigen test (Lefever et. al.). From different studies along with our study we can conclude that regardless the genetic target in RT-PCR, automation process in antigen test and brand of antigen kit used the sensitivity of RAT kit for detection of SARS-CoV-2 decreases with the decrease in the viral load. In our setting RAT are frequently negative in rtRT-PCR positive sample with CT value > 30 which is supported by other studies including (Platten et. al & Paul et.al).

The diagnostic accuracy in our study for SD Biosensor was 95.8%, 95.3%, 89.2% and 79.2% with Cohens kappa 0.91, 0.91, 0.78 and 0.59 respectively displaying the almost perfect agreement with CT value <20 and <25, substantial agreement for CT <30 and moderate agreement for overall (CT <35). In case of Panbio, diagnostic accuracy was 96.5%, 96.4%, 91.3% and 79.3% with Cohen kappa value 0.92, 0.93, 0.83 and 0.6 with similar agreement for CT value <20 and <25 and overall (<35) of SD Biosensor whereas contrast with almost perfect agreement in case of CT value <30. Youden's J statistic (also called Youden's index) is a single statistic that captures the dichotomous diagnostics test performance. For test meeting the empirical benchmark for being administered for diagnostic purpose

it should be more than 0.5 which is obtained in our study with Youden index of 0.912, 0.905, 0.783 and 0.594 in overall study.

Statistically significant results were obtained between RAT positive and negative test when compared with CT values of individual target genes obtained from RT-PCR. A study conducted by Tregiarri et al. and Mandal et. al. too demonstrated the statistically significant result ($p < 0.05$) when Ag-RDT data were compared to that of CT values obtained.

In our study discordant result were obtained between RAT and rtRT-PCR with 13 (1.0%) False positive and 256 (19.77%) false negative. This discrepancy on result is unknown, 62.9% (161/256) of false negative cases had CT ≥ 30 and 25.78% (66/256) have CT ≥ 25 – 30, which indicate the low viral load elucidate the false negative result obtained (Kanji JN et al.2020). According to Robert Koch institute, the individuals with CT value ≥ 30 can be considered non-infectious (Laferal et.al 2020). As the antigen detection in RAT and RNA amplification in RT-PCR have different mechanism and works on different principle where RT-PCR requires gene amplification and RAT simply works on antigen-antibody complex formation which might be the reason for discrepancy between test result of RT-PCR and RAT (Torjesen et. al., 2021). As RAT works on the principle of Antigen-antibody complex formation the pre-zone and post-zone phenomenon might also have masked the detection of high viral load sample and low viral load sample.

According to Routsias et. al., CT value of lower range had higher change of RAT positive result. In our study CT value median was lower in RAT positive (17-19) as compared to CT value was higher in RAT negative sample (31.8) which suggest that there is a high probability RAT give true positive result if CT value is <25 while probability of getting negative result increases when CT value > 25. Similar result of higher CT value in negative RAT test was observed by Young et. al., Mandal et. al. our study also indicated that true positive rate decreases with subsequent increase in the CT value > 25 supported by Takeda et. al. and Kahn et. al subsequently lowering the performance of RAT (Pena et. al. 2021).

Four random samples send for sequencing and submitted to GISAID all of them are VOC Delta GK (B.1.617.2 + AY*) first detected in India. Among them one was

Delta (B.1.617.2-like) + K417N known as Delta plus other three were classified as Delta variant. The related genome to our sequence submitted in GISAID, most frequent genome was found to be from USA after BLAST. Although being Delta variant S-54 and S-55 have related genome and S-53 and S-77 have related genome. All the 4 sequence have common Amino acid substitution like Spike D614G, D950B, L452R, P618R, T19R, T487K in spike protein, M I82T in membrane protein and N D63G and R203M in Nucleo-capsid protein. As compared to reference sequence from Wuhan EPI_ISI_402124 the genetic distance was found to be 36, 34, 32 and 37 for S-53, S-54, S-55 and S-77 respectively.

To our knowledge this is the first study on performance of RAT assay conducted at point of care for 1295 sample and comparing the performance of two kits approved by WHO in the context of Nepal. Both the kits yield an excellent specificity and fairly good overall sensitivity of 64.9% and 63.8% even in the case of low viral load (CT value <35), the latter drastically increase in sensitivity as the viral load increases from 82% to 93.9% with the subsequent increase in the CT value from <30 to <20. This numbers are spectacular and appealing compare to manufacturer as the manufacturer might have incorporated a sample with high viral load in larger fraction.

Limitation of Study

1. Quantitative estimation of RNA was not done on the sample to note down the Viral load.
2. Virus culture was not due to lack of fund and resources.
3. The Clinical detail of symptoms was not noted down properly.

CHAPTER-VI

Conclusion

In summary we found that both the Rapid antigen kit perform well as a point of care testing (POCT) for the early diagnosis and recognition of SARS-CoV-2 infection in health care center. Most important finding from our study is that RT-PCR proven SARS-CoV-2 infection (CT> 30) and negative by RAT are not likely to be infectious. The rapid results within 30 minutes, inexpensive procedure, no requirement of sophisticated equipment, easy to perform and result interpretation makes RAT valuable and potential candidate for the alternative procedure for diagnosis of SARS-CoV-2in the patients with high viral load and help in reducing transmission by early diagnosis and mass screening in the community basis.

Recommendation

1) Comparative study between the rapid antigen test and rtRT-PCR can be done in community level with mass sample for proper validation and get ready for next outbreak.
2) Other Rapid antigen kit can be compared in future along with updated product in future.
3) Proper clinical detail along with days of infectious should be noted down for proper evaluation.

Chapter VII

Reference

Adil MT, Rahman R, Whitelaw D, Jain V, Al-Taan O, Rashid F, Munasinghe A,Jambulingam P (2021). SARS-CoV-2 and the Pandemic of COVID-19. Postgrad Med J; **97** :110–116. doi:10.1136/postgradmedj-2020-138386

Aoki K, Nagasawa T, Ishii Y et al., "Evaluation of clinical utility of novel coronavirus antigen detection reagent, espline® SARS-COV-2," *Journal of Infection and Chemotherapy*, vol. 27, 2021.

Aryal M, Shergill IS, Williamson M, Gommersall L, Aryal N & Patel HR (2005). "Basic principle of real-time quantitative PCR. Expert Rev. Mol. Diagn, 5(2).

Basnet BB, Bishwakarma K, Pant RR, Dhakal S, Pandey N, Gautam D, Ghimire A and Basnet TB (2021). Combating the COVID-19 Pandemic: Experiences of the First Wave From Nepal. Front. Public Health 9:613402. doi: 10.3389/fpubh.2021.613402

Bastola, A., Sah, R., Rodriguez-Morales, A. J., Lal, B. K., Jha, R., Ojha, H. C., Shrestha, B., Chu, D., Poon, L., Costello, A., Morita, K., & Pandey, B. D. (2020). The first 2019 novel coronavirus case in Nepal. The Lancet. *Infectious diseases, 20(3)*, 279–280. https://doi.org/10.1016/S1473-3099(20)30067-0

Cennimo DJ (2022). Coronavirus Disease 2019 (COVID-19). Medscape, https://emedicine.medscape.com/article/2500114-print

Centers for Disease Control and Prevention CDC (2021). COVID-19 (coronavirus disease): people with certain medical conditions. Available at: https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/people-with-medical-conditions.html. Accessed june 2, 2022.

Chaimayo C, Kaewnaphan B, Tanlieng N, Athipanyasilp N Sirijatuphat R, Chayakulkeeree M Angkasekwinai N Suttent R, Puangpunngam N, tharmviboonsri T, Pongraweewan O, Chuthapisth S, Sirivatanauksorn Y kantalamalakul W & Horthongkham N (2020). "Rapid SARS-COV-2 antigen detection assay in comparison with real-time rt-pcr assay for laboratory diagnosis of COVID-19 in Thailand," *Virology Journal*, vol. 17, no. 1, p. 177, 2020.

Chan JF-W, Yip CC-Y, To KK-W, Tang TH-C, Wong SC-Y, Leung K-H, Fung AY-F, Ng AC-K, Zou Z, Tsoi H-W, Choi GK-Y, Tam AR, Cheng VC-C, Chan K-H, Tsang OT-Y, Yuen K-Y (2020) Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse transcription-polymerase chain reaction assay validated in vitro and with clinical specimens. J Clin Microbiol. doi: 10.1128/JCM.00310-20

Chinese National Institute for Viral Disease Control and Prevention. In: Primer Probes Detect. Novel Coronavirus RT-PCR. http://ivdc.chinacdc.cn/kyjz/202001/t20200121_211337.html

CWBIO (2020). Product- Viral DNA/RNA Kit. (online) https://www.cwbiosciences.com/uploads/20200312/605e3ad1016fee1c644ce32154eef7cd.pdf Accessed 21 April 2022.

Dinnes J, Deeks JJ, Adriano A, Berhane S, Davenport C, Dittrich S, Emperador D, Takwoingi Y, Cunningham J, Beese S, Dretzke J, Ferrante di Ru&ano L, Harris IM, Price MJ, Taylor-Phillips S, Hoo L, Leeflang MMG, Spijker R, Van den Bruel A

(2020). Rapid, point-of-care antigen and molecular-based tests for diagnosis of SARS-CoV-2 infection. *Cochrane Database of Systematic Reviews*, Issue 8. Art. No.: CD013705. DOI: 10.1002/14651858.CD013705.

Esakandari, H., Nabi-Afjadi, M., Fakkari-Afjadi, J., Farahmandian, N., Miresmaeili, S. M., & Bahreini, E. (2020). A comprehensive review of COVID-19 characteristics. *Biological procedures online, 22,* 19. https://doi.org/10.1186/s12575-020-00128-2

Fehr, A. R., & Perlman, S. (2015). Coronaviruses: an overview of their replication and pathogenesis. *Methods in molecular biology (Clifton, N.J.)*, *1282*, 1–23. https://doi.org/10.1007/978-1-4939-2438-7_1

Ford L, Lee C, Pray IW, Cole D, Bigouette JP, Abedi GR, Bushman D, Delahoy MJ, Currie DW, Cherney B, Kirby MK, Fajardo GC, Caudill M, Langolf K, Kahrs J, Zochert T, Kelly P, Pitts C, Lim A, Aulik N, Tamin A, Harcourt JL, Queen K, Zhang J, Whitaker B, Browne H, Medrzycki M, Shewmaker PL, Bonenfant G, Zhou B, Folster JM, Bankamp B, Bowen MD, Thornburg NJ, Goffard K, Limbago B, Bateman A, Tate JE, Gieryn D, Kirking HL, Westergaard RP, Killerby ME; CDC COVID-19 Surge Laboratory Group (2021). "Epidemiologic Characteristics Associated With Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigen-Based Test Results, Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) Cycle Threshold Values, Subgenomic RNA, and Viral Culture Results From University Testing." Clin Infect Dis. 15;73(6):e1348-e1355. doi: 10.1093/cid/ciab303. PMID: 33846714; PMCID: PMC8083323.

Foundation for Innovative New Diagnostics. FIND evaluation of Abbott Panbio COVID-19 Ag Rapid Test Device. External Report Version 21, 10 December 2020. Geneva: Foundation for Innovative New Diagnostics; 2020.

Foundation for Innovative New Diagnostics. FIND evaluation of Coris BioConcept COVID-19 Ag Respi-Strip. External Report Version 12, 10 December 2020. Geneva: Foundation for Innovative New Diagnostics; 2020.

Foundation for Innovative New Diagnostics. FIND evaluation of Shenzhen Bioeasy Biotechnology Co. Ltd. 2019-nCoV Ag Rapid Test Kit (Fluorescence). External Report Version 10, 11 February 2021. Geneva: Foundation for Innovative New Diagnostics; 2021.

Ghimire P, Bhandari I, Bhandari R, Bhandari B (2021). "Comparison of COVID-19 Antigen Test and HRCT Chest with RT- PCR Test for Diagnosis of COVID-19."Journal of College of Medical Sciences-Nepal, Vol(17);3 DOI: 10.3126/jcmsn.v17i3.39623

Goldsmith, C. S., Tatti, K. M., Ksiazek, T. G., Rollin, P. E., Comer, J. A., Lee, W. W., Rota, P. A., Bankamp, B., Bellini, W. J., & Zaki, S. R. (2004). Ultrastructural characterization of SARS coronavirus. *Emerging infectious diseases, 10(2),* 320–326. https://doi.org/10.3201/eid1002.030913

Guo YR, Cao QD, Hong ZS, Tan YY, Chen SD, Jin HJ, Tan KS, Wang DY,& Yan Y (2020). The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. Military Medical Research, 7:11 https://doi.org/10.1186/s40779-020-00240-0

Gupta A, Singla M, Bhatia H, Sharma V (2020). Lockdown-the only solution to defeat COVID-19. Int J Diabetes Dev Ctries. 6:1–2. doi: 10.1007/s13410-020-00826-3

Harrison AG, Lin T, Wang P (2020). Mechanisms of SARS-CoV-2 Transmission and Pathogenesis. Trends Immunol. 2020 Dec;41(12):1100-1115. doi: 10.1016/j.it.2020.10.004. Epub 2020 Oct 14. PMID: 33132005

He X, Lau EHY, Wu P, Deng X, Wang J,Hao X, Lau YC, Wong JY, Guan Y, Tan X, Mo X, Chen Y, Liao B, Chen W, Hu F, Zhang Q, Zhong M, Wu Y, Zhao L, ZHAng F, Cowling BJ, Li F & Leung GM (2020). "Temporal dynamics in viral shedding and transmissibility of COVID-19," *Nature Medicine*, vol. 26, no. 5, pp. 672–675.

Higuchi R, Fockler C, Dollinger G & Watson R (1993). "Kinetic PCR analysis: real time monitoring of DNA amplification reactions. Biotechnolgy (Nature Publishing Company), 11(9), 1026-30.

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., Cheng, Z., Yu, T., Xia, J., Wei, Y., Wu, W., Xie, X., Yin, W., Li, H., Liu, M., Xiao, Y., … Cao, B. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet (London, England),395(10223),497–506. https://doi.org/10.1016/S0140-6736(20)30183-5

Islam N, Sharp SJ, Chowell G (2020). Physical distancing interventions and incidence of coronavirus disease 2019: natural experiment in 149 countries. BMJ. 370:27–43. doi: 10.1136/bmj.m2743

Jackson CB, Farzan M, Chen B, Choe H (2022). "Mechanisms of SARS- CoV-2 entry into cells." Nature Reveiws; Molecular cell Biology; vol (23),1-18

Kahn M., Schuierer, L., Bartenschlager C., Zellmer S, Frey R, Freitag M, Dhillon C, Heier M, Ebigbo A, Denzel C, Temizel S, Messmann H, Wehler M, Hoffmann R, Kling E & Rommele C *(2021).* "Performance of antigen testing for diagnosis of COVID-19: a direct comparison of a lateral flow device to nucleic acid amplification based tests." *BMC Infect Dis* **21,** 798. https://doi.org/10.1186/s12879-021-06524-7 .

Kanji J.N., Zelyas,N, MacDonald C, Pabbaraju K, khan MN, Prasad A, Hu J, Diggle M, Berenger BM, Tipples G (2021*).* False negative rate of COVID-19 PCR testing: a discordant testing analysis. *Virol J* **18,** 13. https://doi.org/10.1186/s12985-021-01489-0

Lai, M. M., & Cavanagh, D. (1997). The molecular biology of coronaviruses. *Advances in virus research, 48,* 1–100. https://doi.org/10.1016/S0065-3527(08)60286-9

Lefever S, Indevuyst C, Cuypers L, Dewaele K, Yin N, Cotton F, Padalko E, Oyaert M, Descy J, Cavalier E, Van Ranst M, André E, Lagrou K, Vermeersch P; Royal Belgian Society of Laboratory Medicine(2021). Comparison of the Quantitative DiaSorin Liaison Antigen Test to Reverse Transcription-PCR for the Diagnosis of COVID-19 in Symptomatic and Asymptomatic Outpatients. J Clin Microbiol. Jun 18;59(7):e0037421. doi: 10.1128/JCM.00374-21. Epub 2021 Jun 18.

Lu G, Razum O, Jahn A, Zhang Y, Sutton B, Sridhar D, Ariyoshi K, Seidlein Lv & Muller O (2021). COVID-19 in Germany and China: mitigation versus elimination strategy. Glob. Health Action; 14:1875601. doi: 10.1080/16549716.2021.1875601

Maccura (2020). Product- SARS-CoV-2 Fluorescent PCR Kit. (online). https://www.maccura.com/en/product/vAMA7UmFXAE-.html. Acces 23 Apr 2022

Mak GC, Cheng PK, Lau SS, Wong KK, Lau CS, Lam ET, Chan RC, Tsang DN. Evaluation of rapid antigen test for detection of SARS-CoV-2 virus. J Clin Virol. 2020 Aug;129:104500. doi: 10.1016/j.jcv.2020.104500. Epub 2020 Jun 8.

Mandal DK, Bhattarai BR, Pokhrel S, Chhusyabaga M, Bhandari P, Bhatta MP, Marhatta SB (2022). "Diagnostic Performance of SARS-CoV-2 Rapid Antigen Test in relation to RT-PCR Cq Value." Hindawi Advances in Virology Volume 2022, Article ID 9245248, 8 pageshttps://doi.org/10.1155/2022/9245248

Mariano G, Farthing RJ, Lale-Farjat SLM and Bergeron JRC (2020) Structural Characterization of SARS-CoV-2: Where We Are, and Where We Need to Be. Front. Mol. Biosci. 7:605236. doi: 10.3389/fmolb.2020.605236

Mina MJ, Parker R, Larremore DB (2020). "Rethinking COVID-19 test sensitivity—a strategy for containment." N Engl J Med.;383(22):e120. pmid:32997903

Nalumansi A, Tom Lutalo, John Kayiwa, Christine Watera, Stephen Balinandi, Jocelyn Kiconco, Joweria Nakaseegu, Denis Olara, Emmanuel Odwilo, Jennifer Serwanga, Bernard Kikaire, Deogratius Ssemwanga, Susan Nabadda, Isaac Ssewanyana, Diane Atwine, Henry Mwebesa, Henry Kyobe Bosa, Christopher Nsereko, Matthew Cotten, Robert Downing, Julius Lutwama, Pontiano Kaleebu (2021). "Field evaluation of the performance of a SARS-COV-2 antigen rapid diagnostic test in uganda using nasopharyngeal samples," *InternationalJournal of Infectious Diseases*, vol. 104, pp. 282–286.

Naqvi AAT, Fatima K, Mohammad T, Fatima U, Singh IK, Singh A, Atif SM, Hariprasad G, Hasan GM, Hassan MI. Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. Biochim Biophys Acta Mol Basis Dis. 2020 Oct 1;1866(10):165878. doi: 10.1016/j.bbadis.2020.165878.

Neuman, B. W., Kiss, G., Kunding, A. H., Bhella, D., Baksh, M. F., Connelly, S., Droese, B., Klaus, J. P., Makino, S., Sawicki, S. G., Siddell, S. G., Stamou, D. G., Wilson, I. A., Kuhn, P., & Buchmeier, M. J. (2011). A structural analysis of M protein in coronavirus assembly and morphology. *Journal of structural biology, 174(1),* 11–22. https://doi.org/10.1016/j.jsb.2010.11.021

Nuthalapti N (2021) SARS-CoV-2Genome 10(2).199.

Pal M, Berhanu G, Desalegn C, & Kandi V (2020). Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2): An Update. Cureus, 12(3): e7423. DOI 10.7759/cureus.7423

Panbio (2020). Products- Abbott panbio COVID-19 Ag Rapid Test Device. https://www.globalpointofcare.abbott/en/product-details/panbio-COVID-19-ag-antigen-test-ww.html. Accessed 11 March 2022.

Pang J, Wang MX, Ang IYH, Tan SHX, Lewis RF, Chen JI-P, Gutierrez RA, Gwee SXW, Chua PEY, Yang Q, Ng XY, Yap RK, Tan HY, Teo YY, Tan CC, Cook AR, Yap JC-H, Hsu LY (2020) Potential Rapid Diagnostics, Vaccine and Therapeutics for 2019 Novel Coronavirus (2019-nCoV): A Systematic Review. J Clin Med 9. doi: 10.3390/jcm9030623

Panthee B, Dhungana S, Panthee N, Paudel A, Gyawali S, Panthee S. COVID-19: the current situation in Nepal. New Microbes New Infect. (2020) 37:100737. doi:10.1016/j.nmni.2020.100737

Paul G, Plecko T, Sethi S, Schilling T, Wienand O, Jürgensen J.S, Menzel C.U(2021). "Klinische Performance eines neuen. "SARS-CoV-2-Antigen-Tests in der Notaufnahme eines Maximalversorgers." Epidemiol. Bull., 3, 13–18.

Peña M, Ampuero M, Garcés C, Gaggero A, García P, Velasquez MS, Luza R, Alvarez P, Paredes F, Acevedo J, Farfán MJ, Solari S, Soto-Rifo R, Valiente-Echeverría F(2021). Performance of SARS-CoV-2 rapid antigen test compared with real-time RT-PCR in asymptomatic individuals. Int J Infect Dis. Jun;107:201-204. doi: 10.1016/j.ijid.2021.04.087. Epub 2021 May 1. PMID: 33945868

Pfefferle S, Reucher S, Nörz D, Lütgehetmann M (2020) Evaluation of a quantitative RTPCR assay for the detection of the emerging coronavirus SARS-CoV-2 using a

high throughput system. Eurosurveillance 25:2000152. doi: 10.2807/1560-7917.ES.2020.25.9.2000152

Pilarowski G, Lebel P, Sara Sunshine, Jamin Liu, Emily Crawford, Carina Marquez, Luis Rubio, Gabriel Chamie, Jackie Martinez, James Peng, Douglas Black, Wesley Wu, John Pak, Matthew T Laurie, Diane Jones, Steve Miller, Jon Jacobo, Susana Rojas, Susy Rojas, Robert Nakamura, Valerie Tulier-Laiwa, Maya Petersen, Diane V Havlir, Joseph DeRisi, The CLIAHUB Consortium,(2021). "Performance Characteristics of a Rapid Severe Acute Respiratory Syndrome Coronavirus 2 Antigen Detection Assay at a Public Plaza Testing Site in San Francisco." *The Journal of Infectious Diseases*, Volume 223, Issue 7, Pages 1139–1144, https://doi.org/10.1093/infdis/jiaa802

Platten M, Hoffmann D, Grosser R, Wisplinghoff F, Wisplinghoff H, Wiesmüller G, Schildgen O, Schildgen V (2021). "SARS-CoV-2, CT-Values, and Infectivity—Conclusions to BeDrawn from Side Observations." Viruses 2021, 13, 1459. https://doi.org/10.3390/v13081459

Rahimi A, Mirzazadeh A, Tavakolpour S. Genetics and genomics of SARS-CoV-2: A review of the literature with the special focus on genetic diversity and SARS-CoV-2 genome detection. Genomics. 2021 Jan;113(1 Pt 2):1221-1232. doi: 10.1016/j.ygeno.2020.09.059.

Reusken CBEM, Broberg EK, Haagmans B, Meijer A, Corman VM, Papa A, Charrel R, Drosten C, Koopmans M, Leitmeyer K, ERLI-Net on behalf of E-L and (2020) Laboratory readiness and response for novel coronavirus (2019-nCoV) in expert laboratories in 30 EU/EEA countries, January 2020. Eurosurveillance 25:2000082. doi: 10.2807/1560- 7917.ES.2020.25.6.2000082

Routsias JG, Mavrouli M, Tsoplou P, Dioikitopoulou K, and Tsakris A (2021). "Diagnostic performance of rapid antigen tests (rats) for SARS-COV-2 and their efficacy in monitoring the infectiousness of COVID-19 patients," *Scientific Reports*, vol. 11,no. 1, Article ID 22863.

Safiabadi Tali SH, LeBlanc JJ, Sadiq Z, Oyewunmi OD, Camargo C, Nikpour B, Armanfard N, Sagan SM, Jahanshahi-Anbuhi S. (2021). Tools and techniques for severe acute respiratory syndrome coronavirus 2 (SARS-CoV- 2)/COVID-19 detection. Clin Microbiol Rev 34: e00228-20. https://doi.org/10.1128/CMR.00228-20.

Sahin AR, Erdogan A, Mutlu Agaoglu P, Dineri Y, Cakirci AY, Senel ME,Okyay RA & Tasdogan AM (2020). 2019 Novel Coronavirus (COVID-19) Outbreak: A Review of the Current Literature. EJMO; 4(1):1-7.

Scohy A, Anantharajah A, Bod´eus M, Kabamba-Mukadi K, Verroken A, and Rodriguez-Villalobos H (2020). "Low performance of rapid antigen detection test as frontline testing for COVID-19 diagnosis," *Journal of Clinical Virology*, vol. 129, Article ID 104455.

SD Biosensor (2020). Products-STANDARD Q COVID-19 Ag Test. https://www.sdbiosensor.com/product/product_view?product_no=236. Accessed 11 Mar 2022.

Sharma A, Tiwari S, Deb MK, Marty JL (2020). "Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2): a global pandemic and treatment strategies." *International Journal of Antimicrobial Agents 56; 106054*

Sharma K, Banstola A, and Parajuli RR (2021). "Assessment of COVID-19 pandemic in Nepal: a lockdown scenario analysis."*Frontiers in Public Health*, vol. 9, 302 pages.

Singhal T. (2020). A Review of Coronavirus Disease-2019 (COVID-19). *Indian journal of pediatrics, 87(4),* 281–286. https://doi.org/10.1007/s12098-020-03263-6

Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., Guan, Y., Rozanov, M., Spaan, W. J., & Gorbalenya, A. E. (2003). Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. *Journal of molecular biology, 331(5),* 991–1004. https://doi.org/10.1016/s0022-2836(03)00865-9

Takeda Y, Mori M, and Omi K, (2020). "Sars-Cov-2 QRT-PCR CT value distribution in Japan and possible utility of rapid antigen testing kit." https://www.medrxiv.org/content/10.1101/2020.06.16.20131243v1.full.pdf.

The Lancet (2020). COVID-19: too little, too late. Lancet; 395: P755. doi: 10.1016/S0140-6736(20)30522-5

Torjesen I (2021). "COVID-19: how the UK is using lateral flow tests in the pandemic," *BMJ*, vol. 372, p. n287.

Treggiari D, Piubelli C, Caldrer S, Mistretta M, Ragusa A, Orza P, Pajola B, Piccoli D, Conti A, Lorenzi C, Serafini V, Boni M, Perandin F. SARS-CoV-2 rapid antigen test in comparison to RT-PCR targeting different genes: A real-life evaluation among unselected patients in a regional hospital of Italy. J Med Virol. 2022 Mar;94(3):1190-1195. doi: 10.1002/jmv.27378. Epub 2021 Oct 14.

Trougakos IP, Stamatelopoulos K, Terpos E, Tsitsilonis OE, Aivalioti E, Paraskevis D, Kastritis E, Pavlakis GN, Dimpoulos MA (2021) "Insights to SARS-CoV-2 life cycle, pathophysiology, and rationalized treatments that target COVID-19 clinical complications". Lournal of Biomedical Science; *28:9* https://doi.org/10.1186/s12929-020-00703-5

Undurraga EA, Chowell G, and MizumotoK (2020). "COVID-19 case fatality risk by age and gender in a high testing setting in Latin America: chile, march-august 2020," *Infectious Diseases of Poverty; vo;(10); 1-11*

Wang, C., Horby, P. W., Hayden, F. G., & Gao, G. F. (2020). A novel coronavirus outbreak of global health concern. *Lancet (London, England), 395(10223),* 470–473. https://doi.org/10.1016/S0140-6736(20)30185-9

WHO COVID-19 Technical Guidance. https://www.who.int/emergencies/diseases/novelcoronavirus- 2019/technical-guidance/laboratory-guidance. Accessed 23 Apr 2020

Woo, P. C., Huang, Y., Lau, S. K., & Yuen, K. Y. (2010). Coronavirus genomics and bioinformatics analysis. *Viruses, 2(8), 1804–1820.* https://doi.org/10.3390/v2081803

Worldometer (2020). COVID-19 Coronavirus Pandemic. Available online at: https://www.worldometers.info/coronavirus/ (accessed January 15, 2021).

Worldometer (2020). Nepal Population. Available online at: https://www.worldometers.info/world-population/nepal-population/ (accessed Jan 15, 2021).

Worldometer (2022). COVID-19 Coronavirus Pandemic. Available online at: https://www.worldometers.info/coronavirus/ (accessed June 5, 2022).

Wu F, Zhao S, Yu B, Chen Y-M, Wang W, Song Z-G, Hu Y, Tao Z-W, Tian J-H, Pei Y-Y, Yuan M-L, Zhang Y-L, Dai F-H, Liu Y, Wang Q-M, Zheng J-J, Xu L, Holmes EC, Zhang Y-Z (2020) A new coronavirus associated with human respiratory disease in China. Nature 579:265–269. doi: 10.1038/s41586-020-2008-3

Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J., Sheng, J., Quan, L., Xia, Z., Tan, W., Cheng, G., & Jiang, T. (2020). Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. *Cell host & microbe, 27(3),* 325–328. https://doi.org/10.1016/j.chom.2020.02.001

Xu, H., Zhong, L., Deng, J., Peng, J., Dan, H., Zeng, X., Li, T., & Chen, Q. (2020). High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *International journal of oral science, 12(1),* 8. https://doi.org/10.1038/s41368-020-0074-x

Young S, Taylor SN, Cammarata CL, Varnado KG, Roger-Dalbert C, Montano A, Griego-Fullbright C, Burgard C, Fernandez C, Eckert K, Andrews JC, Ren H, Allen J, Ackerman R, Cooper CK. Clinical Evaluation of BD Veritor SARS-CoV-2 Point-of-Care Test Performance Compared to PCR-Based Testing and versus the Sofia 2 SARS Antigen Point-of-Care Test. J Clin Microbiol. 2020 Dec 17;59(1):e02338-20. doi: 10.1128/JCM.02338-20. PMID: 33023911; PMCID: PMC7771450.

Zhang Z, Bi Q, Fang S, Wei L, Wang X, He J, Wu Y, Lui X, Gao W, Zhang R, Gong W, Su Q, Azman AS, Lessler J, Zou X (2021). "Insight into the practical performance of RT-PCR testing for SARS-COV-2 using serological data: a cohort study," *Lancet Microbe*, 2(2), pp. e79–e87.

Zheng J (2020). SARS-CoV-2: an emerging coronavirus that causes a global threat. Int J Biol Science; 16:1678–85. doi: 10.7150/ijbs.45053

Zhou, X., Chen, K., Zou, J., Han, P., Hao, J., & Han, Z. (2020). Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Frontiers of medicine, 14(2),* 185–192. https://doi.org/10.1007/s11684-020-0754-0

https://heoc.mohp.gov.np/update-on-novel-corona-virus2019_ ncov

https://www.aa.com.tr/en/asia-pacific/nepal-registers-its-first-death-from-COVID-19/1843635

https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports/.