Development of Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) assay as a point-of-care diagnostics for SARS-CoV-2

THESIS SUBMITTED TO THE

NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY MICROBIOLOGY

BY

SHWETA KELKAR

B. Tech. (Dairy Technology)

DAIRY MICROBIOLOGY DIVISION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

KARNAL-132001 (HARYANA), INDIA

2021

Reg. No. 19-M-DM-10

DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) ASSAY AS A POINT-OF-CARE DIAGNOSTICS FOR SARS CoV-2

BY

SHWETA KELKAR

THESIS SUBMITTED TO THE

NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY MICROBIOLOGY

Approved by

( ) **(Dr. RASHMI H.M)**

EXTERNAL EXAMINER **MAJOR ADVISOR (GUIDE)**

MEMBERS OF ADVISORY COMMITTEE

1. **Dr. DIWAS PRADHAN**
   (Scientist, DM Division)
2. **SAURABH KADYAN**
   (Scientist, DM Division)
3. **Dr. SUNEEL KUMAR ONTERU**
   (Principal Scientist, ABC Division)
4. **Dr. J.K KAUSHIK**
   (Principal Scientist, ABT Division)
   (Joint Director's Nominee)

DAIRY MICROBIOLOGY DIVISION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA

Dr. RASHMI H.M.

Scientist (SS)

Dairy Microbiology Division

CERTIFICATE

This is to certify that the thesis entitled **"DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP MEDIATED ISOTHERMAL AMPLIFICATION RT-LAMP ASSAY AS POINT-OF-CARE DIAGNOSTICS FOR SARS CoV-2"** submitted by **Ms. Shweta Kelkar** in partial fulfillment of the requirement for the award of the degree of **MASTER OF TECHNOLOGY in DAIRY MICROBIOLOGY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY),** Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 29th September, 2021 **(Dr. Rashmi H.M.)**

Major Advisor

Telephone / Fax : 0184-2259186/2250042; Email: Rashmi.M @icar.gov.in


"To my beloved parents,
my brave sister &
honourable guide"

ACKNOWLEDGEMENTS

This investigation is the execution of hard work and dedication towards the development of reverse transcription loop mediated isothermal amplification (RT-LAMP) assay as point-of-care diagnostics for SARS CoV-2 and was not possible without the help of many people. I take this privilege to extend my sincere gratitude and appreciation to all those who have contributed directly or indirectly towards the completion of my M.Tech thesis.

First and foremost, I would like to thank God for always supporting and providing me the path to achieve success in my academic career as well as in life.

I wish to express my heartiest gratitude to my major advisor (guide) and in-charge of MBU Lab, ***Dr. Rashmi H.M****, Scientist (Senior Scale), Dairy Microbiology Division, NDRI, Karnal, Haryana. During the whole research work, she always helped me to carry out all the experiments even in the toughest situations involving the majority of risks. I also thank her for providing heartfelt support, guidance, valuable suggestions, creative ideas, and encouragement that I will always remember in my whole life. She always treated me like her own family member and always proved as an ideal mentor and actively participate in all the research activities. I consider myself very fortunate to have worked under her guidance. Without her support and encouragement, this thesis would not have been possible and therefore I am very thankful for all the assistance provided by her.*

I will be forever thankful to the members of my Advisory Committee, ***Dr. Diwas Pradhan****, Scientist, Dairy Microbiology Section,* ***Saurabh Kadyan Sir****, Scientist, Dairy Microbiology Section,* ***Dr. Suneel Kumar Onteru****, Principal Scientist, Animal Biochemistry Section, and* ***Dr. J.K Kaushik****, Principal Scientist, Animal Biotechnology Section for their valuable suggestions, guidance, insightful comments, and heartfelt support. I additionally thank* ***Dr. Suneel Kumar Onteru*** *for providing necessary suggestions to carry out LAMP reactions as well as for helping during the designing of the relevant LAMP primers.*

I extend my gratitude to ***Dr. M. S. Chauhan****, Director, ICAR-NDRI, Karnal,* ***Dr. R. R. B. Singh****, Joint Director (Academic),* ***Dr. Dheer Singh****, Joint Director (Research),* ***Dr. Nitin Tyagi****, Academic coordinator for providing all necessary infrastructure to carry out the present investigation.*

I express my insufficiency to trace out appropriate words to speak highly of ***Dr. A.K. Puniya,*** *Head of Dairy Microbiology Division, for always providing encouragement,*

academic support and guidance throughout my M.Tech programme. My sincere thanks to all scientists, teachers, and staff members of DM Division for their overwhelming help during my stay at NDRI.

I would also like to express the deep sense of gratitude to ***Dr. Sunita Grover*** *for providing expert advice relevant to the research work and I consider myself lucky that I acquired such a piece of wonderful knowledge and inspiration from her. I also wish to thank* ***Saurabh Kadyan Sir****, Scientist, Dairy Microbiology Section for their support in academic career and encouragement.*

I must express special thanks to ***Dr. Prerna Aggarwal , HOD. and Dr. Bhawna Sharma, Assistant Professor, Microbiology Department, Kalpana Chawla Government Medical College, Karnal, Haryana****, for permitting us to carry out the RT- LAMP experiments and handling of RNA virus samples of SARS-CoV-2 under their supervision and also for providing all the necessary help and infrastructure needed at the time of experimentation. I also wish to thank all the* ***staff members*** *of Virus Research and Diagnostic Laboratory (VRDL),* ***KCGMC, Karnal, Haryana*** *for providing their cooperation and support. Special thanks to* ***Dr. Sandeep Kumar****, Research Scientist, VRDL for his help and support during the experimental activities.*

I sincerely acknowledge the valuable help and co-operation extended by all my Labmates and seniors of Molecular Biology Unit, NDRI, Karnal, Haryana, ***A. Monica Rose, Chandrasekhar B and Dr. Gbenga Adewumi*** *for treating me like their own family member and always came forward to help me in any kind of situation related to the research work and also I learned many things from you all that I will be remembering throughout my life. In addition to this I wish to express a deep sense of gratitude to* ***A. Monica Rose****, for providing wonderful memories of happiness, love and care as well as the nice time spent together in NDRI. I wish to thank* ***Gurbajan ji and Ravinder ji****, the supporting staff of MBU Section, for always helping and maintaining a good as well as friendly atmosphere in the laboratory. I wish to thank all the other staffs of ICAR-NDRI who deserves special appreciation for their work during my stay here.*

I would like to express my sincere thanks to all fellow scholars ***Mr. Maharshi, Ms. Kiran Ms. Rolly, Ms. Nikitha, Ms. Bhavani, Mr. Prashanth, Mr. Likhith, Mr. Akshay, Mr. Umang****, for their support during the entire study period and for making it a very memorable journey.*

Words do not suffice to express my gratitude and devotion to my parents who always supported me to fulfill my wishes and dreams and helped me through every thick and thin in my life. I would never have achieved this success without their full support, strength, encouragement, and love given to me by my father ***Mr. Mukesh Kelkar,*** *my mother* ***Mrs. Kiran Kelkar,*** *and my encouraging sister* ***Dr. Sneha Kelkar****, who gave me immense courage to carry out this challenging research work involving the handling of SARS CoV-2 RNA virus. They were always there, supporting, correcting, and encouraging me. It is no one else but to all in my loving family that I dedicate this thesis. I thank them for having faith in me and for their support.*

All are not mentioned but no one is forgotten. Any omission in this brief acknowledgment does not mean a lack of gratitude. Words seem to be inadequate to express my deep sense of gratitude to all my family and hostel friends for their ready help and support in the form of suggestions and encouragement during the period of study.

Date: 29th September, 2021
Place: NDRI**,** Karnal, Haryana

Shwetale.

(Shweta Kelkar)

ABSTRACT

The present study aimed to develop a Reverse-transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay as a "Point-of-**c**are" (PoC) diagnostic solution which can be useful for the detection of SARS CoV-2. To develop this assay, the complete genome sequences of SARS CoV-2 reported from different States and Union Territories of India were downloaded from GISAID (**g**lobal **i**nitiative on **s**haring **a**vian **i**nfluenza **d**ata) database. The sequences were aligned to identify conserved regions for designing RT-LAMP primers. After literature search, three conserved regions with no previously reported primers for detection of SARS CoV-2 were selected and the primers were designed using Primer Explorer V5. A total of six sets (A, B, C, D, E, and F) of primers, two each from the single conserved region were designed. The specificity of these primers were again checked *in silico* with BLAST tool in the NCBI server. The designed primers did not yield any similarity matches with the other human coronavirus except MERS-CoV and Bat SARS-CoV. On the other hand for the development of LAMP assay with these designed primers, the target conserved regions were got synthesized and analysed for quality parameters prior to their use in the LAMP assay. Later, the primers (type, concentration and ratio) and LAMP reaction temperature were optimized in the development of LAMP assay using synthetic gene constructs. Further, the performance of developed assay was evaluated at KCGMC using cDNA synthesized from RNA of SARS-CoV-2 and developed LAMP techniques was found effective in the detection of SARS-CoV-2. The developed LAMP technique was further explored for development of colorimetric/fluorometric LAMP assay for rapid visual detection of SARS-CoV-2. The performance evaluation of developed colorimetric and fluorometric LAMP assays yielded satisfactory results in the detection of SARs-CoV-2 RNA. Hence, the developed Colorimetric and fluorometric LAMP assays can be used as PoC diagnostic test for the detection of SARS-CoV-2. However, its performance directly with RNA or samples needs to be evaluated to make it as an RT-LAMP assay and further validation is required with a larger sample size as per ICMR guidelines.

सारांश

वर्तमान अध्ययन का उद्देश्य सार्स-सीओवी-2 का पता लगाने के लिए आरटी-लैंप परख को पॉइंट-ऑफ-केयर नैदानिक समाधान के रूप में विकसित करना है। इस ऐसे को विकसित करने के लिए भारत के विभिन्न राज्यों और केंद्र शासित प्रदेशों से रिपोर्ट किए गए सार्स-सीओवी-2 के संपूर्ण जीनोम अनुक्रम जी.आई.एस.ए.आईडी (ग्लोबल इनिशिएटिव ऑन शेयरिंग एवियन इन्फ्लुएंजा डेटा) डेटाबेस से डाउनलोड किए गए थे। अनुक्रमों को आरटी-लैंप प्राइमरों को डिजाइन करने तथा संरक्षित क्षेत्रों की पहचान करने के लिए संरेखित किया गया था। इससे संबंधित साहित्य की खोज के बाद सार्स-सीओवी-2 का पता लगाने के लिए पहले से रिपोर्ट किए गए प्राइमरों वाले तीन संरक्षित क्षेत्रों का चयन किया गया था और प्राइमरों को प्राइमर एक्सप्लोरर वी5 का उपयोग कर डिज़ाइन किया गया। प्राइमरों के कुल छह सेट (ए, बी, सी, डी, ई और एफ) एकल संरक्षित क्षेत्र से दो प्रत्येक डिजाइन किए गए थे। इन प्राइमरों की विशिष्टता को एनसीबीआई सर्वर में ब्लास्ट टूल के साथ सिलिको में फिर से जांचा गया। डिज़ाइन किए गए प्राइमरों में मार्सकोरव और सीओवी जैसे बैट एसएआरएस को छोड़कर अन्य मानव कोरोनावायरस के साथ कोई समानता नहीं मिली। दूसरी ओर इन डिज़ाइन किए गए प्राइमरों के साथ लैंप परख के विकास के लिए लैंप ऐसे में उनके उपयोग से पहले लक्ष्य संरक्षित क्षेत्रों को गुणवत्ता मानकों के लिए संश्लेषित और विश्लेषण किया गया था। बाद में, प्राइमर (प्रकार, एकाग्रता और अनुपात) और लैंप प्रतिक्रिया तापमान को कृत्रिम जीन निर्माणों का उपयोग कर लैंप ऐसे के विकास में अनुकूलित किया गया। इसके अलावा, सार्स-सीओवी-2 पॉजिटिव नमूनों के आरएनए से संश्लेषित सीडीएनए का उपयोग करके के.सी.जी.एम.सी. में विकसित ऐसे के प्रदर्शन का मूल्यांकन किया गया और विकसित लैंप तकनीक सार्स-सीओवी-2 का पता लगाने में प्रभावी पाई गई थी। सार्स-सीओवी-2 की तेजी से दृश्य पहचान के लिए वर्णमिति/फ्लोरोमेट्रिक लैंप परख के विकास के लिए विकसित लैंप तकनीक की और खोज की गई। विकसित वर्णमिति और फ्लोरोमेट्रिक लैंप परख के प्रदर्शन मूल्यांकन से सार्स-सीओवी-2 आरएनए का पता लगाने में संतोषजनक परिणाम मिले। इसलिए, विकसित वर्णमिति और फ्लोरोमेट्रिक लैंप एस्सै का उपयोग सार्स-सीओवी-2 का पता लगाने के लिए पी.ओ.सी डायग्नोस्टिक टेस्ट के रूप में किया जा सकता है। हालांकि, आरटी-लैंप परख के रूप में अर्हता प्राप्त करने के लिए आरएनए (सकारात्मक नमूनों से) के साथ सीधे इसके प्रदर्शन का मूल्यांकन करने की आवश्यकता है और आईसीएमआर दिशानिर्देशों के अनुसार बड़े नमूना आकार के साथ आगे सत्यापन की आवश्यकता है।

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION

ACE-2	:	Angiotensin Converting Enzyme-2
ARDS	:	Acute Respiratory Distress Syndrome
ASe	:	Analytical Senstivity
Asp	:	Analytical Specificity
CDC	:	Center of Disease Control
cDNA	:	Complementary DNA
CNR	:	National Reference Center
COVID-19	:	Coronavirus Disease of 2019
Ct	:	Cycle Threshold
CT	:	Computed Tomography
DM	:	Dairy Microbiology Division
DNA	:	Deoxyribonucleic Acid
E	:	Envelope Protein
EDTA	:	Ethylenediaminetetraacetic Acid
ELISA	:	Enzyme-linked Immunoassay
EtBr	:	Ethidium Bromide Dye
GISAID	:	Global Initiative on Sharing all Influenza Data
HNB	:	Hydroxynaphthol Blue
IgG	:	Immunoglobulin M
IgM	:	Immunoglobulin M
KCGMC	:	Kalpana Chawla Government Medical College
LAMP	:	Loop Mediated Isothermal Amplification
LOD	:	Limit of Detection
M	:	Membrane Protein
MBU	:	Molecular Biology Unit
MERS CoV	:	Middle East Respiratory Syndrome
Mpro	:	Main Protease
MSA	:	Multiple Sequence Alignment

N	:	Nucleocapsid
NAAT	:	Nucleic Acid Amplification Test
NDRI	:	National Dairy Research Institute
Nsp	:	Non-structural Protein
Orf	:	Open Reading Frame
PCR	:	Polymerase Chain Reaction
POC	:	Point of Care
RAT	:	Rapid Antigen Test
RdRp	:	RNA-dependent RNA polymerase
RNA	:	Ribonucleic acid
RT-LAMP	:	Reverse Transcription Loop Mediated Isothermal Amplification
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
S	:	Spike Protein
SARS CoV	:	Severe Acute Respiratory Syndrome Coronavirus
SARS CoV -2	:	Severe Acute Respiratory Syndrome Coronavirus 2
SGC	:	Synthetic Gene Construct
Tm	:	Temperature of Melting
VRDL	:	Virus Research and Diagnostics Laboratory
WHO	:	World Health Organization

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Covid-19 pandemic, also known as coronavirus disease caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome –Corona Virus-2) had devastating effects globally as well as in India. This disease affected many countries (World Health Organization, Covid-19 situation report-76, 2020) and brought the health care system of most of the populous countries like India to a halt. The pandemic threw up an unprecedented challenge in terms of catering to the national needs of diagnostic and life support systems in a very short time frame. The second wave again affected countries like Europe (Jun-Aug, 2020), United States (Jun-July, 2020), Germany (April, 2020), including India (since March, 2021). The second wave that caught India off guard, compelled India to strengthen its capacity to develop POC diagnostics to tackle future waves of Covid-19 of much higher magnitudes (on 30th April India became the first country to report 4,08,323 cases in a single day) when only 15 crore individuals were vaccinated (https://www.thehindu.com/news/national/coronavirus-india-becomes-first-country-in-the-world-to-report-over-400000-new-cases-on-april-30-2021/article34453081.ece) But in countries like India, with 138 crore population (Ministry of Statistics and Programme Implementation, 2020), controlling the contagion requires the development of rapid and effective diagnostic devices to handle the crisis through timely diagnosis and treatment. Currently, only two common vaccines namely Covishield and Covaxin are widely available in India with respective efficacy rates of 90% and 81 % (https://www.bbc.com/news/world-asia-india-57264622). Nevertheless, the breakthrough infections are on the rise and there is no concrete data to substantiate the efficacy of vaccine. Hence, a multidimensional approach of test, track and treat is the only alternative available. This three-pronged approach calls for advancements in the diagnostics techniques that should go hand in hand with the development of effective vaccines and vaccination of the large Indian population. This approach can be considered as a gold standard in containing the virus especially when the virus is behaving erratically owing to its rapid mutation. The strain of SARS CoV-2 is getting mutated rapidly and re-infecting 0.04 to 0.2 % of vaccinated people (ICMR). Moreover, only about 59 crore people were vaccinated according to the Officials of Ministry of Health and Family Welfare on 25th August, 2021. WHO, on 11th May, classified the highly contagious triple mutant SARS-CoV-2 variant B.1.617 found in India as “variant

of concern" as well as a "Global Risk". Besides, according to popular predictive models, the third wave of Covid-19 pandemic is also in the offing and slated to hit India in due course of time.

Looking into this scenario, to beat the virus requires effective preparedness both in terms of diagnostics and the health care system before the third wave hits the country. Though RT-PCR kits are widely used for diagnostics, it usually takes 2-3 hours for yielding results and requires sophisticated machinery and skilled professionals. The experiences from the first and the second waves have shown that timely delivery of results was a major impediment for most of the VRDL (Virus Research and Diagnostics Laboratory), which relied invariably on RT-PCR. In few instances, the RT-PCR tests took more than a week to provide results as seen in few states such as Gujarat (The Indian Express) in the second wave. Hence the need for rapid and POC diagnostics is the need of the hour to combat such uncertain medical emergencies and to scale up the entire health care system of India.

As SARS CoV-2 consists of single stranded RNA particles as genetic material and to multiply those specific RNA sequences, reverse transcription loop-mediated isothermal amplification (RT-LAMP) can serve as a promising rapid POC diagnostic method. This technique is generally employed to diagnose RNA virus-induced infectious diseases and combines the LAMP DNA-detection method with reverse transcription and synthesis of Complementary DNA (cDNA) from RNA before running the reaction. This technique takes place at a constant temperature i.e. between 60 and 65°C and does not require any thermal cyclers like RT-PCR technique and provides results in a very short time interval i.e. within 30 min. RT-LAMP is considered a cheaper and faster alternative to RT-PCR for the early detection of people who are infected with COVID-19.

Nevertheless, the RT-LAMP tests can be conducted without prior RNA separation, and viruses can be extracted directly from swabs (nose and throat) or saliva. The technique involves three important processes namely reverse transcription through the utilization enzyme i.e. reverse transcriptase, amplification using DNA polymerase, and read-out process employed to analyze and detect the RT-LAMP products which are frequently colorimetric or fluorometricc. The RT-LAMP technique has been also approved by Food and Drug Administration (FDA) as a molecular rapid diagnostic kit for the detection of SARS CoV-2

named as AQ-TOP^{M} COVID-19 Rapid Detection Kit. The RT-PCR technique is widely performed in India for Covid-19 testing but possesses few drawbacks which could be overcome by the RT-LAMP technique. Despite its ergonomic design, no commercial indigenous RT-LAMP diagnostics kit is available in India till date and only one RT-LAMP diagnostics kit got approval recently from ICMR for its usage in the detection of SARS-CoV-2 in India. Considering the enormous benefits of RT-LAMP, the present study has been undertaken to develop RT-LAMP diagnostics to fasten the testing technologies, which could serve as a potential POC diagnostic technique for effectively combating the present and future medical emergencies of the Nation.

The study was designed with the following objectives

1. Synthesis of artificial genes of SARS CoV-2 and designing of RT-LAMP primers.
2. Development and performance evaluation of RT-LAMP assay

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

This chapter describes the literature related to the objectives of the present study and are discussed under the following groupings:

- Severe Acute Respiratory Syndrome **(**SARS CoV) disease
- Middle East Respiratory Syndrome (MERS CoV) disease
- Severe Acute Respiratory Syndrome (SARS CoV-2) disease
- Structure of SARS CoV-2
- Coronavirus Infections and their origin
- First genome sequencing of SARS CoV-2
- Nucleic Acid Amplification for SARS CoV-2
- Serological detection of SARS CoV-2
- RT-LAMP Technique
- Rapid and visual detection through RT-LAMP assay

2.1 Severe Acute Respiratory Syndrome (SARS CoV) disease

The SARS stands for "Severe Acute Respiratory Syndrome" is a zoonotic disease that resulted in an outbreak during 2002-2004 which significantly affected 26 countries and led to more than 8000 cases. The SARS epidemic was caused by the first identified Severe Acute Respiratory Syndrome Corona Virus (SARS CoV or SARS CoV-1). SARS CoV is considered highly contagious due to its rapid spreading of pneumonia-causing sudden fever and shortness of breath within 3-7 days of infection. The origin of this virus was traced back to Guangdong province located in Southern China in late 2002. The first infection case was reported between January and March 2003, and it majorly caused upper respiratory illnesses to life-threatening infections including pneumonia (Zhao *et al.*, 2003).

Further studies demonstrated the clinical features and epidemiology of the outbreak along with the treatment results of around 190 patients. Some individuals were found to have normal conditions of respiration whereas others exhibited serious breathing issues

including acute respiratory distress syndrome (ARDS) and exhibited no response to antibiotics. Laboratory diagnosis included the sampling of throat swabs and sputum followed by serological detection of antibodies. The medical treatment was offered to patients after dividing them into different groups A, B, C and, D based upon the prescribed antibiotics namely ribavirin, fluoroquinolone with azithromycin, quinolone with azithromycin and levofloxacin, respectively. The best results were observed in group D patients.

The WHO responded globally towards the SARS CoV epidemic 2003 by setting up five priority tasks to function and control the overall scenario. These tasks were assigned to identify the causative agent of novel SARS disease, development of diagnostic kits for proper and accurate detection as well as the management of testing protocols for the reduction of mortality rates. However, a grave problem appeared in the area of accurate and rapid testing along with non-availability of drugs available for treatment. Detection of SARS CoV through RT-PCR technique resulted in very limited sensitivity during the beginning of the infection and suggested that sensitivity could be improved only when the sample is taken from the lower respiratory tract during the occurrence of clinical symptoms.

On the other hand, serological methods were highly sensitive but nearly after 21 days of clinical symptoms (Anderson *et al.,* 2004). The incubation period is referred to as the time from infection up to the occurrence of clinical symptoms and it depends on crucial parameters such as the age of the patient, genetic factors and dose of infecting virus. The distribution of infections occurring in individuals and its statistical information plays a great role during the beginning of such dangerous as well as life-threatening diseases. On the other hand, a key component of these infections is the infectious period which determines the infectiousness of a particular patient and is a responsible factor for the transmission of infection from one individual to another.

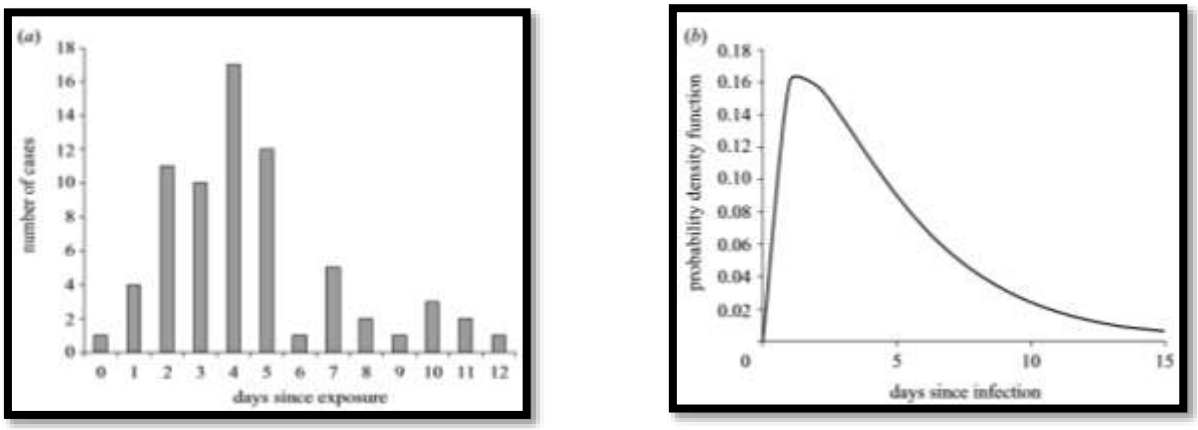
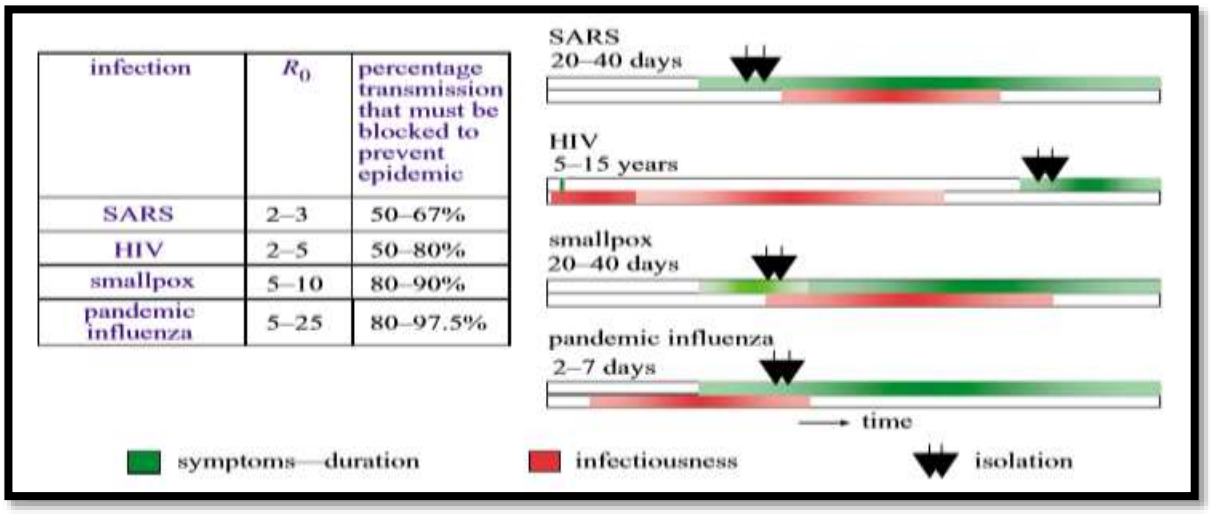


Fig. 2.1: Incubation periods during exposure of SARS and producing symptoms in case of infection (Donnelly *et al.*, 2003)



infection	R_0	percentage transmission that must be blocked to prevent epidemic
SARS	2–3	50–67%
HIV	2–5	50–80%
smallpox	5–10	80–90%
pandemic influenza	5–25	80–97.5%

Fig. 2.2: Schematic diagram showing the incubation and infectious periods in case of SARS and other diseases (Fraser *et al.*, 2004)

2.2 Middle East Respiratory Syndrome (MERS CoV)

MERS stands for Middle East Respiratory Syndrome which also refers to the camel flu, a respiratory disease of viral origin caused by Middle East Respiratory Syndrome Coronavirus (MERS CoV). The MERS CoV is one of the sixth hCoV (human coronavirus), first isolated from a 60-year old patient possessing serious respiratory conditions (Chafekar *et*

al., 2018) and is considered as the dangerous pathogenic virus after the SARS CoV (2003). MERS CoV is a positive-sense single stranded RNA (ssRNA) having 30 kb genomic size (Wernery *et al.,* 2015). This disease was first reported in Saudi Arabia in the year of 2012 and about 35 % of infected patients died (WHO, 2019). The symptoms include fever, shortness of breath, cough, and sometimes pneumonia. MERS is a type of zoonotic disease where viral transmission from camel to human was reported in Saudi Arabia when genome sequencing of isolated MERS from an individual was performed, who was in contact with camel, previously reported as positive for MERS infection (Ramadan *et al.,* 2019). Currently, no effective vaccines or antiviral drug is available for the prevention and proper treatment of MERS infections but the development of these vaccines and treatments are under process.

The laboratory detection of MERS CoV involved mainly three approaches namely molecular techniques to detect MERS CoV RNA, antigen detection, identification of humoral response before MERS infection in the case of humans (Mackay *et al.,* 2015). According to the case definition of MERS provided by WHO, they have suggested RT-PCR targeting two different regions for the confirmation of MERS CoV infection (Shirato *et al.,* 2014). The specific primers and probes used for RT-PCR targeting ORF1a and E gene displayed the highest ranges of sensitivity for the detection of MERS infections (Corman *et al.,* 2012 and Lu *et al.,* 2014). However, the major problem of this technique is that it requires sophisticated equipment and skilled personnel, hence these are not appropriate for rapid and point-of-care diagnosis. Due to this reason, there is a greater need for effective diagnostic kits or assays for the accurate detection of MERS CoV. Yamaoka *et al.,* (2016) have suggested that the tests to detect antigens and antibodies in the infected host would be the most appropriate way for the detection of MERS CoV.

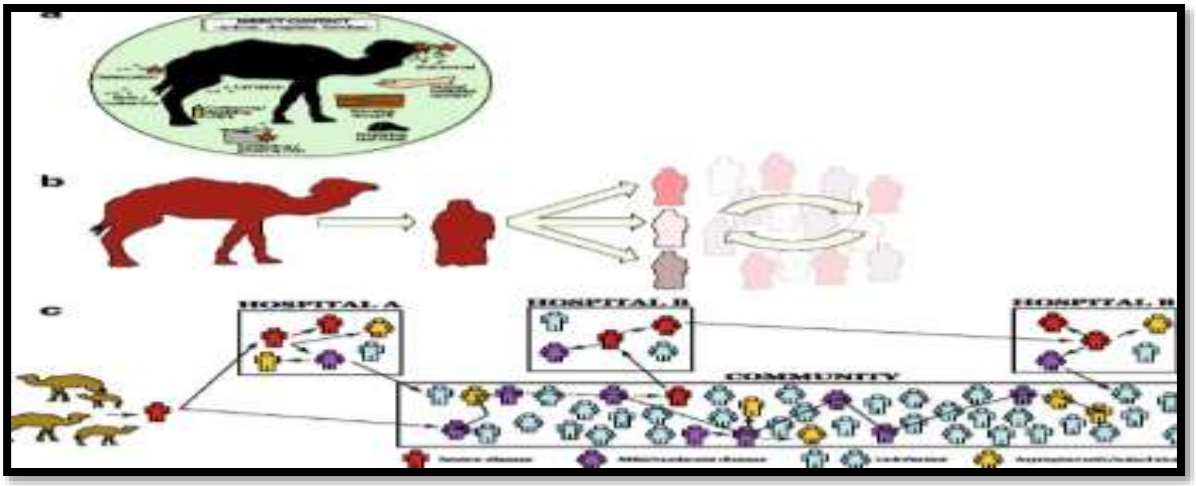


Fig. 2.3: MERS CoV infections transmission from camel to humans (Mackay *et al.*, 2015)

2.3 Severe Acute Respiratory Syndrome (SARS CoV-2)

SARS CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) is a particular strain that belongs to the family Coronaviridae and genus Betacoronavirus, and they share approximately 80-89% homology with all discovered coronaviruses in humans, bat and other wild animals. These viruses belong to the Baltimore Class IV positive-sense single stranded RNA viruses and are highly contagious in humans. The infection involves transmission of virus through aerosols or respiratory droplets from one infected person to another when in close proximity. These strains enter the human via the "Angiotensin Converting Enzyme-2" (ACE-2) and infect the host cells. The most common symptoms are fever, dry cough and tiredness but according to the WHO, nearly 1 out of 5 people suffers from serious illness and breathing difficulties whereas the older people having major medical issues such as high blood pressure, lung and cardiovascular diseases, cancer and diabetes being at higher levels of risk for developing much severe health conditions.

2.4 Structure of SARS-CoV-2

SARS-CoV-2 virion consist of a crown like appearance hence named as corona, having a diameter of 50-200 nm (Wang *et al.,* 2020) and with a largest genomic size ranging from 26-32 kb in length which provides for more plasticity during accommodation and mainly for modifying their respective genes. It specifically consists of six major open reading frames

(ORFs) namely ORF 1a and 1b (replication enzyme coding region), E gene (envelope protein), S gene (spike protein), M gene (membrane protein) and N gene (nucleocapsid gene) which the most abundant and conservative genes found in nearly all coronaviruses. Along with these ORFs, there are several other accessory proteins viz. ORF 3a, 6, 7a, 7b and 8. The E, N, S and M genes are known as the structural proteins of SARS-CoV-2 and are essential for reproducing the complete structure of the corresponding viral particle. On the other side, the ORFs 1a and 1b give rise to 16 non-structural genes (nsp1-16) due to the encoding process of replication enzyme The Spike proteins are responsible for their entry into the human host cell. One of the essential nsp encoded by ORF 1a and 1b is "Main protease" (Mpro, also referred as 3CLpro), which plays a very beneficial role by controlling the replication of SARS-CoV-2 and also helps in the production of polyproteins. RdRp gene (RNA-dependent RNA polymerase), which is also referred as nsp12, is an important replicase enzyme that utilizes the viral genome RNA template for catalyzing and assessing the replication of RNA (Khan *et al.*, 2020).

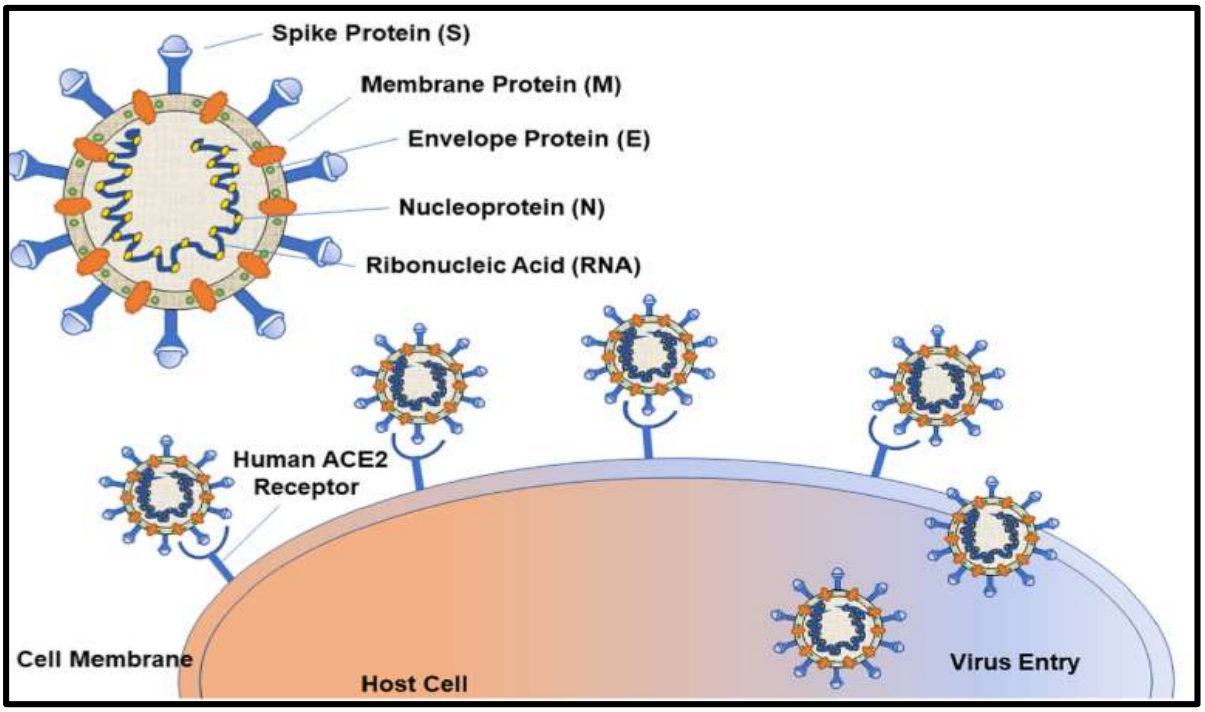


Fig. 2.4: Schematic representation of the SARS CoV-2 structure and depiction of entry into the host cell (A.A.T. Naqvi, *et al.*, 2020)

2.5 Coronavirus infections and their origin

During the last two decades, three coronaviruses were discovered namely SARS-CoV (Severe Acute Respiratory Syndrome), MERS-CoV (Middle East Respiratory Syndrome) and SARS-CoV-2 which majorly caused pneumonia infections in human beings. Earlier in 2002, SARS-CoV emerged in China and the transmission of infection spanned through five continents resulting in more than 774 deaths. Similarly in the year 2012, MERS-CoV emerged in Arabian Peninsula and then spread in 27 different countries. The current pandemic which was confirmed by WHO as pandemic on 11th March 2020 is attributed to SARS-CoV-2 which caused coronavirus illness termed as COVID-19, started in 2019 from the city Wuhan, Hubei Province of China. Certain reports have significantly shown that the SARS-CoV and SARS-CoV-2 have originated from the bats, which serve as a reservoir for both of these viruses, whereas the MERS-CoV have originated from bats but they do not serve as a reservoir for their transmission (Khan *et al*., 2020). The SARS-CoV-2 matches approximately 82% similarity with the previously emerged SARS CoV and MERS CoV and >90% for several essential structural genes as well as enzymes (A.A.T. Naqvi *et al.*, 2020).

Coronavirus generally consists of a large family of viruses, among them four viruses are responsible for mild cold in all age groups. Some of these viruses also come from animals which infects humans and are known as zoonotic species. The SARS CoV-2 species are responsible to cause severe pneumonia and deaths in infected individuals, and thereafter they can transmit these infections to other healthy individuals via the act of coughing, sneezing or due to contact of contaminated surfaces. The most common symptoms of these infections are fever, fatigue, cough as well as rapid shortening of breath, whereas the severe symptoms include severe acute respiratory syndrome, renal failure and ultimately death. A report stated by Faculty of Medicine, University of Bergen on 12th March 2020, approximately 14% of these infections are serious and about 5 % are only critical. The patients who had previous co-morbidities like diabetes, pulmonary disease or any other chronic illnesses suffered greater risk during the pandemic. According to WHO guidelines, some crucial precautions were advised to be followed such as physical distancing, wearing face masks to ensure proper coverage of mouth and nose, hand washing, avoiding crowds and other public gatherings. All

these precautions had prevented or lowered the occurrence of these infections up to some extent and in the cases of individuals who followed all these advisory guidelines.

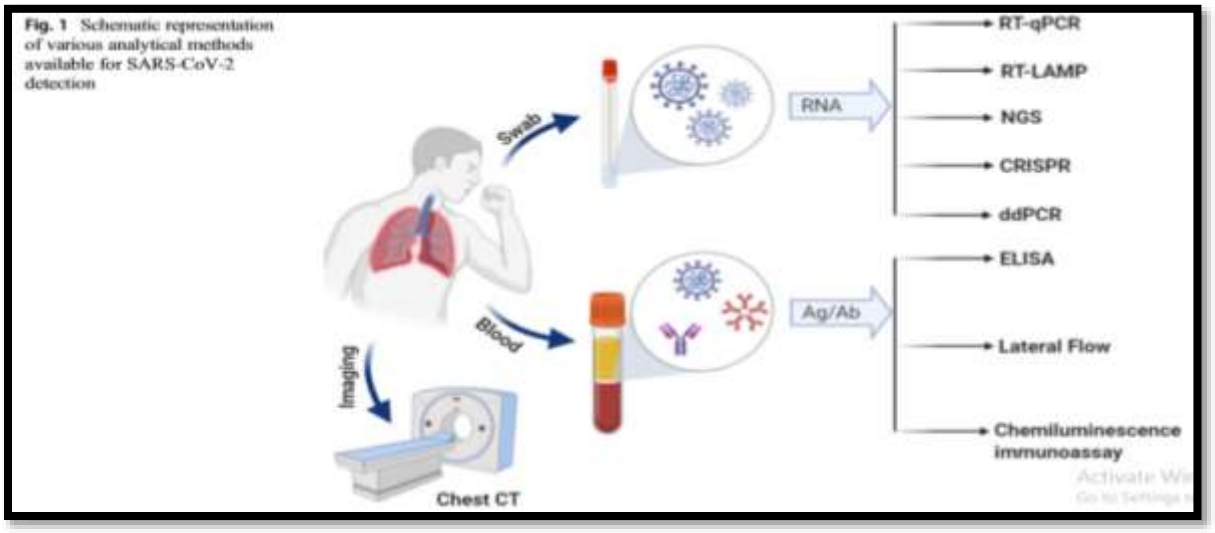


Fig. 2.5: Picture showing different methodologies used for the detection of SARS CoV-2 (Kevadiya *et al.*, 2021)

2.6 First genome sequencing of SARS CoV-2

On 24th January, the French Ministry of Health stated that they have found the first three confirmed positive cases being infected by SARS CoV-2. The Institute Pasteur was the first institution in Europe to sequence the whole genome of coronavirus, also known as "2019 novel coronavirus". The viral sequencing was performed at Mutualized Platform for Microbiology, P2M of Institut Pasteur. The P2M also performs the genome sequencing of other genomes originated from bacteria, fungi and that are received from CNR (National Reference Center), reference laboratory of WHO and other Collaborating Centers of WHO for the aim of disease surveillance. They have sequenced two full-length genomes of SARS CoV-2 were then submitted to the GISAID (Global Initiative on Sharing All Influenza Data) platform (Science Daily, 31st January 2020).

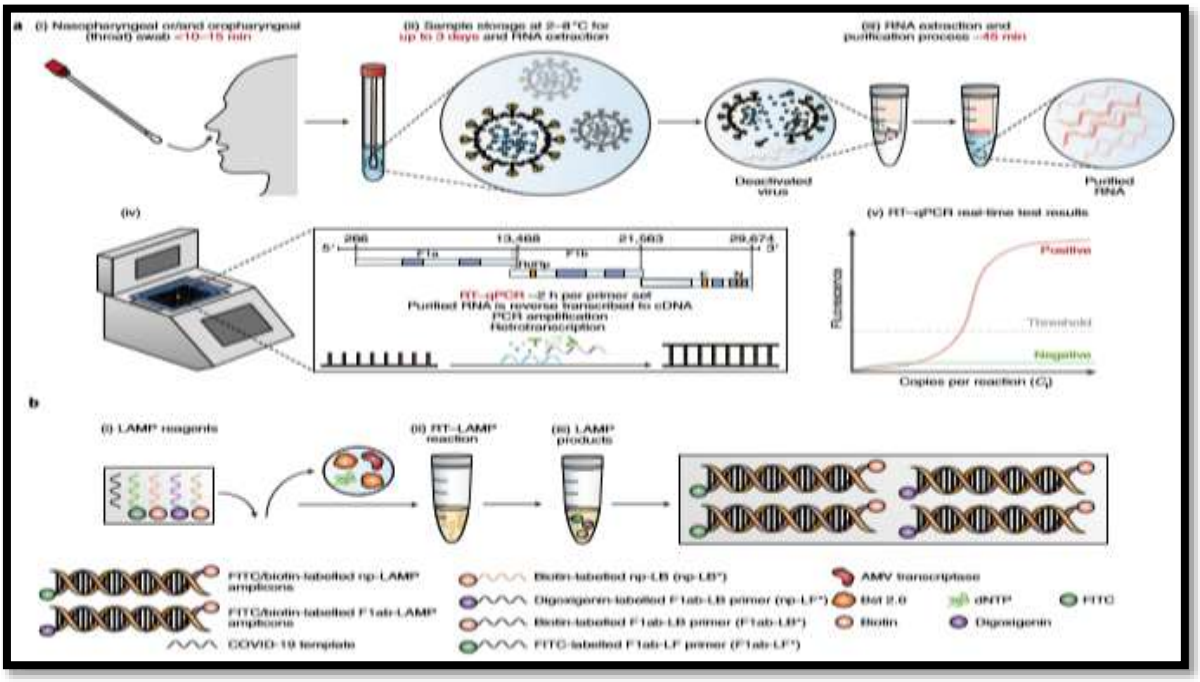


Fig. 2.6: Procedures of RT-PCR and RT-LAMP for the detection of SARS CoV-2 (Kevadiya *et al.*, 2021)

2.7 Nucleic acid amplification technique for the detection of SARS CoV-2

The occurrence of a sudden pandemic involving 2019 novel coronavirus (SARS CoV-2) has demonstrated the need of rapid testing and ultimately the development of diagnostic devices essential to provide quick and accurate test results to control the rising infection rate as well as for better prevention (Nascimento Junior *et al.*, 2020). The Covid-19 testing involves any kind of laboratory techniques namely CT scan, nucleic acid amplification test (NAAT), serological test (Corman *et al.*, 2020 and Wan *et al.*, 2020). NAAT is considered as most sensitive laboratory technique for the early detection of SARS CoV-2.

The various nucleic acid amplification method involves certain techniques namely real time reverse transcriptase-polymerase chain reaction (RT-PCR), reverse transcription loop mediated isothermal amplification (RT-LAMP), etc. However, these tests require good quality of the virus particle. The real time RT-PCR technique is nowadays considered a gold standard for the detection purpose and is most widely used in many countries as recommended by Center of Disease Control (CDC) and World Health Organization (WHO) (Chu *et al.*,

2020) targeting the E and RdRp gene (Corman *et al.*, 2020). On the other hand, RT-LAMP technique involves the use of 4-6 primers and amplification of multiple target regions efficiently at a constant temperature without requiring any sophisticated instrument and skilled personnel.

Table. 2.1: List of nucleic acid-based tests for the detection of SARS CoV-2 (Rai *et al.*, 2021)

S.No.	Name of detection assay	Testing Principle	Company
1.	TaqMan 2019-nCoV Control Kit v1	Real-time RT-PCR	ABI (Applied bio-systems), United States
2.	Protect COVID-19 RT-qPCR Kit	Real-time RT-PCR	JN Medsys Pte Ltd
3.	ePlex SARS-CoV-2 test	Cartridge Based Technology	GenMark Diagnostics, Inc.
4.	SARAGENETM Corona Virus (2019 NCV) Test Kit	Real-time RT-PCR	CoSara Diagnostics Pvt. Limited, Ahmedabad, India
5.	Helini Coronavirus [COVID-19] Real	Real-time RT-PCR	Helini Biomolecules, Chennai, India
6.	Q-line Molecular Coronavirus (COVID-19) RT-PCR kit	Real-time RT-PCR	POCT Services Pvt. Limited, Lucknow, India
7.	Covid 19 Probe-free Real Time PCR Diagnostic Kit	Real-time RT-PCR	Indian Institute of Technology, Delhi, India
8.	cobas SARS-CoV-2	Real-time RT-PCR	Roche Molecular Systems, Inc.
9.	Meril COVID-19 One-step RT-PCR Kit	RT-PCR	Meril Diagnostics, Vapi, Gujarat, India
10.	Patho Detect	RT-PCR	Mylab Discovery Solutions, Pune, India
11	ID NOW COVID-19	Isothermal nucleic acid amplification Technology	Abbott Diagnostics Scarborough, Inc

12.	CRISPR-based tests for SARS-CoV-2	CRISPR-based lateral flow assay isothermal amplification	Cepheid Sherlock Biosciences

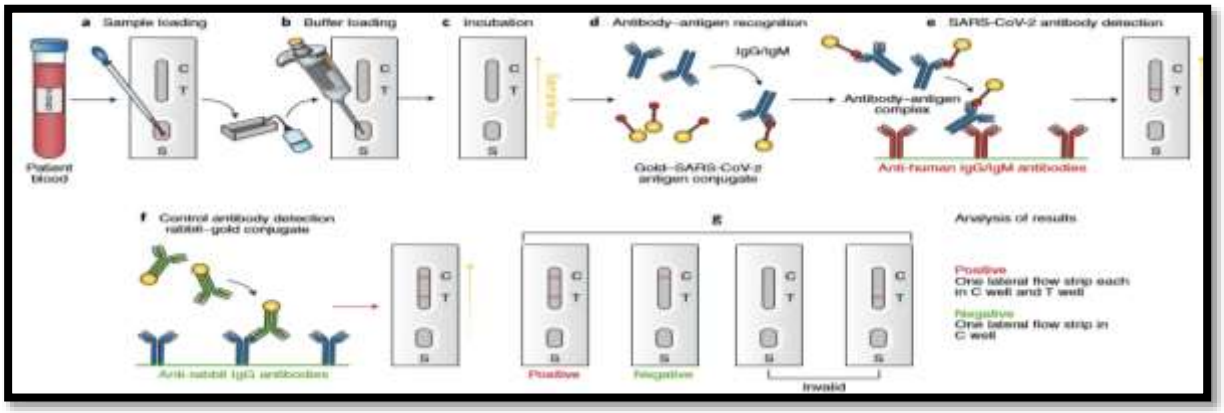


Fig. 2.7: Schematic diagram depicting procedure for serological detection of SARS CoV-2 (Kevadiya *et al.*, 2021)

2.8 Serological detection of SARS CoV-2

The serological detection method includes antibody and antigen testing. ICMR recommended antibody testing in most of the hot spot regions to track the Covid-19 infections. Anti SARS CoV-2 human IgG ELISA testing kit was developed by ICMR-NIV for the purpose of antibody detection with 98.7 % sensitivity and 100 % specificity resulted in external validation. This ELISA test demonstrated merits in testing around 90 samples in a single run with minimal biosafety requirements. On the other hand, antigen testing involves the detection of SARS CoV-2 antigens such as nucleocapsid (N), S1 and S2 regions of the spike (S) protein with a sensitivity in the range of 34 to 80 %.

In September 2020, ICMR has permitted Rapid Antigen Test (RAT) for its utilization in various containment zones. However, the RAT providing negative results has been forced to undergo RT-PCR for the confirmation of infection. These serological-based tests has some disadvantages of producing false-negative results after being influenced by non-active replication and not having sufficient concentrations of SARS CoV-2. Hence, these tests can be utilized only to diagnose early infection cases and their performance largely depend on the testing conditions, personnel and viral load of samples. All these factors arises the urgent need

of reliable and accurate point of care (POC) diagnosis kits or devices to combat the Covid-19 crisis in India (Kumar *et al.*, 2021).

Table. 2.2: List of serology-based tests for the detection of SARS CoV-2 (Rai *et al.*, 2021)

S.No.	Name of detection assay	Testing Principle	Company
1.	SARS CoV-2 Rapid Test	Lateral flow immunoassay	Pharma ACT
2.	Covid Kavach ELISA IgG	ELISA	Zydus Cadila Healthcare Ltd.
3.	qSARS-CoV-2 Rapid Test	Lateral flow immunoassay	Cellex Inc
4.	IgG antibody kit for novel coronavirus 2019	Magnetic particle based chemiluminescence immunoassay	Bioscience (Chongqing) Diagnostic Technology Co. Ltd.
5.	COVID-19 IgG/IgM Point of Care Rapid test	Lateral flow immunoassay	Aytu Biosciences/Orient Gene Biotech
6.	COVID-19 Ag Respi-Strip	Lateral flow immunoassay (dipstick)	Coris Bioconcept
7.	m2000 SARS-CoV-2 assay	Chemiluminescent Microparticle	Abbott Core Laboratory
8.	DEIASL019/020 SARS-CoV-2 IgG ELISA kit	ELISA	Creative Diagnostics
9.	Standard Q COVID-19 Ag	Chromatographic Immunoassay	SD Biosensor
10.	Standard Q COVID-19 IgM/IgG Duo	Lateral flow immunoassay	SD Biosensor

2.9 RT-LAMP Technique

LAMP is a type of isothermal amplification system discovered by Notomi *et al.*, (2000), that can amplify nucleic acid with high specificity, sensitivity and pace at 60-65° C and which does not require any special tool such as thermal cycler. This method relies on self-recycling line displacement DNA synthesis by Bst DNA polymerase. If the template is RNA, the amplification process can take place in one step by adding a reverse transcription process, therefore known as RT-LAMP technique (Yang *et al.,* 2020). The principle of RT-LAMP involves the synthesis of cDNA (complementary DNA) from the template RNA and the through the application of LAMP technology we can amplify the cDNA and detect them. Park *et al.,* 2020 have developed RT-LAMP assay to detect the genomic RNA upto 100 copies and in comparison with RT-PCR shows sensitivity of <10 copies per reaction. Also, the amplification efficiency was high. The leuco crystal violet method was applied to achieve colorimetric detection of LAMP reaction. Ben-Assa *et al.,* (2020) have developed a protocol based on Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) for detection of SARS-CoV-2 and applied directly on SARS-CoV-2 nose and throat swabs, with no RNA purification step required. Lu *et al.,* (2020) developed RT-LAMP assay having a high sensitivity, with a LOD of 118.6 copies of SARS-CoV-2 RNA per 25 µL reaction, and showed good specificity. Lu *et al.,* (2020) by using a mismatch-tolerant method of amplification, built a simple, fast, delicate and visual RT-LAMP assay for detection of SARS CoV-2.

2.10 Rapid and visual detection through RT-LAMP assay

The reading of RT-LAMP test is frequently colorimetric. Two common ways that are based on the measurements of either the pH or magnesium ions. As soon as the amplification takes place, pH lowers and magnesium ions drop immediately. These changes can be perceived by certain indicators such as phenol red for pH and hydroxynaphthol blue (HNB) for magnesium. Ben-Assa *et al.,* (2020) developed the assay and visualized by colour change and considered samples negative if the original pink colour of the phenol red was maintained and positive if the phenol red colour turned orange-yellow. Also, leuco crystal method can be applied to this assay for the colorimetric detection of the reaction (Park *et al.,* 2020). A visual version of this assay was developed by Lu *et al.,* (2020) for convenient use and it produced a clear colour change for all positive samples when tested for about 40 min. Yan *et al.,* (2020)

added 1 mL of fluorescent calcein and observed a colour change from orange to green for a positive reaction. In a visual detection system, cresol red is used as a pH dye indicator due to a strong colour difference between negative (red) and positive (yellow) reactions after 40 min of incubation at 63° C and a colour change from red to orange or yellow is considered a positive reaction (Lu *et al.,* 2020).

Detection methods that allow detection by visual examination without need of instrumentation using dyes that utilize inherent by-products of the extensive DNA synthesis, such as malachite green, calcein and hydroxynaphthol blue (Zhang *et al.,* 2020). Anahtar *et al.,* (2020), have developed a simple and inexpensive RT-LAMP technique having 87.5 % sensitivity and 100 % specificity to test nearly 135 nasopharyngeal samples that they have added directly and performed the RT-LAMP reactions which provided accurate results within 30-40 minutes and suggested that sensitivity increases when the samples were subjected to chemical RNAse inactivation using TPEC/EDTA followed by heat-mediated lysis. Nawattanapaiboon *et al.,* (2021) have developed RT-LAMP having an overall accuracy of 99.86 % based on targeting the viral RdRp gene as much of the laboratories across Europe which provided good results in 1hr included temperature about 65° C when compared to the gold standard, qRT-PCR, requires no sophisticated equipment and facilitated visual detection possible through even naked eyes. They have also incorporated phenol red dye for colorimetric detection leading to a colour change i.e. pink to yellow during positive reaction.

Through their experiments, they have suggested that this RT-LAMP technique for detecting SARS CoV-2 is highly compatible with the resource-limiting settings such as community hospitals and healthcare centers in rural areas as it does not require any sophisticated equipment. Lalli *et al.,* (2021) have developed a colorimetric RT-LAMP assay with a limit of detection 59 (95% confidence interval: 44-110) particle copies per reaction and optimized that on the human saliva samples without the RNA purification step. They have also compared the performance of recently developed primers targeting different sequences of SARS CoV-2 and suggested that the primers targeting the N gene and orf1ab regions shown greater analytical sensitivity and also reduced the chances of false-positive results along with this they showed that there was no cross-reactivity with MERS coronavirus. Jang *et al.,* (2021) developed a multiplex SARS CoV-2 RT-LAMP detection kit targeting mainly three genes

namely, RdRp, N and E genes along with internal control i.e. actin beta. The developed LAMP assay resulted good sensitivity as 93.85% for RdRp, 94.62% for N, 96.92% for RdRp/N. The limits of detection for the RdRP, E and N genes were found out to be $1x10^1$ copies/µL, $1x10^1$ copies/µL and $1x10^2$ copies/µL. This kit ensures a reduced level of contamination and enables diagnosing of mass population at a very shorter period of time. This assay has shown 100 % specificity in the detection process with no cross reactivity with the other known respiratory viruses.

Fowler *et al.,* (2021) developed and simultaneously validated a highly effective RT-LAMP assay that targeted the RdRp gene and suggested that it could replace the conventional RT-PCR technique during an emergency or when a large sample range is to be tested and Direct RT-LAMP can be successfully used as a near-patient screening tool to rapidly detect the presence of highly contagious SARS CoV-2. They have reported 100 % Analytical Specificity (ASp) and 97% Analytical Sensitivity (ASe) of between 1×10^1 and 1×10^2 copies per reaction which corresponds to the overall diagnostic sensitivity and specificity of 97% and 99% respectively.

CHAPTER 3

MATERIALS AND METHODS

Objective 1

Synthesis of artificial SARS-CoV-2 genes and designing of LAMP primers

Activity 1: Alignment of complete genome of SARS-CoV-2 sequenced from different parts (States and Union Territories) of India

Fig. 3.1: Illustration of aligned sequences of SARS-CoV-2 sequenced from different States & Union Territories of India

Activity 2: Identification of conserved regions

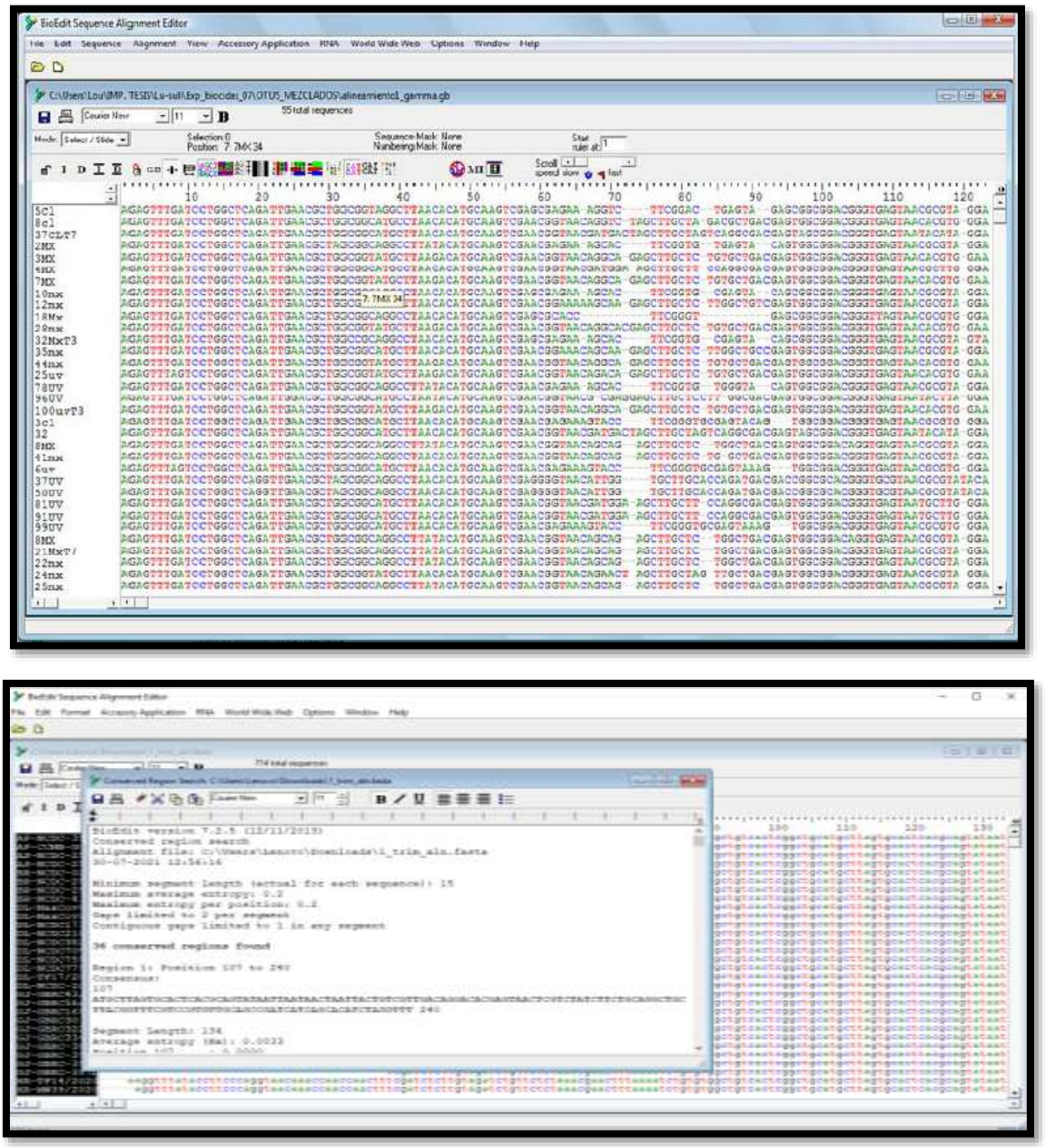


Fig. 3.2: Illustration and identification of conserved regions of SARS CoV-2 after complete alignment of collected sequences

Activity 3: Designing of RT-LAMP primers

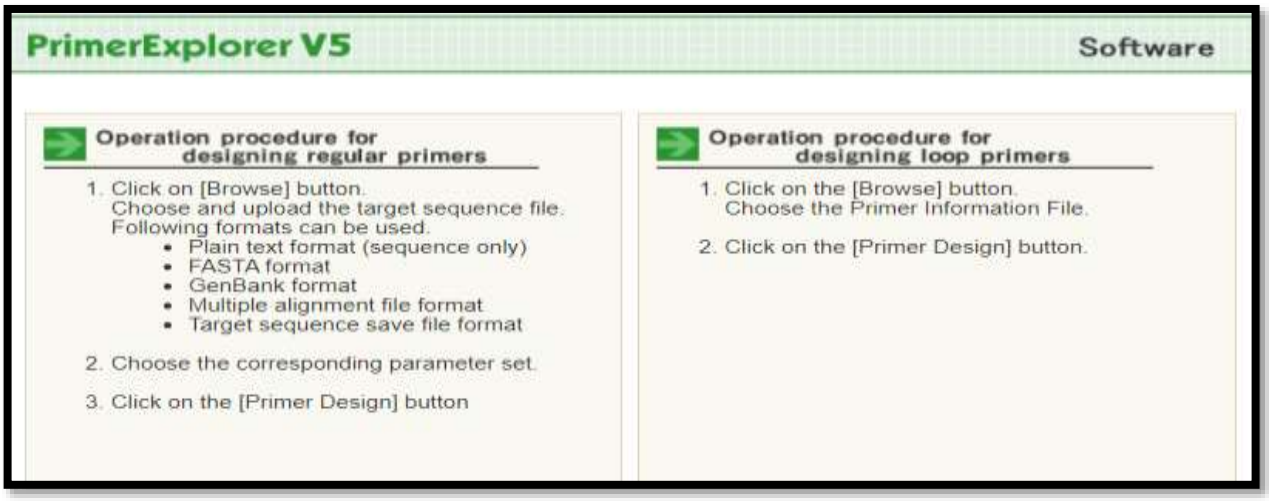


Fig. 3.3: Procedure for designing RT-LAMP primers through the software "Primer Explorer V5"

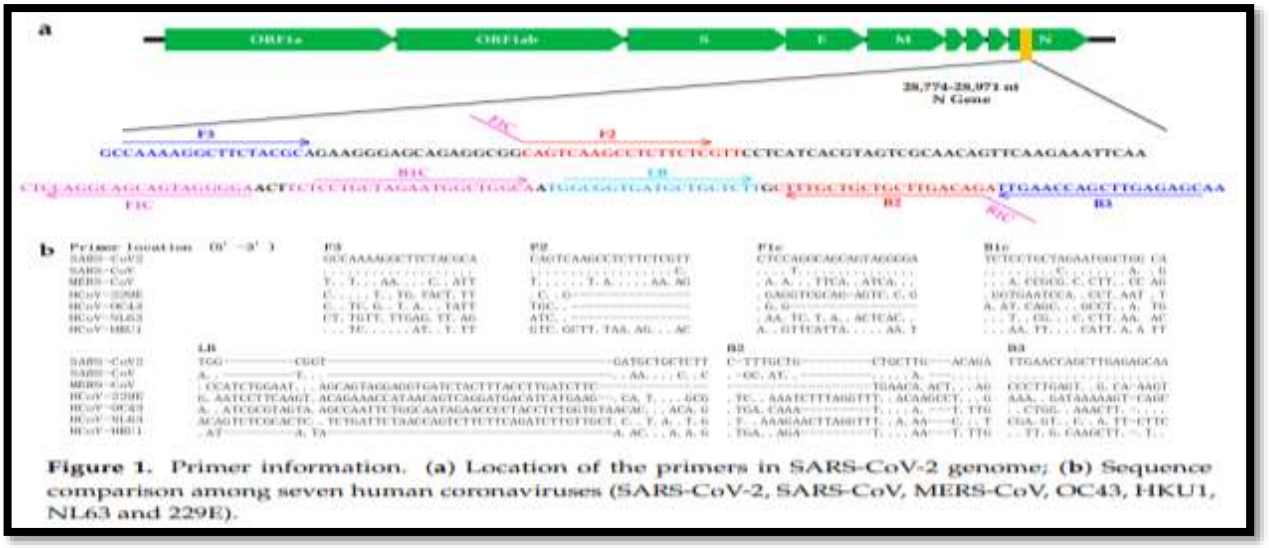


Figure 1. Primer information. **(a)** Location of the primers in SARS-CoV-2 genome; **(b)** Sequence comparison among seven human coronaviruses (SARS-CoV-2, SARS-CoV, MERS-CoV, OC43, HKU1, NL63 and 229E).

Fig. 3.4: (a): Location of primers in SARS CoV-2 genome; (b): Sequence comparison among seven coronaviruses namely SARS-CoV, MERS-CoV, OC43, HKU1, NL63, 229E ***(*Lu *et al.*, 2020*)***

Activity 4: Synthesis of artificial SARS-CoV-2 gene

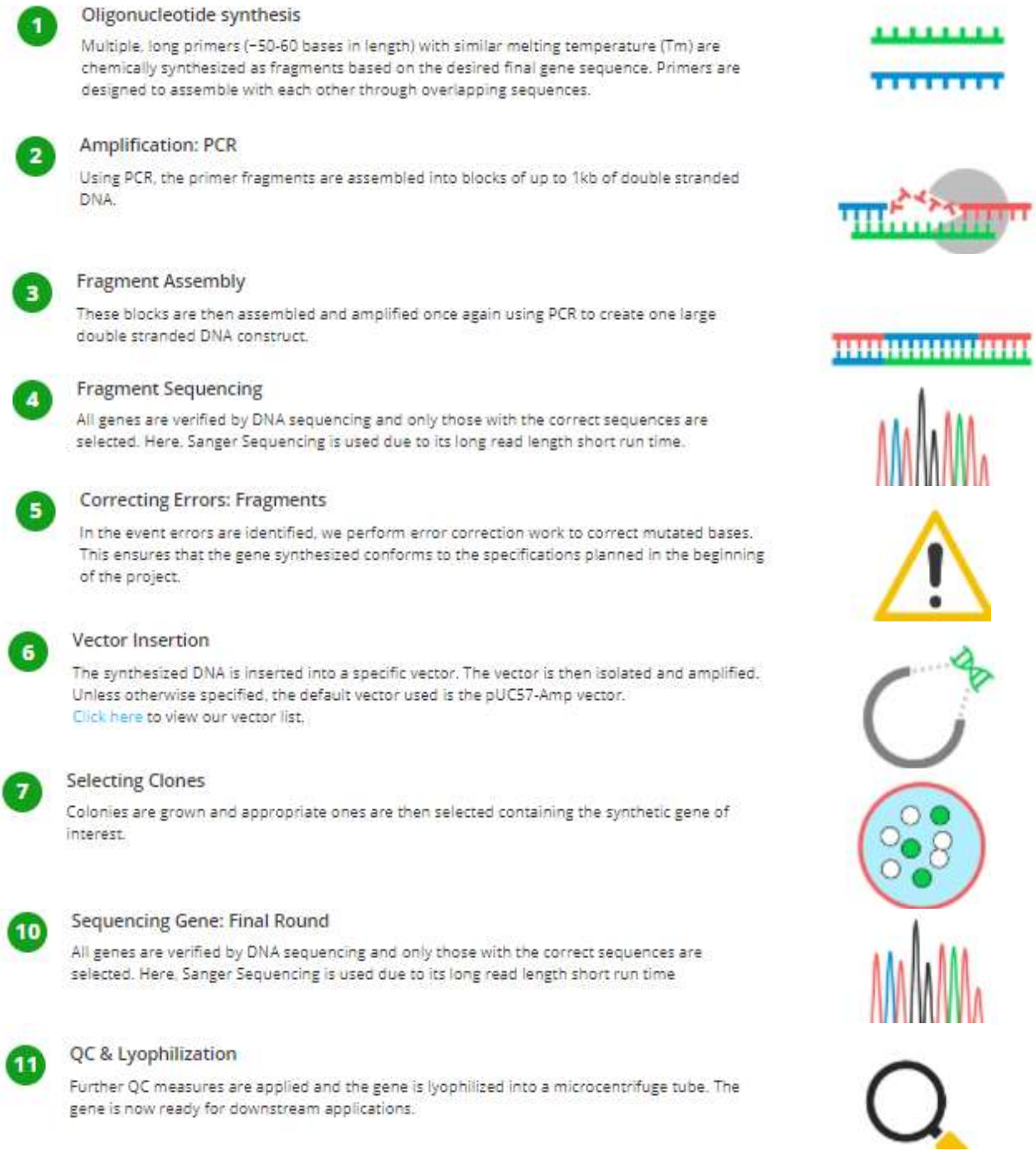
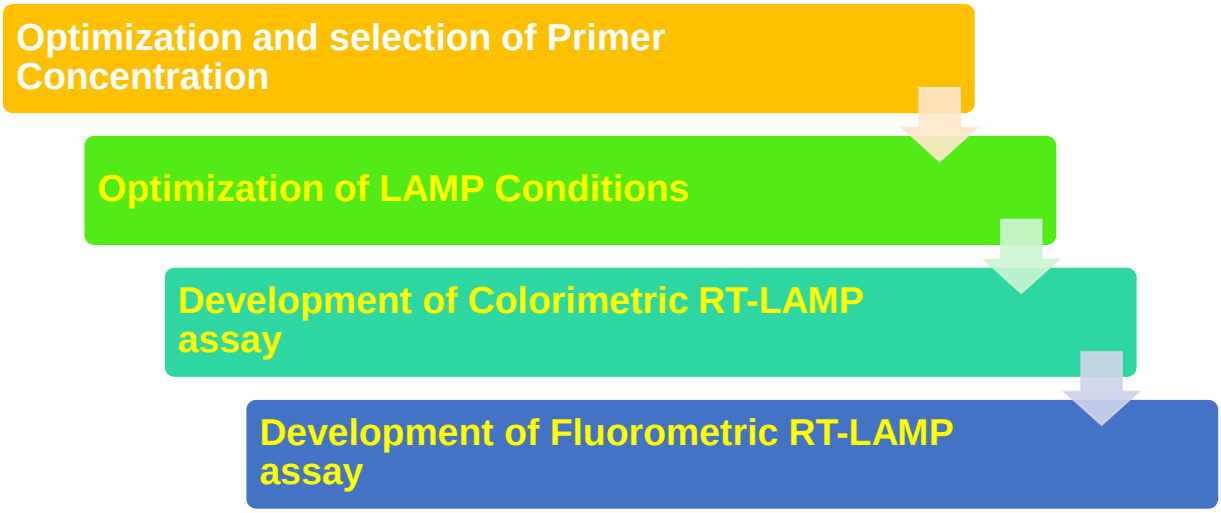


Fig. 3.5: Procedure for synthesis of artificial SARS CoV-2 genes or "Synthetic Gene Construct" (SGC)

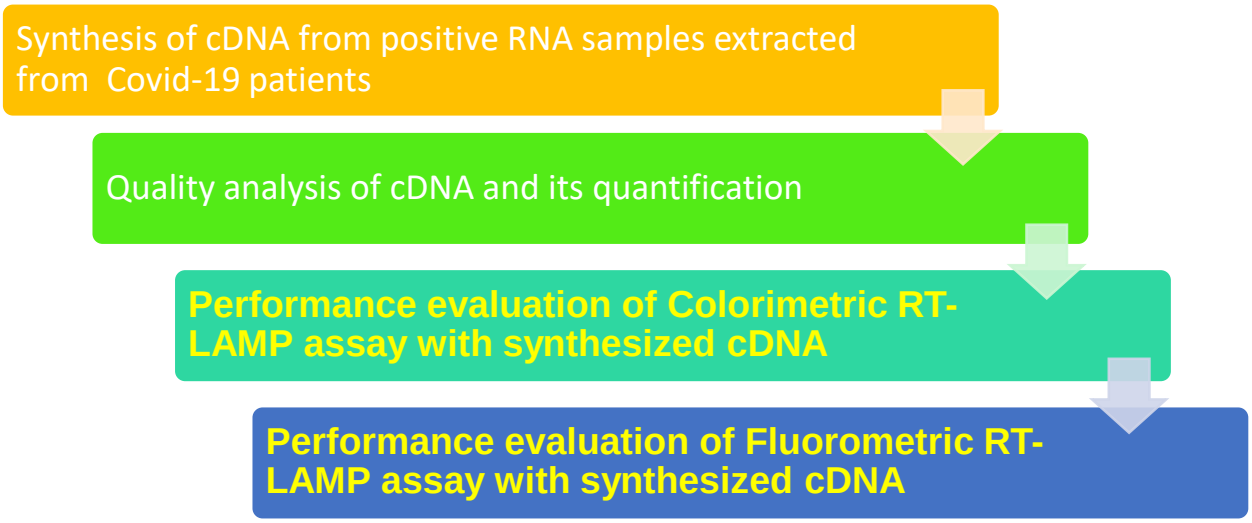
Development and performance evaluation of RT-LAMP assay

Objective 2

Activity 1: Development of colorimetric/flourometric LAMP assay using Synthetic Gene construct (SGC)



Activity 2: Performance evaluation of colorimetric/ flourometric LAMP assay using cDNA synthesized from SARS-CoV-2 RNA at KCGMC



Materials:

A. List of all the equipment used in the current study:

S.No.	Name of the equipment	Model	Company/ Manufacturer Name
1	Autoclave	AC-48	New Brunswick scientific, USA
2	Biosafety level-2 cabinet	Labconco	Labconco, USA
3	Centrifuge	6K15 Sartorius	Sigma, Germany
4	Centrifuge	R8C	REMI Laboratory
5	Centrifuge	Centrifuge 5418	Eppendorf, Germany
6	Deep freezer	R134 A	Vestfrost solutions
7	DNA electrophoresis system	Sub-cell® GT	BIO-RAD, USA
8	Freezer	DAEWOO	AR-310
9	Gel Documentation System	G-Box	Syngene, USA
10	Ice maker	F-200	Icematic, Italy
11	PCR 96 well thermal cycler	Veriti® 96-Well Thermal Cycler	Applied Biosystems/ Genex Life sciences, USA
12	Ultra low deep freezer	C760-86	New Brunswick scientific
13	UV trans-illuminator		
13	Vortex Mixer	SPINX MC-01	Tarsons, India
14	Water purification system	ZMQS5000Y	Merck Millipore, USA
15	Weighing balance	AE-200	Mettler, USA

B. List of all chemicals used in the current study

S. No.	Name of the chemical	Catalogue no.	Company/ Manufacturer Name
1	Agarose	RM187-100G	Hi-Media
2	EDTA	79F-0458	Sigma
3	Ethidium Bromide	BIO 15	Tarsons
4	Ethanol Absolute (99.9 %)	C1211132	Cympran Gludt BV
5	Glacial Acetic Acid	20001 L 25	Sd Fine Chem Ltd.
6	Hydrocloric Acid	32395	Samir Tech Chem, Ltd.
7	Phosphate buffered saline tablets	P4417-100TAB	Sigma
8	Primers (designed)	----------	IDT
9	Sodium hydroxide	RM467-500G	Hi-Media
10	Sulphuric Acid (H_2SO_4)	CK0C600460	Merck
11	Tris base	T153-1KG	Sigma

C. List of all the solutions/ buffers used in the current study

S. No.	Solutions/ Buffers	Composition	Source
1	Sample loading buffer (2X)	1.25 ml of Tris HCl (6.8 pH) 2 ml Glycerol, 4 mg Bromo phenol blue, 4 ml 10% SDS, 0.2 ml of β-mercapto	Lab prepared

		ethanol and make up to 10 ml using milliQ water	
2	50X TAE Buffer	24.2 g Tris base 5.71 ml Glacial Acetic Acid 100 ml 0.5 M EDTA pH-8.0	Lab prepared
3	TE Buffer	10 Mm Tris-HCl 1 Mm EDTA, pH-8.0	Lab prepared

D. List of all the softwares and web-tools used in the current study

S.No.	Software/ web-tool	Application
1	BLAST	For specific analysis of primers
2	Gel Doc	For Capturing gel images
3	GEN5-2.0 Version	For cDNA quantification
4	GISAID & BioEdit	For obtaining SARS CoV-2 Sequences
5	IDT	For designing RT-PCR primers
6	Primer Explorer V5	For designing RT-LAMP primers

Methods

Objective 1

3.1 Synthesis of artificial SARS-CoV-2 genes and designing of LAMP primers

3.1.1 Collection of complete genome sequences of SARS-CoV-2 reported from India

For the selection of target genes conserved among SARS-CoV-2 genomes reported from India, the complete genome sequences of SARS-CoV-2 reported from different parts of India were downloaded from GISAID (https://www.gisaid.org/) after successful registration **(Fig. 3.6).**

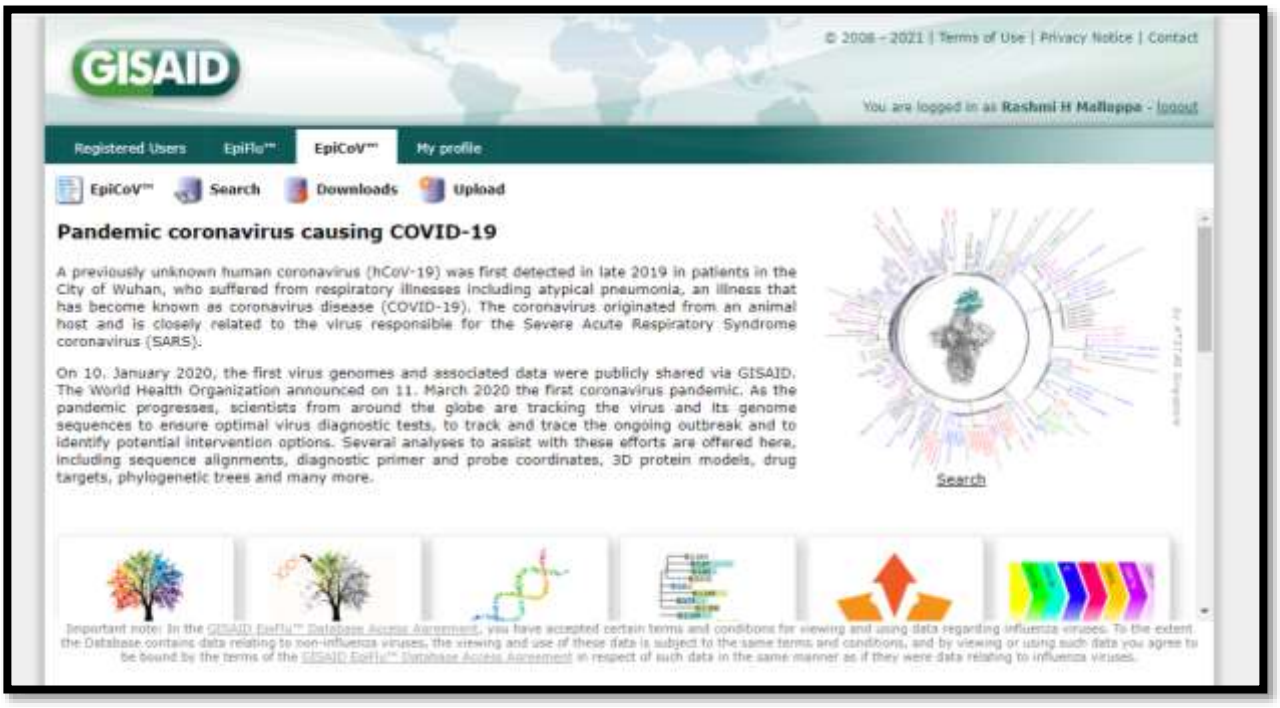


Fig 3.6: The GISAID website outlook used for downloading complete genome sequences of SARS-CoV-2 genomes reported from India

After registration, the complete genome sequences of SARS-CoV-2 with high coverage have been downloaded representing different states (Andhra Pradesh, Assam, Bihar, Gujarat, Haryana, Jammu and Kashmir, Karnataka, Ladakh, Madhya Pradesh, Maharashtra, Odisha, Punjab, Rajasthan, Tamil Nadu, Telangana, Uttar Pradesh and Uttarakhand), Union

territories (Delhi) and clades (G,GH and GR) have been downloaded in FASTA file format for the sequence analysis to identify conserved regions for selection of region for primer designing and subsequent synthesis of synthetic artificial SARS-CoV-2 genes.

3.1.2 Alignment of sequences and identification of conserved regions

The downloaded sequences have been aligned using GUIDANCE2, the server for alignment of sequences with MAFFT multiple sequence alignment (MSA) algorithm. After alignment, the aligned file was visualized in BioEdit, and the conserved and the variable regions of SARS-CoV-2 genomes reported from India were identified (**Fig. 3.7**).

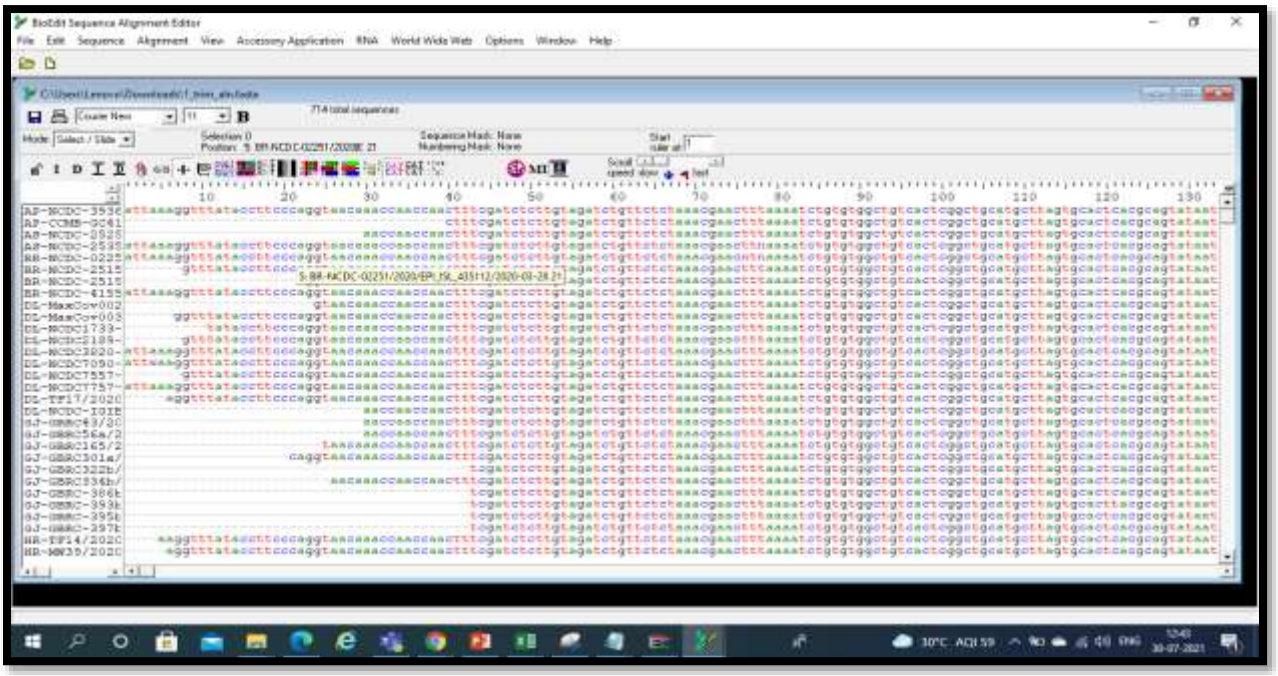


Fig. 3.7: The view of the aligned file used in the identification of conserved regions among SARS-CoV-2 genomes reported from India

3.1.3 Selection of conserved regions for primer designing and synthesis of artificial/synthetic SARS-CoV-2 gene

Before the selection of conserved regions for primer designing, the literature survey has been carried out to collect all sets of primer sequences earlier used in the development of

PCR- based assays for detection of SARS-CoV-2. The collected primer sequences were then searched for their presence in the identified conserved regions. Then, the conserved regions with no reported primer sequences in the literature were selected for designing RT-LAMP primers and subsequent synthesis of artificial/synthetic SARS-CoV-2 gene for the development of RT-LAMP assay. Besides, the size of the region and function of their genes encompassed were taken into consideration before their exploration in primer designing.

3.1.4 Designing of RT-LAMP Primers using "Primer ExplorerV5" software

Primer Explorer is a primer designing software, which is specifically for LAMP, a novel gene amplification method. This software can easily design 4 primers based on 6 regions required by the LAMP method besides designing loop primers. In addition, it uses the "nearest neighbor" method for Tm calculation and installation is unnecessary since it can be used via the web. Hence, the latest version of Primer Explorer software (Primer Explorer V5) was used to design LAMP primers for the development of RT-LAMP assay targeting selected conserved regions by following the procedure given in the website (http://primerexplorer.jp/lampv5e/index.html).

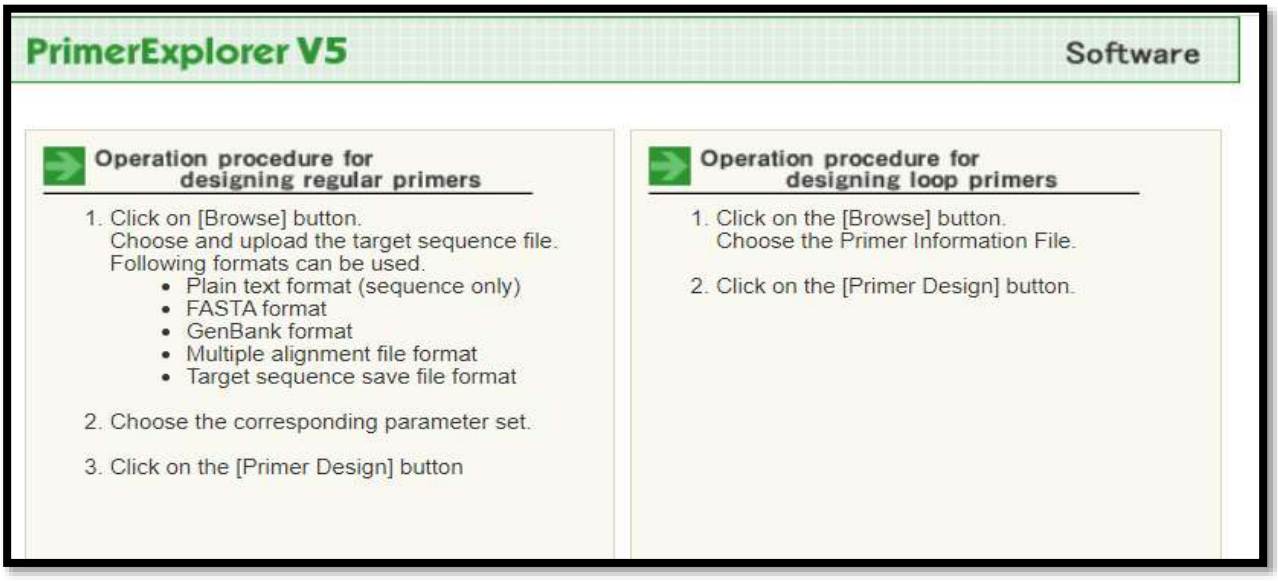


Fig. 3.8: Process of primer designing through the software "Primer Explorer V5"

3.1.5 *In silico* analysis of the specificity of LAMP primers

The designed primers were analyzed for their specificity with the known and previously discovered coronaviruses by the BLAST tool available in NCBI website and obtained information about relative similarity between them.

The designed primers were subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the sequence of specific candidate's target genes were used for similarity search with available genome sequences of coronaviruses (26). Besides the specificity was also checked with the other viral genome sequences available in the NCBI database.

3.1.6. Synthesis of primers

Primers were synthesized by Integrated DNA Technologies, India.

3.1.7 Synthesis of "Synthetic Gene Construct" (SGC) of the selected regions

We procured artificially synthesized gene targets of above selected regions. These artificial genes were synthesized in the laboratory of Bio Basics®- Gene Synt**hesis** by following 5-step procedure namely oligo synthesis, gene assembly, cloning, DNA sequencing and lastly lyophilization. The oligo synthesis included the chemical synthesis of multiple long-length oligos as segments according to the desired final gene to be synthesized. After oligo synthesis, all the resulted oligos are assembled with each other via overlapping sequences. For assembling the whole gene required primers of nearly around 50-60 base pairs (bp) and allowed to undergo PCR reactions to enable the oligos to be arranged in particular blocks of length 1 kb of double stranded DNA. Those double stranded DNA were then assembled followed by amplification once again through PCR reaction to generate large segment of the obtained double stranded DNA. The synthesized complete gene was then cloned into a particular high expression vector, pUC57 having 2710 bp in length. All the obtained and resulted genes were then verified by DNA sequencing (Sanger's sequencing) and only the correct ones were further selected. Only after assuring the quality of the selected genes, they were lyophilized and further downstream applications were performed. The resulted synthetic gene construct (SGC) was further utilized for carrying out the RT-LAMP experiments.

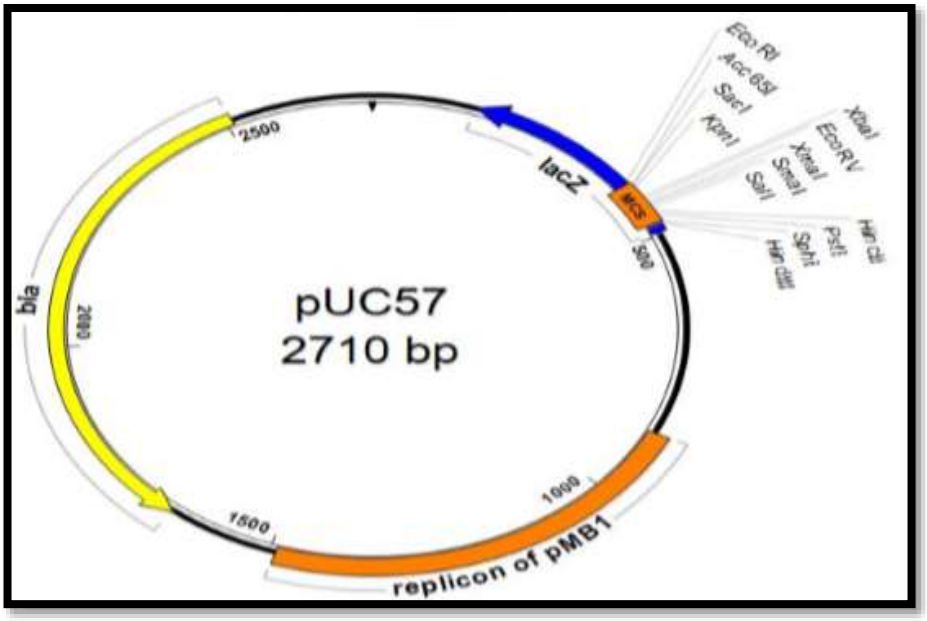


Fig. 3.9: Vector map of pUC57 used for cloning "Synthetic Gene Construct" (SGC)

Objective 2

3.2 Development and performance evaluation of RT-LAMP assay construct (SGC)

3.2.1 Development of colorimetric RT- LAMP assay using SGC

3.2.1.1 Optimization and selection of primer concentrations

A total of six sets of LAMP primers targeting three conserved regi**ons** were designed, two corresponding to each selected conserved region. In each primer sets consists of four primers i.e. two inner loop primers namely FIP (Forward Inner Loop) and BIP (Backward Inner Loop) and two outer loop primers namely F3 (Forward primer) and B3 (Backward primer). In the first step, the concentrations of primers (1:4; 1:6 and 1:8) to be utilized for the performance of RT-LAMP reaction were optimized. Finally, the concentration of primers resulting into the amplification of the target region with a good quantity of amplified products was selected for the development of RT-LAMP assay.

3.2.1.2 Preparation of RT-LAMP reaction mixture with optimized concentration of primer mix

The RT-LAMP reaction mixture was prepared according to the instructions provided by the commercial LMP reaction kit i.e. WarmStart® LAMP Kit (DNA & RNA) from NEB (New England Biolabs). The final prepared reaction includes the specific primer set mixture at optimized level, WarmStart LAMP 2X Master Mix, target DNA (synthetic gene construct) and nuclease-free water to make the required reaction volume (10/12.5/25µl).

3.2.1.3 Optimization of RT-LAMP reaction temperature

We have performed the RT-LAMP reaction after the preparation of the reaction mixture as explained in section **3.2.1.2**. For optimizing the conditions of LAMP experiment, we have employed the gradient the LAMP technique which involved three different temperature-time conditions i.e. 61°, 63° and 65° C for 40 minutes. Then, based on the intensity of bands developed on ethidium bromide staining of agarose gel loaded with LAMP amplified products, the temperature condition was selected for the final performance of LAMP reaction.

3.2.2 Development of colorimetric RT-LAMP assay

To develop a rapid RT-LAMP assay with visible detection of RT-LAMP products after amplification, we have employed three chromogenic dyes namely A, B and C at different concentrations (**Table 3.1**) to prepare LAMP reaction. The visual clear change in the colour or the development of clear distinct colour of the chromogenic dyes either due to change in pH of the LAMP reaction with the release of H^+ ions or due to variation in Mg^{2+} ion concentration during LAMP amplification was observed in comparison to a negative control.

Table 3.1: Details of Chromogenic dyes and their concentrations

chromogenic dye	Concentration
A	X, Y and Z
B	U and V
C	W

The optimal dye concentration was selected on successful and differentiable colour change in each of the LAMP reactions involving particular dyes.

3.2.3 Development of fluorometricc RT-LAMP assay

The fluorometric RT-LAMP assay was developed with the fluorescent dye provided with WarmStart® LAMP Kit (DNA & RNA) and the change in the fluorescence was observed under the UV light after the reaction.

3.2.4 Performance evaluation of colorimetric and fluorometric RT-LAMP assay with cDNA synthesized out of SARS-CoV-2 RNA

The performance evaluation of colorimetric and fluoroimetric RT-LAMP assay in the detection of SARS-CoV-2 was carried out at Viral Research and Diagnostic Laboratory, KCGMC, Karnal, Haryana. In this activity, we have collected around 26 positive Covid-19 RNA samples including a few Low Ct (Cycle threshold) from the staff of VRDL. The practical experiments involved a total of 5 day**s** at the Microbiology Department of KCGMC. The work schedule was followed religiously from morning 10:00 AM to 5 PM to perform all the experiments there. The required equipments, UV trans-illuminator, thermal cycler, gel electrophoresis system were taken from Molecular Biology Unit, Dairy Microbiology Division, ICAR-NDRI for the proper and efficient conduct of the experiments.

3.2.4.1 Collection of positive RNA samples and quality analysis

Each day fresh positive RNA samples were collected at VRDL for quality analysis followed by synthesis of cDNA. Initially, the integrity and quality of RNA samples by agarose gel electrophoresis using agarose gel (2%) and EtBr as DNA binding dye. The agarose gel loaded with RNA after agarose gel electrophoresis was observed for staining intensity RNA bands and any degradation products. The samples that exhibited bands with good intensity without any degraded products after visualization using UV-transilluminator were considered good quality for the extraction of cDNA.

Fig. 3.10: Illustration of work related to the quality analysis positive RNA samples used for the synthesis of cDNA

3.2.4.2 Synthesis of cDNA from the selected positive RNA samples

In the process of cDNA synthesis, RNA serves as a template and involves a major step of "Reverse Transcription" which involves the most important enzyme namely reverse transcriptase. The cDNA is usually formed by viruses and retro transposons for the purpose of integrating RNA into the genomic DNA. In the field of molecular biology, the RNA molecule first needs to be purified and should be free from DNA, protein and several other cellular components and afterwards the synthesis of cDNA from RNA is being done *in-vitro* through reverse transcription step. For the purpose of the synthesis of appropriate cDNA from those RNA samples, we have utilized two different cDNA synthesis kit namely iScript™ cDNA Synthesis Kit (Bio-Rad) and Revert Aid First Strand cDNA Synthesis Kit from Thermo Scientific, India.

As it is well known that PCR technique can be applied for the amplification of RNA molecules in which condition, it is also referred as reverse transcription – PCR or much widely followed and commonly known as RT-PCR. For RT-PCR, the molecule of RNA i.e. mRNA should first converted into the cDNA (complementary DNA) through the action of unique enzyme namely reverse transcriptase. Further, the obtained cDNA then acts as a template for PCR. The synthesis of first strand of cDNA, requires several different types of primers which

includes random primer, oligo dT primer with some specifically designed sequence primers. The **fig. 3.11** below shows the mechanism of the first strand synthesis of cDNA through the use of several primers and also the primers are depicted in purple colour in the figure given below and table depicts the PCR cycling conditions for cDNA synthesis.

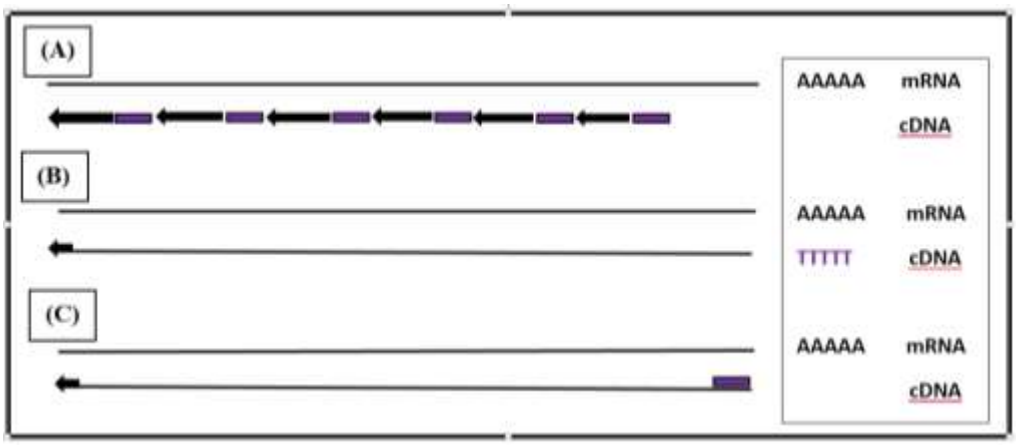


Fig. 3.11: Synthesis of first strand of cDNA in RT-PCR (reverse transcription PCR) through different primers. (A) Random primers (B) oligo dT primer (C) Sequence specific primer. (Reference Book- Biotechnology: Author- U. Satyanarayana)

Table 3.2: PCR cycling conditions for cDNA synthesis

S. No.	Step	Temperature Condition
1	Priming	5 min at 25°C
2	Reverse Transcription	20 min at 46°C
3	RT inactivation	1 min at 95°C
4	Optional Step	Hold at 4°C

3.2.4.3 Quality analysis of cDNA samples by agarose gel electrophoresis

The synthesis of cDNA from RNA was confirmed by Agarose Gel Electrophoresis. The appearance of smeared bands on visualization under UV-trans illuminator confirmed the synthesis of cDNA from RNA to be used as template for the performance evaluation of developed colorimetric or flourimetic RT-LAMP assays.

Fig.3.12: Gel Electrophoresis performed for visualization of synthesized cDNAs from positive RNA samples

3.2.4.4 Performance evaluation of colorimetric and fluorometric RT-LAMP assay using cDNA synthesized from SARS-CoV-2 RNA at KCGMC

The performance evaluation of developed colorimetric and fluorometric RT-LAMP assay was carried out by using cDNA synthesized from positive RNA samples as template, while the synthetic gene construct served as positive control. All the colorimetric and fluorometric assays were carried out as explained earlier with optimized concentration of chromogenic and fluorogenic dyes. At the end, the obtained results were compared to assess the performance of developed colorimetric and fluorometric RT-LAMP assays.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Effective management and prevention of any disease require robust diagnostic techniques that can aid in the early detection and diagnosis of the disease. The necessity of these diagnostic tools have been very strongly felt in the current pandemic scenario where the entire management of the pandemic is based on the three Ts i.e. Test, Track and Treat. The prominence accorded to Testing as the first T shows the crucial role of Diagnostic testing, emphasizing that these are the need of the hour. These diagnostic tools can prove to be a crucial weapon in our fight against the novel Coronavirus. Rapid and accurate diagnostic tests are imperative for identifying and managing infected individuals, contact tracing, epidemiologic characterization, and public health decision making. Laboratory testing may be performed based on symptomatic presentation or for screening of asymptomatic people. Nucleic acid amplification tests (NAAT) are generally the gold standards for confirming SARS-CoV-2 infection, RT-PCR being the widely used one. However, RT-PCR requires specialized equipment, skilled personnel training and may be particularly challenging in resource-limited settings. Besides, long turn-around time (24 h) are not feasible for local and referral laboratories.

Hence, in the current investigation, an attempt was made to develop colorimetric/fluorometric loop mediated isothermal amplification, also known as LAMP, to do away with expensive technologies, like real-time PCR and to shorten the turn-around time to up to 40 min. In this investigation, we intended to develop RT-LAMP assay as Point of Care (PoC) diagnostic tool for the detection of human SARS CoV-2 with the objectives of development of colorimetric or fluorometric RT-LAMP assay and evaluation of its performance. Initially, the complete genome sequences of human SARS CoV-2 sequenced from different parts of India were used to identify the target gene or region which can serve as a potential target for designing RT-LAMP primers. Besides, the same target gene was also got synthesized in the form of synthetic gene construct which could serve as positive control in the lab for the development of assay. The designed primers were then explored for the development of RT-LAMP assay using synthetic gene construct as template. Besides, initially several parameters such as primer concentration and their ratio, LAMP reaction conditions were optimized for the successful amplification of the target region.

Later, the concentrations of various chromogenic dyes were optimized for the development of colorimentric LAMP assays. Further, the same conditions were used to develop fluorometric LAMP assay. Finally, the performance of developed colorimentric/fluorometric RT-LAMP assay was evaluated with the cDNA synthesized out of RNA extracted from SARS CoV-2. The results obtained during this course of investigation were compiled and discussed in this section.

Objective 1

4.1 Designing of LAMP primers and Synthesis of artificial SARS-CoV-2 genes

4.1.1 Collection of complete genome sequences of SARS-CoV-2 reported from India

The GISAID (Global **I**nitiative on **S**haring Avian **I**nfluenza **D**ata; (https://www.gisaid.org/)) is basically a consortium in which its member release databases publically up to a period of six months after appropriate analysis and validation. Even though, several databases for genome sequences exist, GISAID is by far the most popular database for SARS-CoV-2. This consortium shared the first complete genome sequence SARS CoV-2 and more than 1.2 million coronavirus genome sequences from 172 countries and territories have been shared in this popular online data platform, which is a testament to the hard work of researchers around the world during the pandemic (**Fig. 4.1**). Hence GISAID database was chosen and get registered to download sequences reported from different parts of India (Yadav *et al.*, 2021).

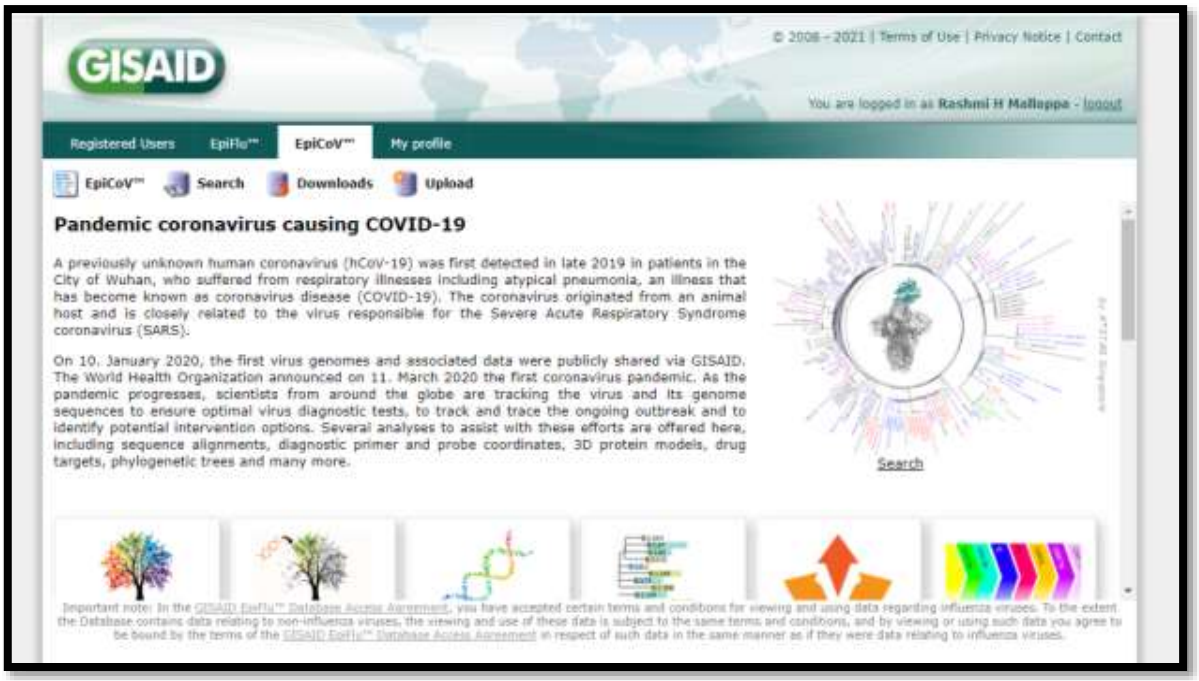


Fig 4.1: Registration in GISAID to collect genome sequences of SARS CoV-2 reported

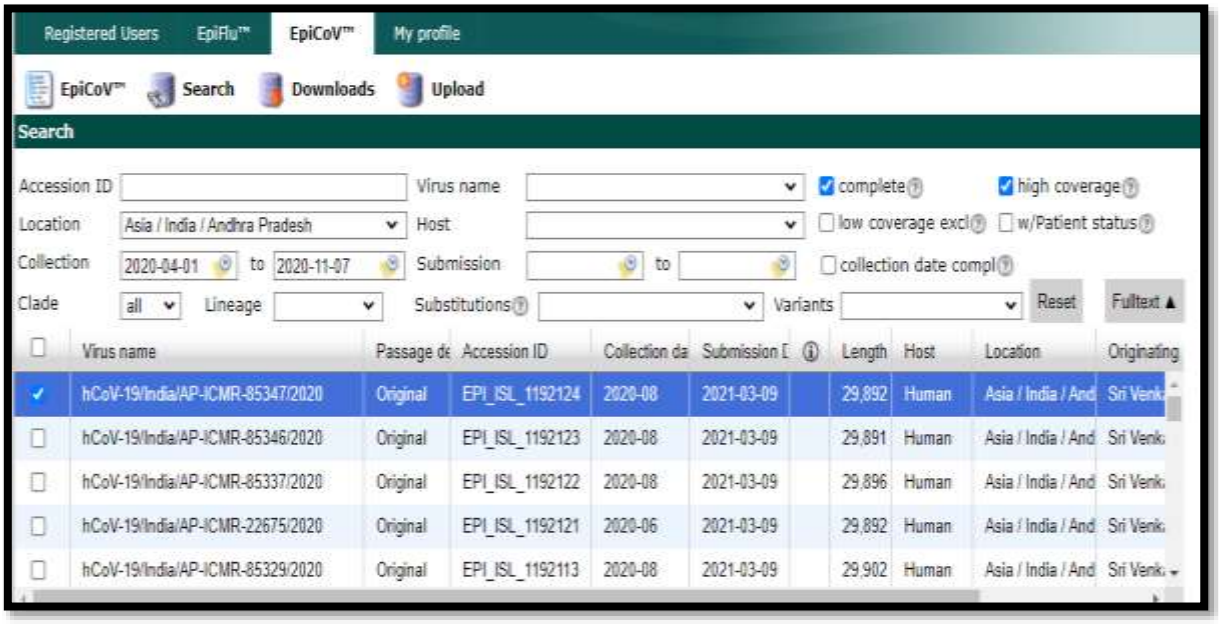


Fig. 4.2: Collection of genome sequences of SARS-CoV-2 from GISAID database

After registration, a total of around 142 sequences of SARS CoV-2 were selected from several Indian States and Union Territories namely, Andhra Pradesh, Assam, Bihar, Delhi, Gujarat, Haryana, Jammu and Kashmir, Karnataka, Ladakh, Madhya Pradesh, Maharashtra, Odisha, Punjab, Rajasthan, Tamil Nadu, Telangana, Uttar Pradesh and Uttarakhand (**Fig. 4.2**) and downloaded in the FASTA format to select the target region for designing RT-LAMP primers and subsequent synthesis of the primer binding regions (synthetic gene/artificial gene construct) for the development of RT-LAMP assay (**Table 4.1**).

Efforts have also been made earlier by several investigators to explore GISAID dataset for designing different primer sets and these investigators proved the ability of selected nucleic acid sequences obtained out of GISAID dataset to separate SARS-CoV-2 from different virus strains with near-perfect accuracy (Lanza *et al.*, 2020 ; Lopez-Rincon *et al.*, 2021). Besides, the designed primers were able to deliver highly sensitive results in routine diagnostic methods with 100% specificity (Lopez-Rincon *et al.*, 2021)

Table 4.1: Selection of complete genome sequences obtained from different regions of India

S. No	State or Union Territory	No. of complete genome sequences selected for analysis
1	Andhra Pradesh	2
2	Assam	2
3	Bihar	4
4	Delhi	10
5	Gujarat	10
6	Haryana	8
7	Jammu Kashmir	1

8	Karnataka	10
9	Ladakh	6
10	Madhya Pradesh	9
11	Maharashtra	13
12	Odhisha	10
13	Punjab	5
14	Rajasthan	6
15	Tamil Nadu	10
16	Telangana	10
17	Uttar Pradesh	16
18	Uttarakhand	10
	Total	**142**

4.1.2 Alignment of sequences and identification of conserved regions

All the 142 sequences were aligned including the first genome sequence reported from China to identify the conserved region. Initially, the sequences were aligned using GUIDANCE2, the server for alignment of sequences with MAFFT ((Multiple Alignment using Fast Fourier Transform) multiple sequence alignment (MSA) algorithm after alignment, the aligned file was visualized using BioEdit programme to identify conserved regions (**Fig. 4.3**).

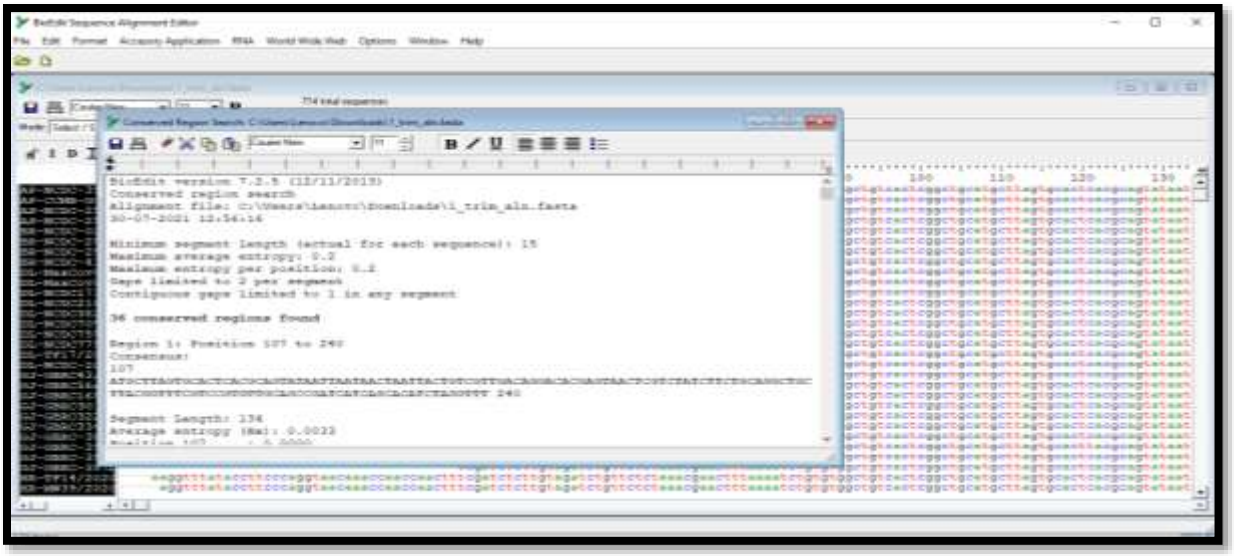


Fig. 4.3: The view of identified conserved regions of SARS CoV-2 from aligned sequences

A total of 36 conserved regions were identified after the alignment and their position and respective size have been shown in the **Table 4.2**. Further, the reported primer sequences that have been designed earlier for the detection of SARS-CoV-2 by RT-PCR and RT-LAMP assays have been collected from the literature and their positions have been located in the conserved regions as indicated in **Table 4.2**.

Previously, investigators have also used several multiple sequence alignment (MSA) tools (ClustalW, MUSCLE and ClustalO) including MAFTT to align and subsequently infer the genetic variability in Indian SARS-CoV-2 genomes by identifying the mutations as substitution, deletion, insertion and SNP in the complete genome sequences of SARS-CoV-2. (Saha *et al.,* 2020). Similarly, in our study, MAFTT tool was able to identify 36 conserved regions without any mutations (substitution, deletion and insertion) among 142 reported sequences of SARS-Cov-2, thereby giving non-variable (conserved) regions for designing highly sensitive primers for the development of nucleic acid based diagnostic assays.

Table 4.2: Details of conserved regions identified among the SARS-CoV-2 genome sequences reported from India

Conserved Region	Genes	Position	Size (bp)	No. of Reported Sequences
1	Nsp1	107-240	133	Nil
2	Nsp1	242-312	70	Nil
3	Nsp1 & Nsp2	314-883	569	3
4	Nsp2	885-1946	1061	Nil
5	Nsp2 & Nsp3	1948-3036	1088	Nil
6	Nsp3	3038-5699	2661	5
7	Nsp3	5701-6309	608	1
8	Nsp3	6313-8021	1708	2
9	Nsp3 & Nsp4	8023-8916	893	Nil
10	Nsp4, Nsp5 & Nsp6	8918-11082	2164	Nil
11	Nsp6	11084-11234	150	Nil
12	Nsp6, Nsp7, Nsp8 & Nsp9	11236-12684	1448	Nil
13	Nsp9 & Nsp10	12686-13729	1043	7
14	Nsp10 & Nsp11	13731-14407	676	4
15	Nsp11 & Nsp12	14409-15323	914	4
16	Nsp12	15325-15434	109	2
17	Nsp12 & Nsp13	15436-16992	1556	4
18	Nsp13 & Nsp14	16994-18876	1882	1
19	Nsp 14 & Nsp15	18878-19302	424	1

20	Nsp15, Nsp16 & S	19549-21990	2441	5
21	S	21994-22443	449	1
22	S	22445-22514	69	Nil
23	S	22516-23402	886	4
24	S	23404-23928	524	Nil
25	S	23930-24388	458	4
26	S & orf3a	24391-25562	1171	3
27	Orf3a	25564-26064	500	Nil
28	M	26066-26157	91	Nil
29	M	26162-26350	188	2
30	M	26357-26708	351	1
31	M	26710-26734	24	Nil
32	M, orf7a, orf8 & N	26736-28310	1574	3
33	N	28312-28687	375	5
34	N	28689-28853	164	4
35	N	28855-28880	25	Nil
36	N & orf10	28884-29686	802	11

4.1.3 Selection of conserved regions for primer designing and synthesis of artificial/synthetic SARS-CoV-2 gene

In order to get accurate results the selection of target genes/regions and designing suitable primers is imperative for the development of an RT-LAMP detection kit with enhanced precision and accuracy. It is important to ensure that the primers scoped through these approaches can be able to detect the most important genomic segment or gene of interest

of SARS CoV-2 for better efficacy. The genome segments should be conserved and involved in the main function of the virus such that the mutations should not have any influence on the genome segments targeted for the study.

The selection of the most relevant gene to be targeted to detect SARS CoV-2 is very crucial step for the development of RT-LAMP diagnostic kit. For the effective selection, we have searched all the relevant and essential information about the most abundant structural and non-structural genes of SARS CoV-2 to get an idea of the repertoire of genes of SARS CoV-2 that participate in the process of replication and induce pathogenicity inside the human host cells after entering the human body upon illness or infection. After a thorough literature review of all the known facts and details helpful for the selection of potential target genes and other proteins available in the literature, we selected a total of three conserved regions to be targeted by the RT-LAMP assay kit for accurate detection of SARS CoV-2. Also, corresponding to these selected regions, no primers were reported or designed to detect SARS CoV-2 infections, previously.

Out of 36 conserved regions, a total of 13 conserved regions were found to be not yet explored for primer designing, as was evident from the literature survey. However, three conserved regions were found associated with replication of the viral genome and are comprised of non-structural proteins with least and no variations (mutations). Hence these three conserved regions covering non-structural proteins were selected to design RT-LAMP primers.

4.1.4 Designing of RT-LAMP primers using "Primer ExplorerV5" software

As explained earlier, Primer Explorer is a primer designing software, widely used for designing primers for LAMP, a novel gene amplification method (Yoshikawa *et al.*, 2020). Hence, in the present investigation, LAMP primers were designed through Primer Explorer V5 software (Eiken; http://primerexplorer.jp/) as shown in **Fig. 4.4**. The RT-LAMP assay requires a set of 6 primers: 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP), and 2 loop primers (LF and LB). FIP consists of a complementary sequence of F1 and a sense sequence of F2, whereas BIP includes a complementary sequence of B1 and a sense sequence of B2 (10). The detailed primer sequences used for SARS-CoV-2 amplification are shown in **Table 4.3.**

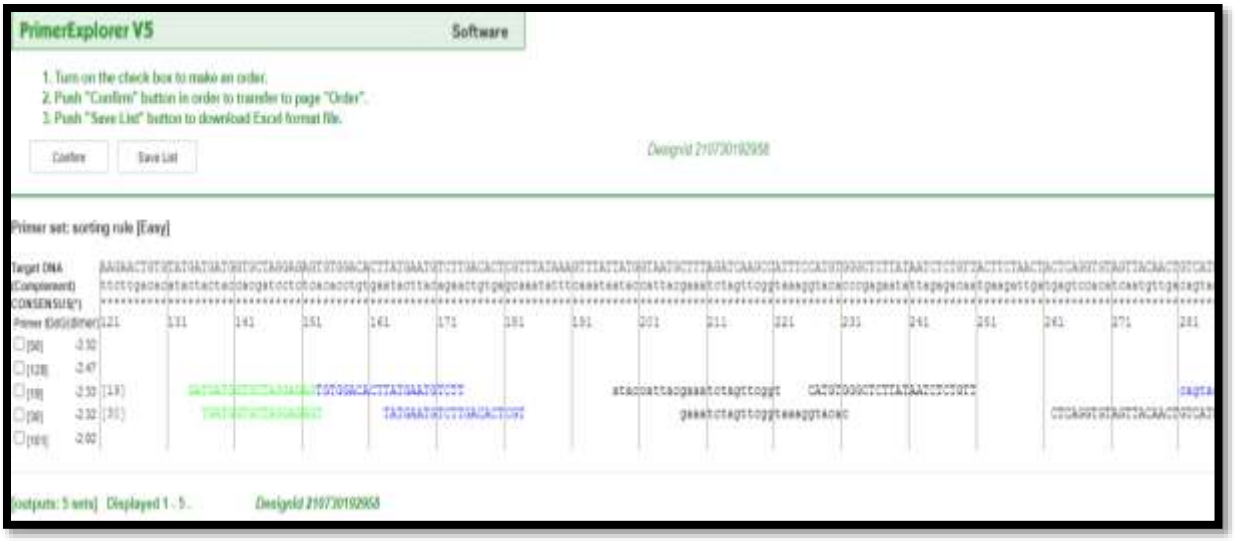


Fig. 4.4: Designed LAMP primer sets being generated through PrimerExplorerV5

Table 4.3: Targeted conserved regions and respective sets of primes selected for the development of RT-LAMP assays.

Targeted conserved Regions	No of LAMP primer sets designed	Primer Sets
X	3 sets	A
		B
Y	3 sets	C
		D
Z	3 sets	E
		F

A total of nine sets of primers were designed targeting three conserved regions. However, only six sets of primers (**Table 4.4**), two each from a single conserved region were selected for the development of RT-LAMP assay due to the limitation of time.

Table 4.4: Details of primer sets designed for the development of RT-LAMP assay

Name	Type	Sequence
12AF3	F3	To be patented
12AB3	B3	To be patented
12AFIP	FIP	To be patented
12ABIP	BIP	To be patented
12AF1c	LF	To be patented
12AB1c	LR	To be patented
12F3	F3	To be patented
12BF3	B3	To be patented
12BFIP	FIP	To be patented
12BBIP	BIP	To be patented
12BF1c	LF	To be patented
12BB1c	LR	To be patented
15AF3	F3	To be patented
15AB3	B3	To be patented
15AFIP	FIP	To be patented
15ABIP	BIP	To be patented
15AF1c	LF	To be patented

15AB1c	LR	To be patented
15BF3	F3	To be patented
15BB3	B3	To be patented
15BFIP	FIP	To be patented
15BBIP	BIP	To be patented
15BF1c	LF	To be patented
15BB1c	LR	To be patented

Earlier investigators (Yoshikawa *et al.,* 2020) were also successful in designing LAMP primers targeting ORF1b region of SARS-CoV-2 using Primer Explorer V5 software (Eiken; http://primerexplorer.jp/). Besides, several investigators have also explored different gene targets (Orf1ab, spike glycoprotein (S), nucleocapsid protein (N)) for designing LAMP primers. However, the primers designed in the present investigation were different from the previous sets of primers reported by several other investigators (Huang *et al.,* 2020**;** Yoshikawa *et al.,* 2020, Rabe *et al.,* 2020**).**

4.1.4.1 Evaluation of primer specificity

The specificity of the designed primer sets was evaluated via sequence alignment with other previously reported human coronaviruses. We also performed the homology search with the help of BLAST tool available in NCBI website and we have found that the designed primer sets have matched only with the genome sequences of MERS CoV and Bat SARS-like coronavirus (SARS-CoV) and no similarity was detected in the genome sequences of other human viruses like HIV, HCoV-OC43, HCoV-229E, HCoV-HKU1, Hepatitis B type Virus, Hepatitis Delta Virus, Dengue, Human orthopneumovirus, Enterovirus A, Enterovirus D, Hepacivirus C, Chikungunya Virus, Zaire Ebolavirus, Orthohepevirus A, Novovirus G II, Mumps rubulavirus, Zika Virus, Measles morbilivirus, BtCoV-HKU10, BtCoV/133/2005, BtCoV/22/29, BtCoV/335/2005 and Human rotavirus **(Table 4.5).**

Table 4.5: Similarity and homology analysis with all previously discovered coronaviruses

Viruses	Region 12	Region 15	Region 17
MERS-CoV	Only primers matched	Only primers matched	Only Primers matched
Bat SARS like Coronavirus	Matched (sequences and primers both)	Matched (sequences and primers both)	Matched (sequences and primers both)
HIV	No Match	No Match	No Match
HCoV-OC43	No Match	No Match	No Match
HCoV-229E	No Match	No Match	No Match
HCoV-HKU1	No Match	No Match	No Match
Hepatitis B type Virus	No Match	No Match	No Match
Hepatitis Delta Virus	No Match	No Match	No Match
Dengue	No Match	No Match	No Match
Human orthopneumovirus	No Match	No Match	No Match
Enterovirus A	No Match	No Match	No Match
Enterovirus D	No Match	No Match	No Match
Hepacivirus C	No Match	No Match	No Match
Chikungunya Virus	No Match	No Match	No Match

Zaire Ebolavirus	No Match	No Match	No Match
Human Respirovirus 3	No Match	No Match	No Match
Orthohepevirus A	No Match	No Match	No Match
Novovirus G II	No Match	No Match	No Match
Mumps rubulavirus	No Match	No Match	No Match
Zika Virus	No Match	No Match	No Match
Measles morbilivirus	No Match	No Match	No Match
BtCoV-HKU10	No Match	No Match	No Match
BtCoV/133/2005	No Match	No Match	No Match
BtCoV/22/29	No Match	No Match	No Match
BtCoV/335/2005	No Match	No Match	No Match
Human rotavirus	No Match	No Match	No Match

Hence, all the selected six primers sequences were found selective for the detection of SARS-CoV-2 including MERS-CoV and Bat SARS like Coronavirus.

In *in-silico* analysis for specificity, the investigators Lopez-Rincon *et al.,* 2021, also compared all the primers sets' sequences with the NCBI-B and NGDC dataset and the results displayed the non-specific nature of designed SARS-CoV-2 primers with that of other SARS-CoV.

After specificity analysis, the selected primers were get synthesized by Integrated DNA Technologies, India.

4.1.5 Synthesis of "Synthetic Gene Construct" (SGC) of the selected regions

The selected conserved regions were synthesized from Bio Basic, India and quality report including restriction digestion analysis report of the synthesized gene was given **Table 4.6** and **Fig. 4.5.**

Table 4.6: Gene synthesis report of selected conserved regions

BBI ID	BioL279		
Work No.		Gene Name	X
Length (bp)	1449	Order Date	
Vector	**pUC57**	Delivery Date	
Plasmid No.	BioL279-043021	Storage	-20°C
Cloning Site	SmaI	Host	TOP10
Resistance	Amp	Plasmid Qty.	4ug/Vial
Growth Condition/		Competent Cells	*E. coli*

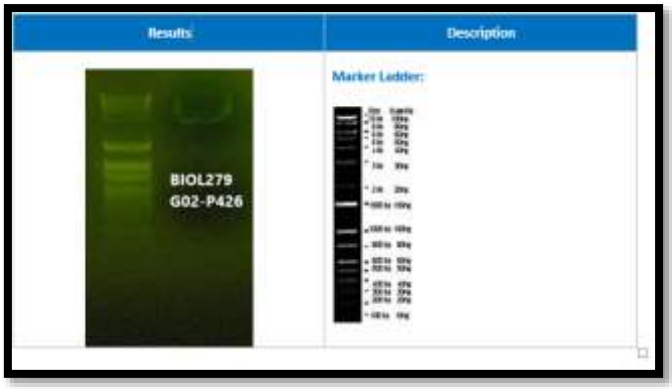


Fig 4.5: Restriction enzyme digestion analysis of Synthetic Gene Cosntruct (SGC)

After getting sufficient quantity of SGC with satisfactory quality, the lyophilized plasmid SGC was dissolved in TE buffer to the final concentration is ~100 ng/µl and was stable at -20°C for downstream application i.e. for its use as positive template in the development of LAMP assay with selected set of primers.

Objective 2

4.2 Development and performance evaluation of RT-LAMP assay

4.2.1 Development of colorimetric RT- LAMP assay using SGC

4.2.1.1 Optimization and selection of primer concentrations and ratio

In the first step, we have optimized the concentration and ratio of primers (A, B and C) to be utilized for the performance of RT-LAMP reaction. Finally, the optimized concentration of primers with B ratio which resulted in amplification of the target region with a good quantity of amplified products was selected for the development of RT-LAMP assay.

4.2.1.2 Optimization of RT-LAMP reaction temperature

For, optimization of RT-LAMP reaction temperature, we employed the gradient LAMP technique which involved three different temperature-time conditions i.e. 61°, 63° and 65° C for 30-40 minutes. Also, in some of the reactions, a final inactivation step at a specific temperature of 80° C for 10 minutes was employed (**Fig. 4.6**). With these conditions, all the primer sets at optimal concentration and ratio were used to check at what temperature they will be able to bind effectively and yield the highest concentration of amplified products by using synthetic gene constructs (SGC) of interest as templates. The yield of LAMP products at different annealing temperatures was analyzed through agarose gel electrophoresis and the results of the same were shown in **Fig.4.7.**

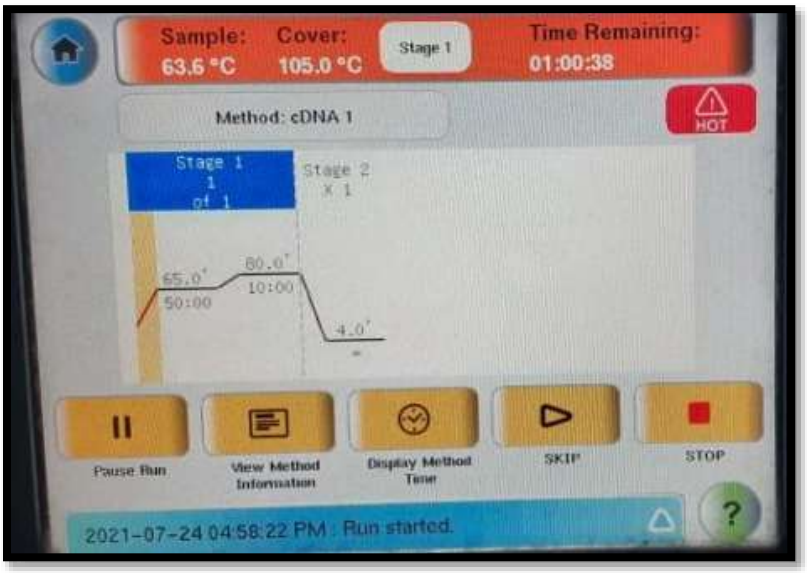


Fig. 4.6: Gradient LAMP assay conditions being performed using thermal cycler

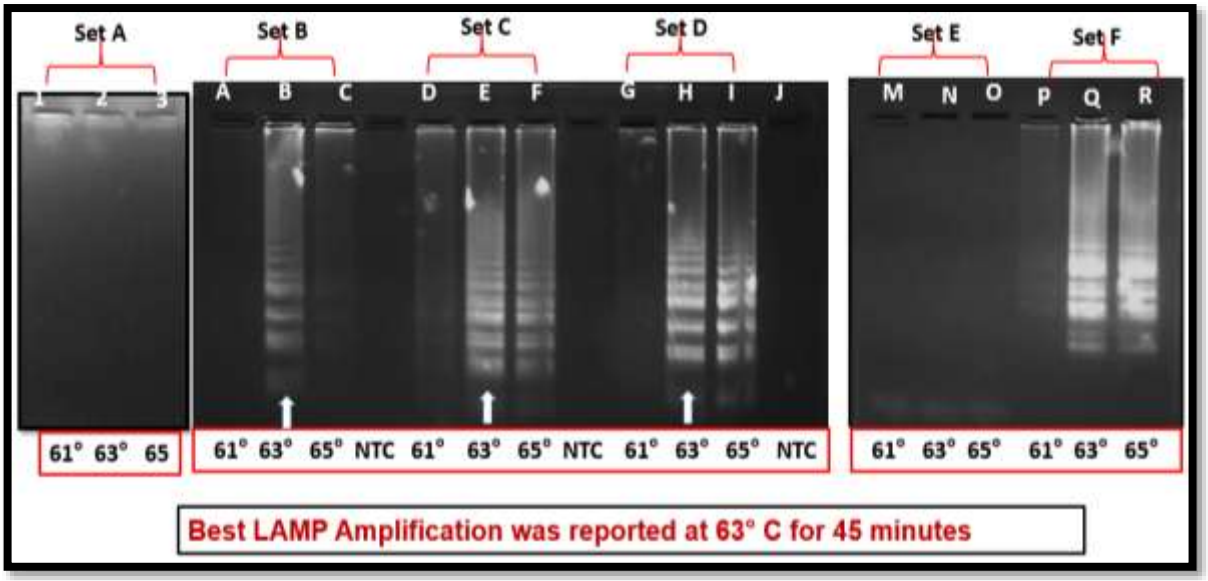


Fig. 4.7: Agarose Gel Electrophoresis confirmed the best amplification at 63^0C with high intensity of bands visualized after loading respective LAMP products

From the above figure, it is clearly evident that out of six set of primers (A, B, C, D, E and F), four sets of primers (B, C, D and F) were able to yield LAMP products using synthetic gene constructs. Besides, we found 63°C optimum temperature and 40 min as the minimal time to generate a large number of LAMP products which can be clearly visualized on examination of EtBr stained agarose gel under UV light. Hence, LAMP temperature and time combination of 63°C for 40 min was selected for the development of LAMP assay.

Though all the four sets of primers (B, C, D and F) were able to yield LAMP products at aforesaid temperature and time conditions, we selected primer set B for the development of colorimetric LAMP assay due to time constraint and limitation of resources like the sufficient availability of SGC for optimization of colorimetric LAMP assay (**Fig. 4.8**).

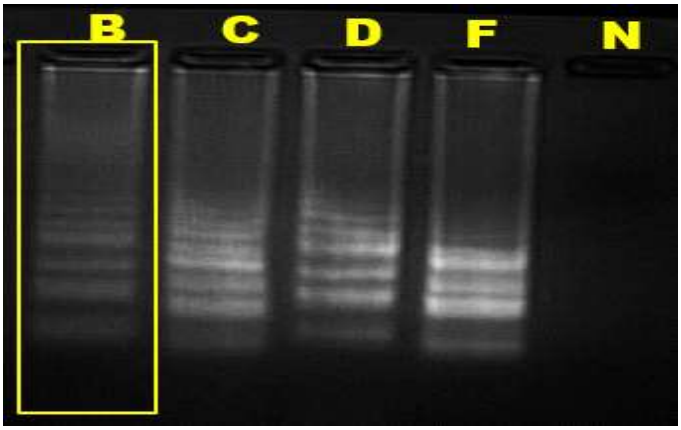


Fig. 4.8: Selection of Primer set 'B' for the performance of final RT-LAMP

Earlier investigators have also reported temperature and time combinations of 63°C/40min for generation of LAMP products with good intensity (Pandey *et al.*, 2018). Our results in this regard are very much similar to those of earlier developed LAMP methods with the designed set of LAMP primers

4.2.1.3 Performance evaluation of selected primer (B) with cDNA synthesized from SARS-CoV-2 RNA

After optimizing LAMP conditions with the help of synthetic gene construct, we proceeded to evaluate the performance of the developed LAMP assay cDNA being synthesized from SARS CoV-2 RNA at Kalpana Chawla Government Medical College (KCGMC), situated in Karnal, Haryana. In performance evaluation, we observed a similar kind of amplification (band pattern) being produced at the end of LAMP assays carried out with cDNA (A1 and A2) and SGC (B1 and B2) as template with alike intensity under the UV trans-illumination of EtBr stained agarose gel located with the LAMP amplified products. **(Fig. 4.9)**

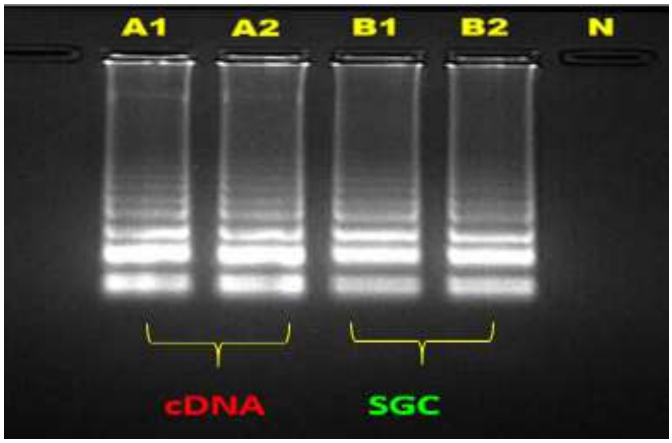


Fig. 4.9: Evaluation of the performance of the developed RT-LAMP assay with cDNA

4.2.2 Development of colorimetric RT-LAMP assay

We have performed the colorimetric LAMP reactions to develop a rapid diagnostic point-of-care (POC) colorimetric assay that could provide visible detection of SARS CoV-2 infections. For the development of these assays, we have utilized three chromogenic dyes namely A, B and C. After development of colorimetric LAMP assay, its performance was evaluated with cDNA synthesized from SARS CoV-2 RNA at Kalpana Chawla Government Medical College (KCGMC), Karnal, Haryana.

4.2.2.1 Optimization and selection of concentrations of the chromogenic dyes

All the three chromogenic dyes namely A, B and C were added at varied concentrations to optimize the reaction conditions of LAMP assay. For dye A, three different concentrations i.e. X, Y and Z µM was employed to be used in the LAMP reaction to found the intensity of colour change being produced after the reaction. The efficient colour change being observed in the case of Y µM concentration. Similarly dye B at U and V concentrations and C dye at W concentration were selectively added in the LAMP assay to find out the colour change. Visible colour change was observed only in the case of dye A at Y concentration and dye B at U concentration (**Fig. 4.10, 4.11)**. However, no colour change was observed W concentration in the case of dye C (**Fig. 4.12**), which needs further optimization with respect to the concentration.

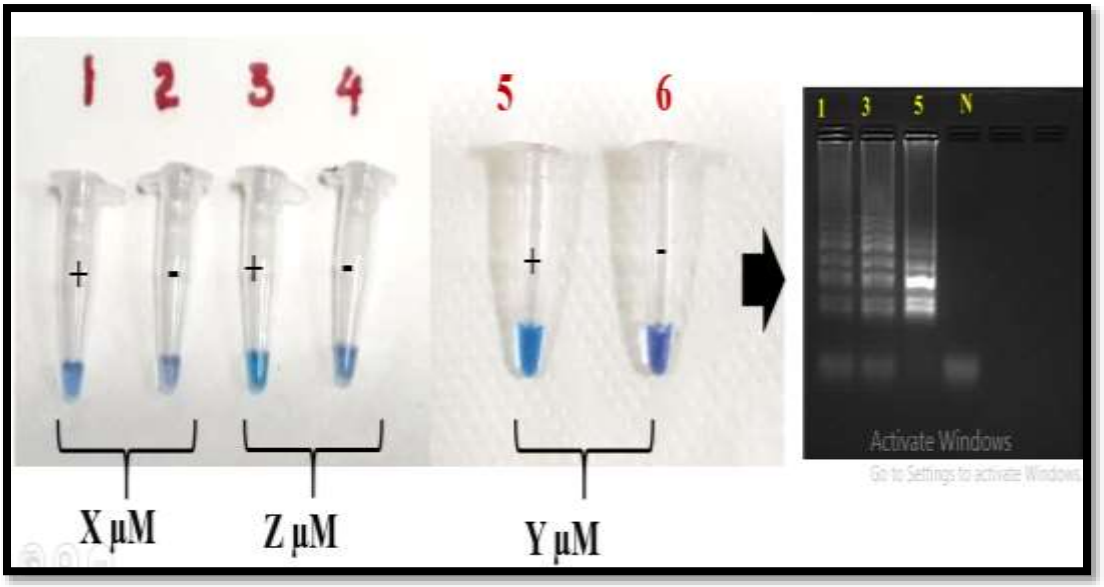


Fig. 4.10: LAMP reaction being performed with the addition of dye A

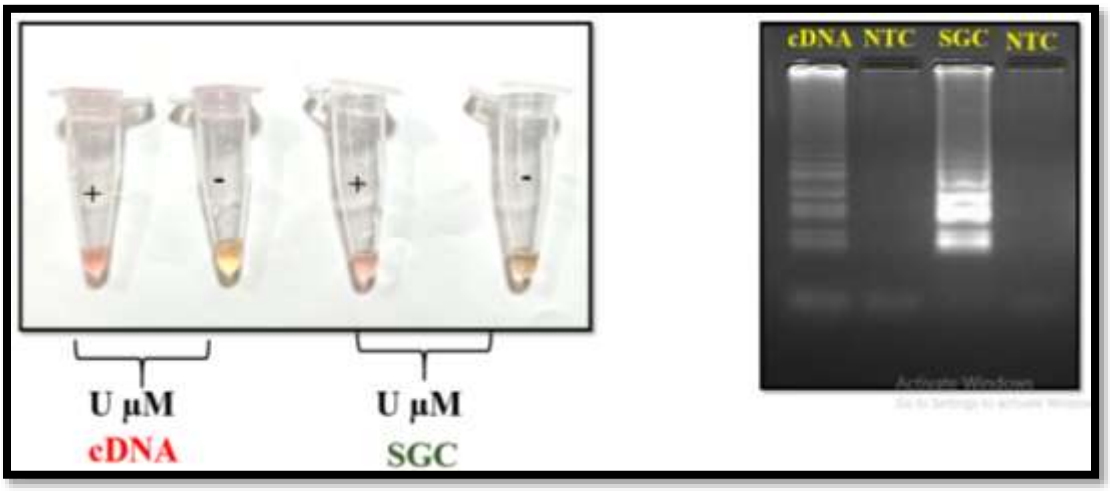


Fig. 4.11: LAMP reaction being performed with the addition of dye B

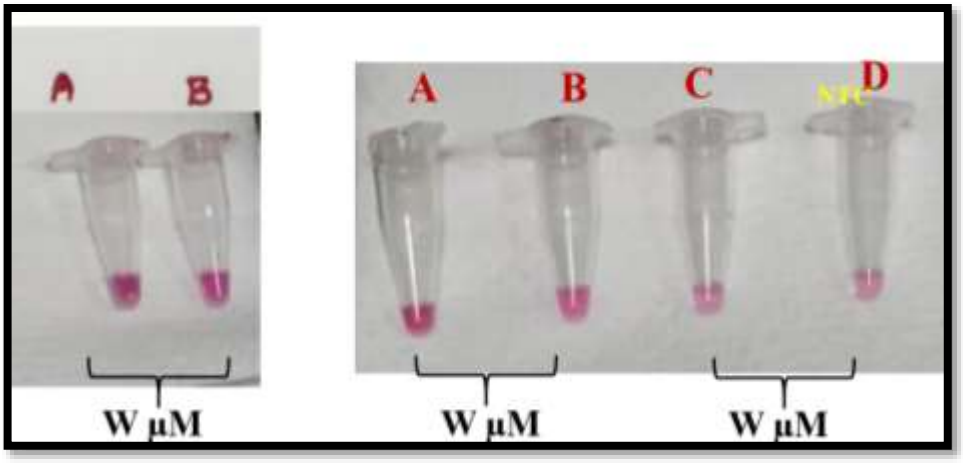


Fig. 4.12: LAMP reaction being performed with the addition of dye C

4.2.2.2 Evaluation of performance of the developed colorimetric RT-LAMP assay with cDNA

Performance evaluation of colorimetric RT-LAMP assay using cDNA synthesized from SARS-CoV-2 RNA was done at KCGMC, Karnal, Haryana. Here, cDNA synthesized from RNA SARS-CoV-2 was used as template to take part in the LAMP reaction and the results were compared with SGC as positivetemplate. After LAMP reaction, the colorimetric observation of sample with cDNA as templated was compared with theat of LAMP reaction involving SGC as templated DAN. From the visible observations, we found similar kind of colour change in both cDNA and SGC samples sets. The resutls were further confirmed with agarose gel electrophoresis of LAMP products generated out of cDNA and SGC, where the amplified products were visible in both the sample sets. From the aforesaid results, it was very clear that the developed LAMP assay was able to yield resutls with the cDNA synthesized from the clinical samples of RNA extracted from SARS-CoV-2 virus.

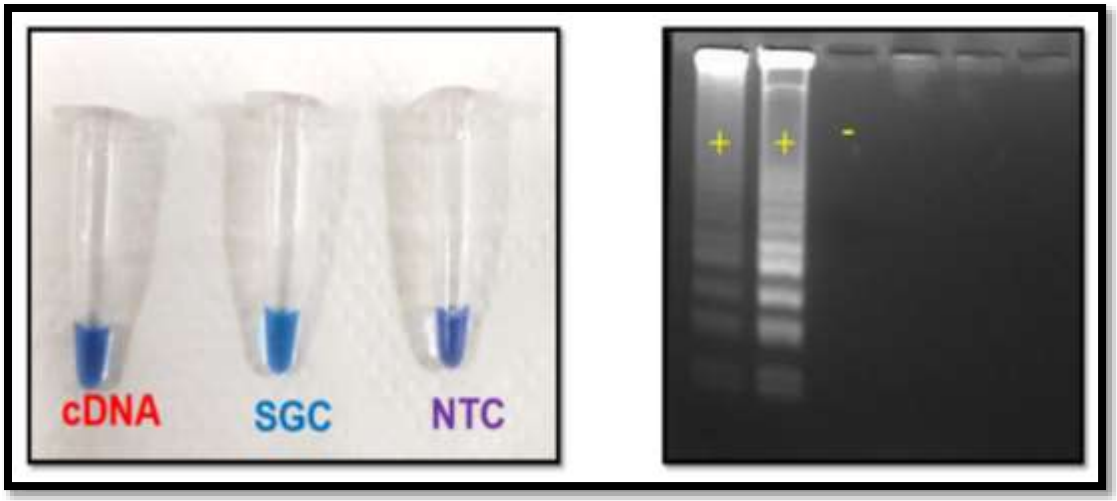


Fig. 4.13 Performance of LAMP reaction being evaluated with the addition of dye A

Earlier investigators have also suggested that the colorimetric RT-LAMP assay could serve as a potential and rapid testing methodology for the detection of SARS CoV-2 (Papadakis *et al.,* 2021 and Kellner *et al.,* 2020). The LAMP reactions were successfully carried out at an optimized temperature of 65° C for 60 minutes and a colour change was observed from pink colour to yellow (Haq *et al.,* 2021). These developed assays were able to demonstrate sensitivity of 90.2% and specificity of 92.4 % (Lim *et al.,* 2021). The other investigators utilized WarmStart colorimetric RT-LAMP 2X Master Mix to carry out the LAMP reactions at 65°C for 15-60 min followed by cooling at 4 for 30 sec to intensify the colour changes being appeared in the tubes (Dao thi *et al.,* 2020). Recently, Nawattanapaiboon *et al.,* (2021) have developed a simple and less expensive colorimetric LAMP assay targeting the RdRp gene with the use of phenol red as pH indicator dye showing 95.74 % sensitivity and 99.95 % specificity.

Yu *et al.,* (2021) have utilized unique pH dye known as LAMP Shade Violet instead of other conventional dyes such as phenol red and HNB dye which contributed to a better intensity of colour change i.e. from purple to clear. Our results in this investigation were found similar with respect to the above-mentioned studies involving chromogenic dyes during the LAMP reactions that we have carried out at 65°C for 30 min. Therefore, we can recommend this study to be explored further for the successful as well as rapid detection of SARS CoV-2. However, further validation of the developed Colorimetric RT-LAMP assay is required

with a larger sample size of RNA extracts of SARS CoV-2 to evaluate the sensitivity and specificity of the above-developed proof of concept for the RT-LAMP assay.

4.3 Development of fluorometric LAMP assay and its performance evaluation with fluoroscent dye

The fluorometric LAMP assay was carried out along with the addition of fluorescent dye in the similar way as in the case of chromogenic dye. The fluorometric LAMP assay developed with provided fluorescent dye from WarmStart® LAMP Kit (DNA & RNA) was able to yield results as increased fluorescence was observed in the reaction tubes with synthetic gene and cDNA as template in comparison with the control without any template DNA as shown in **Fig. 4.14** . Hence, the LAMP developed assay can be used a fluorometricc LAMP assay where the provision of reading or measuring fluorescence is available.

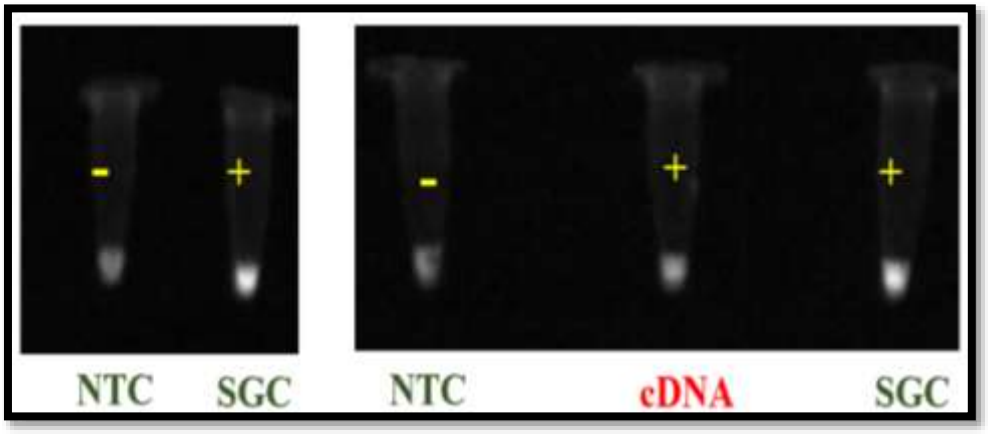


Fig. 4.14: Development and performance evaluation of fluorometric LAMP assay

Earlier investigations have also recommended the fluorometricc RT-LAMP assay as a rapid and scalable testing methodology for the detection of SARS CoV-2 (Xun *et al.,* 2021; Brown *et al.,* 2021). Bikos *et al.,* (2021) have developed rapid LAMP method demonstrating a sensitivity of 91% and specificity of 98% wherein they employed NEB protocol to perform the RT-LAMP reactions at 60-65° C for 45 min along with the addition of as fluorescent dye to observe the fluorescence after the amplification. An inexpensive real time fluorometric RT-

LAMP detection device was developed using the SYTO-9 fluorescent dye which provided results within 30 min (Hayden *et al.*, 2021). Garcia-Venzor *et al.,* (2021) have developed the scalable LAMP device to target the N gene and to amplify the specific regions and obtained a LOD of 100% during real time detection after 30 min. Our results in this regard were found similar at 63 C followed for 40 min and observed appropriate fluorescence after the amplification.

CHAPTER 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

The conventional methods of testing involved in the detection of SARS CoV-2 are time-consuming, expensive and, require the need of more-sophisticated equipment as well as skilled personnel. The increasing rate of coronavirus cases has led to the need to develop an accurate and rapid testing facility that can provide results in a very short period of time. Hence, the RT-LAMP technique which can serve as a suitable point-of-care diagnostics for the rapid and accurate detection of SARS CoV-2 infections was developed in the current investigation and the results obtained during this course of investigation have been summarized below under the specific objectives.

Objective 1: Synthesis of artificial genes of SARS CoV-2 and designing of RT-LAMP primers.

- The approval from the Review Committee on Genetic Manipulation (RCGM) to carry out research and development work on RT-LAMP assay as point-of-care diagnostic for SARS-CoV-2 (IBKB UAC: ICARDLAMM2010) was obtained.
- After GISAID registration, a total of 142 sequences of SARS CoV-2 were selected from several Indian States and Union Territories namely, Andhra Pradesh (), Assam, Bihar, Delhi, Gujarat, Haryana, Jammu and Kashmir, Karnataka, Ladakh, Madhya Pradesh, Maharashtra, Odisha, Punjab, Rajasthan, Tamil Nadu, Telangana, Uttar Pradesh and Uttarakhand.
- All the 142 sequences were successfully aligned with the MAFFT server for alignment of sequences with multiple sequence alignment (MSA) to identify conserved regions for designing RT-LAMP primers.
- A total of 36 conserved regions were identified after the alignment and literature survey was carried out for the selection of potential target genes and respective regions which were not previously explored for designing RT-LAMP primers.
- A total of nine sets of primers were designed targeting three conserved regions. However, only six sets of primers (A, B, C, D, E and F), two each from a single

conserved region were selected for the development of RT-LAMP assay due to the limitation of time.

- The specificity of these designed primer sets was evaluated via sequence alignment with other previously reported human coronaviruses before getting synthesized for development of RT-LAMP assay.
- For development of RT-LAMP assay, the target conserved region was got synthesized from Bio Basic in the form of synthetic gene construct which served as positive template for the development of RT-LAMP assay.

Objective 2: Development and performance evaluation of RT-LAMP assay

- In the development of RT-LAMP assay, out of six sets of LAMP primers, four (B,C, D and F) sets were able to amplify the target region under the optimized LAMP conditions.
- Primer set "B" was finally selected for the development of colorimetric/fluorometric LAMP assay due to the limitation of time and resources.
- Colorimetric LAMP assay was successfully developed with two chromogenic dyes namely A and B.
- The performance of developed colorimetric assay was evaluated with cDNA synthesized from SARS-CoV-2 RNA as template, which yielded satisfactory results at 65°C within time period of 40 min.
- Similarly, the fluorometric LAMP assay was successfully developed with fluorescent dye provided with WarmStart® LAMP Kit (DNA & RNA).
- The performance evaluation of developed colorimetric and fluorometric LAMP assays yielded satisfactory results with cDNA synthesized from SARs-CoV-2 RNA.

Conclusion

- The developed RT-LAMP assay could serve as a potential point-of-care diagnostic solution to test the SARS CoV-2 infections. However, due to the limitation of time and availability of meagre amount of RNA samples of SARS CoV-2, its performance evaluation with a larger sample size was not possible as earlier planned and scheduled. Therefore, we recommend that the developed LAMP assay needs to be performed

directly with RNA extracted from SARS-COV-2 and its performance needs to be evaluated for it to qualify as RT-LAMP assay.

BIBLIOGRAPHY

BIBLIOGRAPHY

Anahtar, M. N., McGrath, G. E., Rabe, B. A., Tanner, N. A., White, B. A., Lennerz, J. K., Branda, J. A., Cepko, C. L. and Rosenberg, E. S. **2021**, February. Clinical Assessment and Validation of a Rapid and Sensitive SARS-CoV-2 Test Using Reverse Transcription Loop-Mediated Isothermal Amplification Without the Need for RNA Extraction. In *Open Forum Infectious Diseases* (Vol. **8**, No. **2:** p. ofaa631). US: Oxford University Press.

Anderson, R. M., Fraser, C., Ghani, A. C., Donnelly, C. A., Riley, S., Ferguson, N. M., Leung, G.M., Lam, T.H. and Hedley, A. J. **2004.** Epidemiology, transmission dynamics and control of SARS: the 2002–2003 epidemic. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **359**(**1447**)**:** 1091-1105.

Aoki, M. N., de Oliveira Coelho, B., Góes, L. G. B., Minoprio, P., Durigon, E. L., Morello, L. G., Marchini, F. K., Riediger, I. N., Debur, M. D. C., Nakaya, H.I. and Blanes, L. **2021.** Colorimetric RT-LAMP SARS-CoV-2 diagnostic sensitivity relies on color interpretation and viral load. *Scientific Reports*, **11(1):** 1-10.

Ben-Assa, N., Naddaf, R., Gefen, T., Capucha, T., Hajjo, H., Mandelbaum, N., Elbaum, L., Rogov, P., Daniel, K. A., Kaplan, S., Chowers, M., Szwarcwort-Cohen, M., Paul, M., Geva-Zatorsky, N. and Rotem, A. **2020.** SARS-CoV-2 On-the-Spot Virus Detection Directly From Patients. *MedRxiv.*

Bikos, D. A., Hwang, C., Brileya, K. A., Parker, A., Loveday, E. K., Rodriguez, M., LeFevre, T., Thornton, I., Wilking, J. N., Dills, M., Walk, S. T., Adams, A. K., Plowright, R., Hoegh, A. B., Carter, J. R., Morrow, J., Taylor, M., Keil, D., Fields, M. W. and Chang, C. B. **2021.** SLAMP: A Rapid Fluorometric RT-LAMP Assay for Sensitive and Specific Detection of SARS-CoV-2 from Human Saliva. *MedRxiv*

Bibliography

Brown, T. A., Schaefer, K. S., Tsang, A., Yi, H. A., Grimm, J. B., Lemire, A. L., Jradi, F. M., Kim, C., McGowan, K., Ritola, K., Armstrong, D. T., Mostafa, H. H., Korff, W., Vale,R. D. and Lavis, L. D. **2021.** Direct detection of SARS-CoV-2 RNA using high-contrast pH-sensitive dyes. *MedRxiv*, 2020-12.

Chafekar, A., and Fielding, B. C. **2018.** MERS-CoV: understanding the latest human coronavirus threat. *Viruses*, **10(2):** 93.

Chu, D. K., Pan, Y., Cheng, S. M., Hui, K. P., Krishnan, P., Liu, Y., Ng, D. Y. M., Wan, C. K. C., Yang, P., Wang, Q., Peiris, M., and Poon, L. L. **2020.** Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clinical Chemistry*, **66(4):** 549-555.

Corman, V. M., Eckerle, I., Bleicker, T., Zaki, A., Landt, O., Eschbach-Bludau, M., Boheemen, S.V., Gopal, R., Ballhause, M., Bestebroer, T. M., Muth, D., Müller, M. A., Drexler, J. F., Zambon, M., Osterhaus, A. D., Fouchier, R. M. and Drosten, C. **2012.** Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. *Eurosurveillance*, **17(39):** 20285.

Corman, V. M., Rabenau, H. F., Adams, O., Oberle, D., Funk, M. B., Keller-Stanislawski, B., Drosten, C., Timm, J. and Ciesek, S. **2020.** SARS-CoV-2 asymptomatic and symptomatic patients and risk for transfusion transmission. *Transfusion*.

Dao Thi, V. L., Herbst, K., Boerner, K., Meurer, M., Kremer, L. P., Kirrmaier, D., Freistaedter, A., Papagiannidis, D., Galmozzi, C., Stanifer, M. L., Boulant, S., Klein, S., Chlanda, P., Khalid, D., Miranda, I. B., Schnitzler, P., Kräusslich, H., Knop, M. and Anders, S**. 2020.** A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Sci Transl Med* **12:** eabc7075.

Donnelly, C. A., Ghani, A.C., Leung, G. M., Hedley, A. J., Fraser, C., Riley, S., Abu-Raddad, L. J., Ho, L-M., Thach T.Q., Chau P., Chan K-P., Lam, T-H., Tse L-Y., Tsang, T., Liu, S-H., Kong, J. H. B., Lau, E. M. C., Ferguson N. M. and Anderson, R. M. **2003.**

Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong. *Lancet* **361:**1761–1766.

Fowler, V. L., Armson, B., Gonzales, J. L., Wise, E. L., Howson, E. L., Vincent-Mistiaen, Z., Fouch, S., Maltby, C. J., Grippon, S., Munro, S., Jones, L., Holmes, T., Tillyer, C., Elwell, J., Sowood, A., Peyer, O. D., Dixon, S., Hatcher, T., Patrick, H., Laxman, S., Walsh, C., Andreou, M., Morant, N., Clark, D., Moore, N., Houghton, R., Cortes, N. and Kidd, S. P. **2021**. A highly effective reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for the rapid detection of SARS-CoV-2 infection. *Journal of Infection*, **82(1):** 117-125.

Fraser, C., Riley, S., Anderson, R. M. and Ferguson, N. M. **2004.** What makes and infectious disease outbreak controllable? Proc. Natl Acad. *Sci. USA* **101:** 6146–6151.

Garcia-Venzor, A., Rueda-Zarazua, B., Marquez-Garcia, E., Maldonado, V., Moncada-Morales, A., Olivera, H., Lopez, I., Zuñiga, J. and Melendez-Zajgla, J. **2021**. SARS-CoV-2 Direct Detection Without RNA Isolation With Loop-Mediated Isothermal Amplification (LAMP) and CRISPR-Cas12. *Frontiers in Medicine*, **8:** 125.

Haq, F., Sharif, S., Khurshid, A., Ikram, A., Shabbir, I., Salman, M., Ahad, A., Rana, M. S., Raja, A., Badar, N., Tashkandi, H., Amri, T. A., Azhar, E. I., Almuhayawi, M. S., Harakeh, S. and Malik, M. F. A. **2021.** Reverse transcriptase loop-mediated isothermal amplification (RT-LAMP)-based diagnosis: A potential alternative to quantitative real-time PCR based detection of the novel SARS-COV-2 virus. *Saudi Journal of Biological Sciences*, **28(1):** 942-947.

Hayden, A., Kuentzel, M., and Chittur, S. V. **2021**. Rapid, Affordable and Scalable SARS-CoV-2 Detection from Saliva. *MedRxiv*.

Huang, W. E., Lim, B., Hsu, C. C., Xiong, D., Wu, W., Yu, Y., Jia, H., Wang, Y., Zeng, Y., Ji, M., Chang, H., Zhang, X., Wang, H. and Cui, Z. **2020.** RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microbial Biotechnology*, **13(4):** 950-961.

Jang, W. S., Lim, D. H., Yoon, J., Kim, A., Lim, M., Nam, J., Yanagihara, R., Ryu, S-W., Jung, B. K., Ryoo, N-H. and Lim, C. S. **2021.** Development of a multiplex Loop-

Mediated Isothermal Amplification (LAMP) assay for on-site diagnosis of SARS CoV-2. *Plos One*, **16(3):** e0248042.

Jiang, M., Pan, W., Arasthfer, A., Fang, W., Ling, L., Fang, H., Daneshnia, F., Yu, J., Liao, W., Pei, H., Li, X. and Lass-Flörl, C. **2020.** Development and validation of a rapid, single-step reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) system potentially to be used for reliable and high-throughput screening of COVID-19. *Frontiers in Cellular and Infection Microbiology*, **10:** 331.

Kellner, M. J., Ross, J. J., Schnabl, J., Dekens, M. P., Heinen, R., Tanner, N. A., Grishkovskaya, I., Bauer, B., Stadlmann, J., Menéndez-Arias, L., Fritsche-Polanz, R., Traugott M., Seitz,T., Zoufaly, A., Födinger M., Wenisch, C., Zuber, J. and Vienna Covid-19 Diagnostics Initiative. **2020.** Scalable, rapid and highly sensitive isothermal detection of SARS-CoV-2 for laboratory and home testing. *BioRxiv*.

Kevadiya, B. D., Machhi, J., Herskovitz, J., Oleynikov, M. D., Blomberg, W. R., Bajwa, N., Soni, D., Das, S., Hasan, M., Patel, M., Senan, A. M., Gorantla, S., McMillan, J., Edagwa, B., Eisenberg, R., Gurumurthy, C. B., Reid, S. P. M., Punyadeera, C., Chang, L. and Gendelman, H. E. **2021.** Diagnostics for SARS-CoV-2 infections. *Nature Materials*, **20(5):** 593-605.

Khan, M. I., Khan, Z. A., Baig, M. H., Ahmad, I., Farouk, A. E., Song, Y. G., and Dong, J. J. **2020.** Comparative genome analysis of novel coronavirus (SARS-CoV-2) from different geographical locations and the effect of mutations on major target proteins: An in silico insight. *PLoS One*, **15(9):** e0238344.

Kumar, R.K.S., Mufti, S.S., Sarathy, Vinu., Hazarika, Diganta., and Naik, Radheshyam. **2021.** An Update on Advances in COVID-19 Laboratory Diagnosis and Testing Guidelines in India.

Lalli, M. A., Langmade, J. S., Chen, X., Fronick, C. C., Sawyer, C. S., Burcea, L. C., Wilkinson, M. N., Fulton, R. S., Heinz, M., Buchser, W. J., Head, R. D., Mitra, R. D. and Milbrandt, J. **2021.** Rapid and extraction-free detection of SARS-CoV-2 from

saliva by colorimetric reverse-transcription loop-mediated isothermal amplification. *Clinical Chemistry*, **67(2):** 415-424.

Lanza, D. C., Lima, J. P., and Jerônimo, S. M. **2020.** Design and in silico validation of polymerase chain reaction primers to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Lim, B., Ratcliff, J., Nawrot, D. A., Yu, Y., Sanghani, H. R., Hsu, C. C., Peto, L., Evans, S., Hodgson, S. H., Skeva, A., Adam, M., Panopoulou, M., Zois, C. E., Poncin, K., Vasudevan, S. R., Dai, S., Ren, S., Chang, H., Cui, Z., Simmonds, P., Huang, W. E. and Andersson, M. I. (**2021**). Clinical validation of optimised RT-LAMP for the diagnosis of SARS-CoV-2 infection. *Scientific Reports*, **11(1):** 1-11.

Liu, X., Feng, J., Zhang, Q., Guo, D., Zhang, L., Suo, T., Hu, W., Guo, M., Wang, X., Huang, Z., Xiong, Y., Chen, G., Chen, Y., and Lan, K. **2020.** Analytical comparisons of SARS-COV-2 detection by qRT-PCR and ddPCR with multiple primer/probe sets. *Emerging Microbes & Infections*, ***9*(1):** 1175-1179.

Lopez-Rincon, A., Tonda, A., Mendoza-Maldonado, L., Mulders, D. G., Molenkamp, R., Perez-Romero, C. A., Claassen, E., Garssen, J. and Kraneveld, A. D. **2021.** Classification and specific primer design for accurate detection of SARS-CoV-2 using deep learning. *Scientific Reports*, ***11*(1):** 1-11.

Lu, R., Wu, X., Wan, Z., Li, Y., Jin, X., and Zhang, C. **2020.** A novel reverse transcription loop-mediated isothermal amplification method for rapid detection of SARS-CoV2. *International Journal of Molecular Sciences*, **21(8):** 2826.

Lu, X., Whitaker, B., Sakthivel, S. K. K., Kamili, S., Rose, L. E., Lowe, L., Mohareb, E., Elassal, E. M., Al-sanouri, T., Haddadin, A., and Erdman, D. D. **2014.** Real-time reverse transcription-PCR assay panel for Middle East respiratory syndrome coronavirus. *Journal of Clinical Microbiology*, **52(1):** 67-75.

Mackay, I. M., and Arden, K. E. **2015.** MERS coronavirus: diagnostics, epidemiology and transmission. *Virology Journal*, **12(1):** 1-21.

Mautner, L., Baillie, C. K., Herold, H. M., Volkwein, W., Guertler, P., Eberle, U., Ackermann, N., Sing, A., Pavlovic, M., Goerlich, O., Busch, U., Wassill, L., Huber, l. and Baiker, A. **2020.** Rapid point-of-care detection of SARS-CoV-2 using reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Virology Journal*, **17(1):** 1-14.

Naqvi, A. A. T., Fatima, K., Mohammad, T., Fatima, U., Singh, I. K., Singh, A., Atif, S. M., Hariprasad, G., Hasan, G. M. and Hassan, M. I. **2020.** Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1866(10):** 165878.

Nascimento Junior, J. A. C., Santos, A. M., Oliveira, A. M. S., Guimarães, A. G., Quintans-Júnior, L. J., Coutinho, H. D. M., Martins, N., Borges, L. P., and Serafini, M. R. **2020.** Trends in MERS-CoV, SARS-CoV, and SARS-CoV-2 (COVID-19) diagnosis strategies: a patent review. *Frontiers in Public Health*, **8:** 663.

Nawattanapaiboon, K., Pasomsub, E., Prombun, P., Wongbunmak, A., Jenjitwanich, A., Mahasupachai, P., Vetcho, P., Chayrach, C., Manatjaroenlap, N., Samphaongern, C., Watthanachockchai, T., Leedorkmai, P., Manopwisedjaroen, S., Akkarawongsapat, R., Thitithanyanont, A., Phanchana, M., Panbangred ,W., Chauvatcharin,S., and Srikhirin, T. **2021.** Colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a visual diagnostic platform for the detection of the emerging coronavirus SARS-CoV-2. *Analyst*, **146(2):** 471-477.

Pandey, M., Singh, D., and Onteru, S. K. **2018.** Reverse transcription loop-mediated isothermal amplification (RT-LAMP), a light for mammalian transcript analysis in low-input laboratories. *Journal of Cellular Biochemistry*, **119(6):** 4334-4338.

Papadakis, G., Pantazis, A. K., Fikas, N., Chatziioannidou, S., Tsiakalou, V., Michaelidou, K., Pogka, V., Megariti, M., Vardaki, M., Giarentis, K., Heaney, J., Nastouli, E., Karamitros, T., Mentis, A., Zafiropoulos, A., Sourvinos, G., Agelaki, S. and Gizeli, E. **2021.** Portable real-time colorimetric LAMP-device for rapid quantitative detection of nucleic acids in crude samples. *BioRxiv*, 2020-07.

Park, G. S., Ku, K., Baek, S. H., Kim, S. J., Kim, S. I., Kim, B. T., and Maeng, J. S. **2020.** Development of reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays targeting SARS-CoV-2. *The Journal of Molecular Diagnostics*

Rabe, B. A., and Cepko, C. **2020.** SARS-CoV-2 detection using isothermal amplification and a rapid, inexpensive protocol for sample inactivation and purification. *Proceedings of the National Academy of Sciences*, **117(39):** 24450-24458.

Rai, P., Kumar, B. K., Deekshit, V. K., Karunasagar, I., and Karunasagar, I. **2021.** Detection technologies and recent developments in the diagnosis of COVID-19 infection. *Applied Microbiology and Biotechnology*, 1-15.

Ramadan, N., and Shaib, H. **2019.** Middle East respiratory syndrome coronavirus (MERS-CoV): *A review. Germs*, **9(1):** 35.

Saha, I., Ghosh, N., Maity, D., Sharma, N., and Mitra, K. **2020.** Inferring the genetic variability in Indian SARS-CoV-2 genomes using consensus of multiple sequence alignment techniques. *Infection, Genetics and Evolution*, **85:** 104522.

Shirato, K., Yano, T., Senba, S., Akachi, S., Kobayashi, T., Nishinaka, T., Notomi, T. and Matsuyama, S. **2014.** Detection of Middle East respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Virology Journal*, **11(1):** 1-11.

Wan, W. Y., Seng, E. H., and Lim, S. H. **2020.** Cross-reaction of sera from COVID-19 patients with SARS-CoV assays. *MedRxiv*.

Wang, Y. C., Yang, W. H., Yang, C. S., Hou, M. H., Tsai, C. L., Chou, Y. Z., Hung, M. C., & Chen, Y. **2020**. Structural basis of SARS-CoV-2 main protease inhibition by a broad-spectrum anti-coronaviral drug. *American journal of cancer research*, ***10*(8):** 2535.

Wernery, U., Corman, V. M., Wong, E. Y., Tsang, A. K., Muth, D., Lau, S. K., Khazanehdari, K., Zirkel, F., Ali, M., Nagy, P., Juhasz, J., Wernery, R., Joseph, S., Syriac, G., Elizabeth, S. K., Patteril, N. A. G., Woo, P. C. Y. and Drosten, C. **2015.** Acute middle

East respiratory syndrome coronavirus infection in livestock Dromedaries, Dubai, 2014. *Emerging Infectious Diseases, **21*(6):** 1019.

Xun, G., Lane, S. T., Petrov, V. A., Pepa, B. E., and Zhao, H. **2021.** A rapid, accurate, scalable, and portable testing system for COVID-19 diagnosis. *Nature communications*, **12(1):** 1-9.

Yadav, P. D., Nyayanit, D. A., Majumdar, T., Patil, S., Kaur, H., Gupta, N., Shete, A. M., Pandit, P., Kumar, A., Aggarwal, N., Narayan, J., Vijay, N., Kalawat, U., Sugunan, A. P., Munivenkatappa, A., Sharma, T., Devi, S., Majumdar, T., Jaryal, S., Bakshi, R., Joshi, Y., Sahay, R., Shastri, J., Singh, M., Kumar M., Rawat, V., Dutta, S., Yadav, S., Krishnasamy, K., Raut, S., Biswas, D., Borkakoty, B., Verma, S., Rani, S., Deval, H., Patel, D., Turuk, J., Malhotra, B., Fomda, B., Nag, V., Jain, A., Bhargava, A., Potdar, V., Cherian, S., Abraham, P., Gopal, A., Panda, S. and Bhargava, B. **2021.** An Epidemiological Analysis of SARS-CoV-2 Genomic Sequences from Different Regions of India. *Viruses*, **13(5):** 925.

Yamaoka, Y., Matsuyama, S., Fukushi, S., Matsunaga, S., Matsushima, Y., Kuroyama, H., Kimura, H., Takeda, M., Chimuro, T. and Ryo, A. **2016.** Development of monoclonal antibody and diagnostic test for Middle East respiratory syndrome coronavirus using cell-free synthesized nucleocapsid antigen. *Frontiers in Microbiology*, **7:** 509.

Yang, W., Dang, X., Wang, Q., Xu, M., Zhao, Q., Zhou, Y., Zhao, H., Wang, L., Xu, Y., Wang, J., Wang, M., Li, H., Pe, F., Wang, Y. and Han, S. **2020.** Rapid and accurate detection of three genes in SARS-CoV-2 using RT-LAMP method

Yoshikawa, R., Abe, H., Igasaki, Y., Negishi, S., Goto, H., and Yasuda, J. **2020.** Development and evaluation of a rapid and simple diagnostic assay for COVID-19 based on loop-mediated isothermal amplification. *PLoS Neglected Tropical Diseases*, **14(11):** e0008855.

Yu, A. D., Galatsis, K., Zheng, J., Le, J. Q., Ma, D., Perlman, S., and Rosbash, M. **2021.** Development of a saliva-optimized RT-LAMP assay for SARS-CoV-2. *MedRxiv*, 2020-12.

Zhao, Z., Zhang, F., Xu, M., Huang, K., Zhong, W., Cai, W., Yin, Z., Huang, S., Deng, Z., Wei, M., Xiong, J., and Hawkey, P. M. **2003.** Description and clinical treatment of an early outbreak of severe acute respiratory syndrome (SARS) in Guangzhou, PR China. *Journal of Medical Microbiology*, **52(8):** 715-720.

*****

ANNEXURE

Annexure-I

Supplementary Table 1: Details of human SARS-CoV-2 genome sequences collected for the analysis (alignment, designing primers and synthesizing artificial gene construct

S. No	State or Union Territory	No. of complete genome sequences selected for analysis	SARS-CoV-2 viral genome ID
1	Andra Pradesh	2	>hCoV-19/India/AP-NCDC-3936/2020|EPI_ISL_436440|2020-04-09 >hCoV-19/India/AP-CCMB-GC41/2020|EPI_ISL_528809|2020-05-29
2	Assam	2	>hCoV-19/India/AS-NCDC-2525/2020|EPI_ISL_436421|2020-03-31
3	Bihar	4	>hCoV-19/India/BR-NCDC-02251/2020|EPI_ISL_435112|2020-03-28 >hCoV-19/India/BR-NCDC-2515/2020|EPI_ISL_436417|2020-03-31 >hCoV-19/India/BR-NCDC-2519/2020|EPI_ISL_436419|2020-03-31 >hCoV-19/India/BR-NCDC-4155/2020|EPI_ISL_436449|2020-04-12
4	Delhi	10	>hCoV-19/India/DL-MaxCov0021-CSIR-IGIB/2020|EPI_ISL_459923|2020-05-08 >hCoV-19/India/DL-MaxCov0035-CSIR-IGIB/2020|EPI_ISL_459937|2020-05-10 >hCoV-19/India/DL-NCDC1733-CSIR-IGIB/2020|EPI_ISL_482503|2020-05-18 >hCoV-19/India/DL-NCDC2189-CSIR-IGIB/2020|EPI_ISL_482546|2020-05-18 >hCoV-19/India/DL-NCDC3820-CSIR-IGIB/2020|EPI_ISL_482574|2020-05-20 >hCoV-19/India/DL-NCDC7090-CSIR-IGIB/2020|EPI_ISL_482628|2020-05-27

			>hCoV-19/India/DL-NCDC7557-CSIR-IGIB/2020|EPI_ISL_482643|2020-05-27 >hCoV-19/India/DL-NCDC7757-CSIR-IGIB/2020|EPI_ISL_482669|2020-05-28 >hCoV-19/India/DL-TF17/2020|EPI_ISL_508495|2020-06-17 >hCoV-19/India/DL-NCDC-IGIB-D1/2020|EPI_ISL_581507|2020-08-25
5	Gujarat	10	>hCoV-19/India/GJ-GBRC43/2020|EPI_ISL_444474|2020-04-29 >hCoV-19/India/GJ-GBRC56a/2020|EPI_ISL_447030|2020-05-03 >hCoV-19/India/GJ-GBRC165/2020|EPI_ISL_461500|2020-05-27 >hCoV-19/India/GJ-GBRC301a/2020|EPI_ISL_495046|2020-06-15 >hCoV-19/India/GJ-GBRC322b/2020|EPI_ISL_500950|2020-06-17 >hCoV-19/India/GJ-GBRC334b/2020|EPI_ISL_512071|2020-06-12 >hCoV-19/India/GJ-GBRC-386b/2020|EPI_ISL_524748|2020-07-11 >hCoV-19/India/GJ-GBRC-393b/2020|EPI_ISL_524761|2020-07-06 >hCoV-19/India/GJ-GBRC-395b/2020|EPI_ISL_524765|2020-07-06 >hCoV-19/India/GJ-GBRC-397b/2020|EPI_ISL_525422|2020-06-25
6	Haryana	8	>hCoV-19/India/HR-TF14/2020|EPI_ISL_508493|2020-06-17 >hCoV-19/India/HR-MW39/2020|EPI_ISL_511928|2020-06-10 >hCoV-19/India/HR-THSTI2010D/2020|EPI_ISL_528385|2020-04-27 >hCoV-19/India/HR-IMT-CB44/2020|EPI_ISL_547590|2020-08-27 >hCoV-19/India/HR-IMT-AE21/2020|EPI_ISL_560318|2020-05-28 >hCoV-19/India/HR-IMT-BZ119/2020|EPI_ISL_560323|2020-08-25 >hCoV-19/India/HR-IMT-CB117/2020|EPI_ISL_560324|2020-08-27 >hCoV-19/India/HR-IMT-BZ164/2020|EPI_ISL_561341|2020-08-25

7	Jammu Kashmir	1	>hCoV-19/India/JK-NCDC-02326/2020|EPI_ISL_435090|2020-03-29
8	Karnataka	10	>hCoV-19/India/KA-nimh-0113/2020|EPI_ISL_428479|2020-04-06 >hCoV-19/India/KA-nimh-1071/2020|EPI_ISL_428487|2020-04-14 >hCoV-19/India/KA-InStem-NCBS-0097/2020|EPI_ISL_486841|2020 >hCoV-19/India/KA-IB14/2020|EPI_ISL_508292|2020-06-18 >hCoV-19/India/KA-IB9/2020|EPI_ISL_508337|2020-06-19 >hCoV-19/India/KA-nimh-13855/2020|EPI_ISL_515954|2020-05-09 >hCoV-19/India/KA-nimh-19688/2020|EPI_ISL_515970|2020-05-16 >hCoV-19/India/KA-nimh-19696/2020|EPI_ISL_515971|2020-05-16 >hCoV-19/India/KA-nimh-19716/2020|EPI_ISL_515973|2020-05-16 >hCoV-19/India/KA-NIV-QC-1801/2020|EPI_ISL_577715|2020-09
9	Ladak	6	>hCoV-19/India/LA-NCDC-01441/2020|EPI_ISL_435101|2020-03-15 >hCoV-19/India/LA-NCDC-01444/2020|EPI_ISL_435102|2020-03-15 >hCoV-19/India/LA-NCDC-01604/2020|EPI_ISL_435103|2020-03-17 >hCoV-19/India/LA-NCDC-01614/2020|EPI_ISL_435104|2020-03-17 >hCoV-19/India/LA-NCDC-01616/2020|EPI_ISL_435105|2020-03-17 >hCoV-19/India/LA-NCDC-01760/2020|EPI_ISL_435106|2020-03-18
10	Madhya Pradesh	9	>hCoV-19/India/MP-NCDC-4877/2020|EPI_ISL_436461|2020-04-20 >hCoV-19/India/MP-NIHSAD-0105-77/2020|EPI_ISL_452792|2020-04-30 >hCoV-19/India/MP-DRDE-3302/2020|EPI_ISL_476854|2020-05-11 >hCoV-19/India/MP-DRDE-4093/2020|EPI_ISL_476887|2020-05-17

			>hCoV-19/India/MP-DRDE-4105/2020|EPI_ISL_476892|2020-05-17 >hCoV-19/India/MP-DRDE-4624/2020|EPI_ISL_476896|2020-05-25 >hCoV-19/India/MP-DRDE 759/2020|EPI_ISL_476848|2020-04-11 >hCoV-19/India/MP-DRDE-4022/2020|EPI_ISL_476890|2020-05-16 >hCoV-19/India/MP-NCDC-4874/2020|EPI_ISL_436459|2020-04-20
11	Maharashtra	13	>hCoV-19/India/MH-NIV-12019/2020|EPI_ISL_452199|2020-04-20 >hCoV-19/India/MH-NIV-47470/2020|EPI_ISL_479553|2020-05-31 >hCoV-19/India/MH-AFMC-4958/2020|EPI_ISL_497887|2020-05-19 >hCoV-19/India/MH-GA39/2020|EPI_ISL_508233|2020-06-20 >hCoV-19/India/MH-GA9/2020|EPI_ISL_508284|2020-06-17 >hCoV-19/India/MH-NIV-191205/2020|EPI_ISL_541723|2020-09-02 >hCoV-19/India/MH-NIV-229360/2020|EPI_ISL_577711|2020-09-29 >hCoV-19/India/MH-AFMC-5458/2020|EPI_ISL_496529|2020-05-23 >hCoV-19/India/MH-AFMC-4954/2020|EPI_ISL_496537|2020-05-19 >hCoV-19/India/MH-GA97/2020|EPI_ISL_511940|2020-06-17 >hCoV-19/India/MH-1-27/2020|EPI_ISL_413522|2020-01-27 >hCoV-19/India/MH-1-31/2020|EPI_ISL_413523|2020-01-31 >hCoV-19/India/MH-NIV-7558/2020|EPI_ISL_454549|2020-04-05
12	Odhisha	10	>hCoV-19/India/OR-ILSCV24694/2020|EPI_ISL_463087|2020-05-18 >hCoV-19/India/OR-ILSCV35482/2020|EPI_ISL_481201|2020-06-11 >hCoV-19/India/OR-ILSCV35615/2020|EPI_ISL_481204|2020-06-12 >hCoV-19/India/OR-ILSCV28594/2020|EPI_ISL_481126|2020-05-22

			>hCoV-19/India/OR-ILSCV32893/2020|EPI_ISL_481173|2020-06-05 >hCoV-19/India/OR-ILSCV35750/2020|EPI_ISL_481205|2020-06-12 >hCoV-19/India/OR-ILSCV32465/2020|EPI_ISL_481156|2020-05-30 >hCoV-19/India/OR-ILC-RMRC123/2020|EPI_ISL_455311|2020-05-07 >hCoV-19/India/OR-ILSCV32670/2020|EPI_ISL_481162|2020-06-03 >hCoV-19/India/OR-MW30/2020|EPI_ISL_508434|2020-05-26
13	Punjab	5	>hCoV-19/India/PB-NCDC-01538/2020|EPI_ISL_435062|2020-03-16 >hCoV-19/India/PB-IMT-I14/2020|EPI_ISL_539483|2020-04-27 >hCoV-19/India/PB-IMT-L176/2020|EPI_ISL_539486|2020-04-30 >hCoV-19/India/PB-IMT-L178/2020|EPI_ISL_539487|2020-05-30 >hCoV-19/India/PB-IMT-L169/2020|EPI_ISL_561344|2020-04-30
14	Rajasthan	6	>hCoV-19/India/RJ-NCDC-2522/2020|EPI_ISL_436420|2020-03-31 >hCoV-19/India/RJ-SMSCOV109/2020|EPI_ISL_454830|2020-04-23 >hCoV-19/India/RJ-SMSCOV141/2020|EPI_ISL_454831|2020-04-29 >hCoV-19/India/RJ-SMSCOV161/2020|EPI_ISL_454832|2020-04-21 >hCoV-19/India/RJ-SMSCOV175/2020|EPI_ISL_454833|2020-04-27 >hCoV-19/India/RJ-S25/2020|EPI_ISL_455655|2020-04-30
15	Tamil Nadu	10	>hCoV-19/India/TN-CCMB-C20/2020|EPI_ISL_471583|2020-04-29 >hCoV-19/India/TN-CCMB-C6/2020|EPI_ISL_458031|2020-04-26 >hCoV-19/India/TN-CCMB-C10/2020|EPI_ISL_458036|2020-04-29 >hCoV-19/India/TN-CCMB-C19/2020|EPI_ISL_458040|2020-05-06 >hCoV-19/India/TN-CCMB-C17/2020|EPI_ISL_458044|2020-05-06

			>hCoV-19/India/TN-NCDC-02311/2020|EPI_ISL_435078|2020-03-29 >hCoV-19/India/TN-NCDC-02333/2020|EPI_ISL_435087|2020-03-29 >hCoV-19/India/TN-NCDC-02327/2020|EPI_ISL_435094|2020-03-29 >hCoV-19/India/TN-CCMB-C1-12/2020|EPI_ISL_447584|2020-04-16 >hCoV-19/India/TN-CCMB-C21/2020|EPI_ISL_471584|2020-05-06
16	Telangana	10	>hCoV-19/India/TG-CCMB-AA328/2020|EPI_ISL_539738|2020-08-11 >hCoV-19/India/TG-CCMB-AC512/2020|EPI_ISL_539748|2020-08-19 >hCoV-19/India/TG-CCMB-L1026/2020|EPI_ISL_458050|2020-05-11 >hCoV-19/India/TG-CCMB-M446/2020|EPI_ISL_495205|2020-06-09 >hCoV-19/India/TG-CDFD-C1132/2020|EPI_ISL_528548|2020-05-30 >hCoV-19/India/TG-CCMB-AB352/2020|EPI_ISL_539632|2020-08-13 >hCoV-19/India/TG-CCMB-AB226/2020|EPI_ISL_539713|2020-08-14 >hCoV-19/India/TG-CCMB-L912/2020|EPI_ISL_471644|2020-05-09
17	Uttar Pradesh	16	>hCoV-19/India/UP-NCDC-01257/2020|EPI_ISL_435060|2020-03-12 >hCoV-19/India/UP-NBRI-N20-TDU302/2020|EPI_ISL_516949|2020-05-14 >hCoV-19/India/UP-NBRI-N17-TCP057/2020|EPI_ISL_516946|2020-03-11 >hCoV-19/India/UP-NIV-32/2020|EPI_ISL_547876|2020 >hCoV-19/India/UP-AR20/2020|EPI_ISL_508168|2020-06-02 >hCoV-19/India/UP-NBRI-N40-TCQ729/2020|EPI_ISL_516969|2020-03-28 >hCoV-19/India/UP-NBRI-N53-TEH628/2020|EPI_ISL_516981|2020-05-25 >hCoV-19/India/UP-NBRI-N58-TEG811/2020|EPI_ISL_516986|2020-05-24 >hCoV-19/India/UP-NCDC-02321/2020|EPI_ISL_435100|2020-03-29

Annexure-I

			>hCoV-19/India/UP-NBRI-N14-TCR324/2020|EPI_ISL_516943|2020-04-02
18	Uttarakhand	10	>hCoV-19/India/UT-AR28/2020|EPI_ISL_508175|2020-06-29 >hCoV-19/India/UT-AR6/2020|EPI_ISL_508196|2020-05-22 >hCoV-19/India/UT-IMT-BB45/2020|EPI_ISL_560321|2020-07-17 >hCoV-19/India/UT-AR19/2020|EPI_ISL_508166|2020-05-30 >hCoV-19/India/UT-AR35/2020|EPI_ISL_511909|2020-05-28 >hCoV-19/India/UT-AR23/2020|EPI_ISL_508171|2020-05-21 >hCoV-19/India/UT-AR42/2020|EPI_ISL_508186|2020-05-28 >hCoV-19/India/UT-AR62/2020|EPI_ISL_508199|2020-06-08 >hCoV-19/India/UT-AR57/2020|EPI_ISL_511921|2020-06-02 >hCoV-19/India/UT-IMT-BY60/2020|EPI_ISL_547584|2020-08-24
	Total	**142**	

Annexure-II

Dear Sir/ Madam,

Submission with UAC: ICARDLAM0210 has been granted authorization. Kindly see your email for further follow-up.

The information about further updates on your request shall be conversed through e-mails as well as by notifications at your organization's dashboard of IBKP.

We thank you for exploring this online submission system.

Yours sincerely,

If you need further assistance with your submission, please write to ibkp2019@dbt.nic.in or call at 011-24360057....

{{ Important: Please do not reply to this email. This mailbox is not set up to receive email. }}

RCGM application for the approval to conduct research work for the detection of Covid-19

Annexure-III

भारत सरकार
विज्ञान और प्रौद्योगिकी मंत्रालय
जैव प्रौद्योगिकी विभाग
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

ब्लॉक-2, 7वां तल, सी0जी0ओ0 कम्पलेक्स
लोधी रोड, नई दिल्ली-110003
Block-2, 7th Floor, C.G.O. Complex
Lodhi Road, New Delhi-110003
Tele : 011-24365071 Fax : 011-24362884
Website : http://www.dbtindia.nic.in

No. BT/IBKP/344/2020 **Dated: 09.09.2021**

To

Dr. Rashmi H M
Scientist (Senior Scale)
ICAR-National Dairy Research Institute
Molecular Biology Unit, Dairy Microbiology Division
ICAR-National Dairy Research Institute,
Karnal-132001, Haryana
Email: - rashmi.ndri@gmail.com

Subject: Application submitted by ICAR-National Dairy Research Institute, Karnal for the information and records of Review Committee on Genetic Manipulation (RCGM) to carry out research and development work on Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay as point-of-care diagnostic for SARS-CoV-2. (IBKP UAC: ICARDLAM0210)

Sir/Madam,

It is informed that the application to carry out research and development work on Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay as point-of-care diagnostic for SARS-CoV-2 was considered and noted by the Review Committee on Genetic Manipulation (RCGM) in its 213th meeting held on 19.08.2021.

2. You are required to comply with the Regulations and Guidelines for Recombinant DNA Research and Biocontainment, 2017.

3. The applicant should follow the "Interim guidance document on laboratory biosafety to handle COVID – 19 specimens" issued by DBT.

4. Any other specific instruction: National and WHO guidelines/recommendations/advisory for research and development work on COVID-19 should be followed.

Please acknowledge the receipt of the letter.

Yours faithfully,

(Dr. Nitin K. Jain)
Member Secretary, RCGM &
'Scientist-'F', DBT

Annexure-IV

Curriculum Vitae

SHWETA KELKAR

D/o : Mr. Mukesh Kelkar

DOB : 8th June, 1998

Address : New Deepak Nagar, Durg, Chhattisgarh-491001

Contact No. : +91 9407983763

E-mail Id : shwetakelkar86@gmail.com

CAREER OBJECTIVE

To work with an organization that offers opportunities to demonstrate my aptitude and skills for achieving the organizational as well as my personal career goals

ACADEMIC QUALIFICATIONS

DEGREE	YEAR	SCHOOL/ COLLEGE	BOARD/ UNIVERSITY	%/ CGPA
M. Tech* (Dairy Microbiology)	2019-2021	ICAR-National DairyResearch Institute, Karnal, Haryana	National Dairy ResearchInstitute (Deemed University), Karnal	8.36/10
B. Tech. (Dairy Technology)	2015-2019	College of Dairy Science &Food Technology, Raipur	ttisgarh Kamdhenu University	7.38/10
12^{th}	2015	Mar Baselios Vidhya Bhavan, Bhilai	CBSE	72.40%
10^{th}	2013	Mar Baselios VidhyaBhavan, Bhilai	CBSE	8.80/10

Annexure-IV

WORK EXPERIENCE

- **Assistant Professor (Dairy Microbiology)** at **Parul University, Vadodara, Gujarat**
- **Content Developer** at **Foodyaari (Linkedin)**

INDUSTRIAL EXPOSURE

1. **Banaskantha District Cooperative Milk Producers's Union Ltd., (GCMMF-AMUL)**
    - Fully automated UHT and Powder Plant and **ASRS** to apply FIFO method
2. **Chhattisgarh State Cooperative Dairy Federation Limited (Devbhog Dairy Plant)**
    - Energy consumption in milk processing and production section of various dairy products
3. **Hands on training and experimental learning at CoDSFT, Raipur, C.G.**
    - Learnt **Marketing Strategies** while **manufacturing and selling** of dairy products in teams
4. Industrial Visits-**Banas Dairy** (Palanpur), **Vidhya Dairy** (Anand), **Mother Dairy** (Delhi), **VerkaDairy** (Ludhiana, Amritsar & Jalandhar), **Experimental Dairy Plant** (GADVASU, Ludhiana), **Yakult Danone India**

M.TECH THESIS TITLE

"Development of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay as Point-of-Care diagnostics for SARS CoV-2"

COMPUTER PROFICIENCY AND LANGUAGES KNOWN

- Hindi, English, Gujarati, Marathi
- MS-office
- Adobe Photoshop and Illustrator
- Basics of SAP (System, Application and Products)
- Origin lab - Data analysis, Graphing software and Java Net Beans (Informatics Practices)

Annexure-IV

AWARDS/ACHIEVEMENTS

1. **Certified Food Safety Supervisor** by **FSSAI-FOSTAC (Advanced Manufacturing Level-2)**
2. Achieved **AIR-16** in **ICAR-AIEEA 2019** and Recipient of **National Talent Scholarship** awarded by **ICAR**
3. **Winner** of **Salt Challenge Every Pinch Counts** organized by **FSSAI and NetProFaN**
4. Achieved **3rd prize** in **Poster Competition** on the topic **Clean and Green Environment** on **150th Birth Anniversary of Mahatma Gandhi**, 2019
5. Awarded **Gold Medal** during **Sports Activity** in foundation program held **at NDRI, Karnal, Haryana**
6. **Received** the **Food Safety Prefect Title** in **Asia Quiz Competition** conducted by **FSSAI CII-FACE Surakshit Khadya Abhiyan** on 18th September, 2018
7. **Finalist** in **National Dairy and Food Quiz Contest** at **AAU, Anand, Gujarat,** 2018

EXTRA-CURRICULAR ACTIVITITES

I. Successfully completed **Six** online courses with **distinction** on

1. **QMS Auditor / Lead Auditor and ISO 9001:2005** course on **Udemy** E-learning platform
2. **Functional Foods: Concept, Technology & Health Benefits** organized by **IIT, Kanpur**
3. **Detection, Diagnosis and Management of Plant Diseases** by **IIT, Kanpur & CoL, Canada**
4. **Vacuum Packaging, Modified Atmospheric Packaging of Food** and **Food allergy** by **FSA**
5. **LinkedIn 5-Day workshop (Marketing and Sales through Social Media)** by Vaibhav Sisinty
6. **Six Sigma Yellow Belt Professional** conducted by **Master of Project Academy**

II. Attended **National & International Webinar** on

1. **Food Safety Policies and Regulations** organized by **Assam Agricultural University** and **NAHEP**
2. **Comprehensive solutions on Safety & Quality in Dairy Industry** organized by the **PerkinElmer**
3. **Current Trends in the application of HPLC and GC-MS techniques for Food and ChemicalTesting** organized by **Department of Food Technology, Haldia Institute of Technology**
4. **Pharmacological Potential of A1 and A2 Milk: Myths and Facts** by Dr. Anil

Kumar Srivastava

5. **Immunity Boosting through Dairy Products** organized by **MAFSU- Pusad and IDA**
6. **Impact of Covid-19 on Dairy Industry** organized by **SHAUTS**

HOBBIES & PERSONAL ATTRIBUTES

- Travelling, Reading, Technical and Creative Writing, Leadership, Working in team and Time management

*****