DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR RAPID DETECTION OF SARS-COV-2

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
δ	Delta
γ	Gamma
λ	Lambda
μ	Mu
®	registered trademark
™	Trademark
U	Unit
V	Volts
X	Times or multiplication

LIST OF ABBREVIATIONS

2019-nCoV	2019 novel coronavirus
ΔG	Gibb free energy
µg	Microgram
µl	Microliter
µM	Micro molar
β-CoV	Betacoronavirus
ACE2	Angiotensin converting enzyme 2
ARD	Acute respiratory distress
ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage
B3	Backward outer primer
BHI	Brain Heart Infusion broth
BIP	Backward inner primer
BLAST	Basic local alignment search tool
bp	Base pair
Bst	*Bacillus stearothermophilus*
Cas	CRISPR-associated proteins
CDC	Centres of Disease Control and Prevention
cDNA	Complement deoxyribonucleic acid
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CMIA	Chemiluminescence Microparticle Immunoassay
CI	Confidence interval
CLIA	Chemiluminescent immunoassay
COVID-19	Coronavirus Disease 2019
crRNA	Cas-guide RNA
Ct	Cycle threshold
DETECTR	DNA endonuclease-targeted CRISPR trans reporter
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
hCoV	Human coronavirus
ELISA	Enzyme-linked Immunoassay
et al.	Et alii (and others)
EUA	Emergency use authorization
F3	Forward outer primer
FDA	Food and Drug Agency

FIP	Forward inner primer
GAVI	Global Alliance for Vaccines and Immunization
GISAID	Global Initiative on Sharing Avian Influenza Data
HDA	Helicase-dependent amplification
HICs	High income countries
HNB	Hydroxynaphtol blue dye
HMNV	Human metapneumovirus
HUSM	Hospital Universiti Sains Malaysia
ICTV	International committee on taxonomy of viruses
IDT	Integrated DNA technologies
IFN-β	Interferon Beta
IFN-λ	Interferon Lambda
JEPeM	Jawatankuasa Etika Penyelidikan Manusia USM
JHU&M	John Hopkins University and Medicine
LAMP	Loop-mediated isothermal amplification
LB	Loop backward primer
LF	Loop forward primer
LFS	Lateral flow strip
LMICs	Low and middle income countries
LRT	Lower respiratory tract
Ltd.	Limited
M	Molar
MERS-CoV	Middle East respiratory syndrome coronavirus
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHC-I	Major histocompatibility complex class I
min	Minute
MIP-1α	macrophage inflammatory protein-1α
ml	Milliliter
mM	Millimolar
MOHM	Ministry of Health Malaysia
MCP-1	Monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
nCoV	Novel coronavirus
NCBI	National Center for Biotechnology Information
NPV	Negative predictive value
NSPs	Non-structural proteins
nsp14-ExoN	nsp14 exoribonuclease
OD	Optical density

OD_{230}	Absorbence at 230 nm
OD_{260}	Absorbence at 260 nm
OD_{280}	Absorbence at 280 nm
$OD_{260:230}$	Ratio of absorbence at 260 nm and 230 nm
$OD_{260:280}$	Ratio of absorbence at 260 nm and 280 nm
OD_{600}	Absorbence at 600 nm
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Potential hydrogen
PHEIC	Public Health Emergency of International Concern
PPV	Positive predictive value
RBD	Receptor binding domain
RdRp	RNA-dependent RNA polymerase
RTx	reverse transcriptase enzyme
RNA	ribonucleic acid
RPA	Recombinase polymerase amplification
RT-PCR	Real-time reverse-transcription polymerase chain reaction
RT-PCR	Real-time quantitative reverse-transcription polymerase chain reaction
RT-LAMP	Reverse-transcription loop-mediated isothermal amplification
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SHERLOCK	Specific high-sensitivity enzymatic reporter unlocking
ssDNA	Single-stranded deoxyribonucleic acid
Ta	Annealing temperature
TBE	Tris-Borate-EDTA
$TCID_{50}$	50 % tissue-culture infectious dose
Tm	Melting temperature
TNF- α	Tumour necrotic factor alpha
TMPRSS2	Transmembrane protease serine 2
UK	United Kingdom
UM	Universiti Malaya
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
VHC	Variants of High Consequence
VOC	Variants of Concern
VOI	Variants of Interest
VTM	Viral transport medium

WGS	Whole genome sequencing
WHO	World Health Organization
x g	Relative centrifugal force

LIST OF APPENDICES

PEMBANGUNAN UJIAN TRANSKRIPSI BERBALIK AMPLIFIKASI ISOTERMA BERPENGANTARA GELUNG BAGI PENGESANAN PANTAS SARS-COV-2

ABSTRAK

COVID-19 bermula di Wuhan, China pada penghujung tahun 2019 sebagai penyakit baharu yang tidak diketahui asalnya yang menunjukkan gejala radang paru-paru tipikal. Dalam masa yang singkat, penyakit ini telah merebak ke hampir seluruh pelosok dunia dengan jangkitan melebihi 238 juta dan kematian melebihi 4.8 juta yang dilaporkan sehingga 12 Oktober 2021. Ujian RT-qPCR ialah ujian piawaian utama untuk mengesahkan penyakit tersebut, tetapi kaedah ini mempunyai kelemahan yang berkaitan dengan aliran kerja yang rumit, penghasilan keputusan yang mengambil masa panjang, peralatan yang mahal dan keperluan kemahiran tinggi mengehadkan penggunaannya, terutamanya di negara yang mempunyai sumber yang terhad. Kit ujian pantas antigen dan antibodi adalah lebih cepat dan murah, tetapi dilaporkan menpunyai sensitiviti yang rendah, dan dapat bertindakbalas silang dengan virus lain. Kajian ini bertujuan untuk membangunkan ujian alternatif yang sesuai bagi mengesan SARS-CoV-2 dengan menggunakan kaedah transkripsi berbalik amplifikasi isoterma berperantara gelung (RT-LAMP). Primer LAMP yang menyasarkan gen nukleokapsid (N) SARS-CoV-2 dan gen RNase P (RNAP) manusia (kawalan amplifikasi dalaman, IAC) telah direka menggunakan PrimerExplorer V5. Primer yang dipilih telah disaring untuk menentukan spesifisiti dan kecekapan primer tersebut dengan menggunakan gen N dan RNAP sintetik yang dikesan dengan kaedah PCR dan LAMP konvensional. Pemilihan primer gen N adalah berdasarkan kecepatan amplifikasi, kespesifikan terhadap SARS-CoV-2 dan ketiadaan amplifikasi latar belakang dalam sampel

kawalan negatif. Optimisasi telah dilakukan terhadap beberapa parameter seperti kepekatan primer (luaran, dalaman dan gelung), magnesium sulfat ($MgSO_4$), betaine, dNTPs, enzim DNA polimerase *Bst* 2.0, enzim transkriptase balik dan juga suhu inkubasi. Optimisasi ujian RT-LAMP seterusnya dilakukan dengan menggunakan RNA yang ditranskrip secara *in vitro* (IVT) bagi gen N daripada SARS-CoV-2. Produk amplikon yang terhasil dikesan secara visual dengan menambahkan pewarna biru hidroksinaftol (HNB), dan juga dengan jalur aliran sisi terpasang (LFS) yang secara serentak mengesan gen N SARS-CoV-2 dan RNAP IAC. Kedua-dua kaedah dapat mengesan sehingga 1 salinan RNA IVT dalam masa 40 minit pada suhu 65°C. Prestasi ujian diagnostik ini seterusnya diuji dengan menggunakan 162 sampel klinikal (81 kes positif dan 81 kes negatif) yang telah disahkan dengan ujian RT-PCR. Sensitiviti dan spesifisiti bagi ujian RT-LAMP kolorimetrik adalah 97.53 % (95% CI: 91.36 - 99.70 %), manakala pengesanan dengan kaedah LFS adalah 97.53 % (95 % CI: 91.36 - 99.70 %) sensitif dan 90.12 % (95 % CI: 91.46 % - 95.64 %) spesifik. Kedua-dua ujian HNB-RT-LAMP dan LFS-RT-LAMP masing-masing menunjukkan ketepatan sebanyak 97.53 % dan 93.83 %. Secara keseluruhannya, 97.5 % daripada sampel klinikal yang disahkan dengan ujian RT-PCR (yang mempunyai nilai Ct 12.87 – 41.10), dapat dikesan dengan kedua-dua ujian yang dibangunkan. Berdasarkan analisis *in silico,* primer RT-LAMP juga boleh mengesan strain varian Alpha, Beta, Delta, Gamma, Lambda dan Mu SARS-CoV-2 dari dalam dan luar Malaysia. Walaupun kajian lanjut untuk ujian RT-LAMP masih diperlukan, namun ujian ini boleh digunakan bersama dengan ujian RT-PCR sebagai ujian saringan komuniti, terutamanya di kawasan bersumber terhad tatkala wabak COVID-19 masih berterusan.

DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR RAPID DETECTION OF SARS-COV-2

ABSTRACT

COVID-19 began in Wuhan, China as a disease of unknown origin, presenting with typical pneumonia in late 2019. Within a short time, the disease spread to almost every part of the world with over 238 million infections and over 4.8 million mortalities as of 12th October 2021. RT-PCR is the gold standard test for confirming the disease, but issues relating to its tedious workflow, lengthy time-to-result, expensive equipment need, and high skill requirements limit its use especially in resource-limited countries. Rapid antigen and antibody test kits are faster and cheaper, but less sensitive, and cross-reactivity with related viruses have been reported. This work aimed at developing a suitable alternative assay for detecting SARS-CoV-2 using reverse transcription loop-mediated isothermal amplification (RT-LAMP). LAMP primer sets targeting the nucleocapsid (N) gene of SARS-CoV-2, and the human RNase P (RNAP) gene (internal amplification control, IAC) were designed using PrimerExplorer V5. Primers were screened for specificity and efficiency using synthetic N and RNAP genes by PCR and conventional LAMP reactions. The final choice of N gene primer was based on amplification speed, specificity for SARS-CoV-2 and absence of background amplification in negative control. Concentrations of primers (outer, inner and loop), magnesium sulfate ($MgSO_4$), betaine, dNTPs, *Bst* 2.0 DNA polymerase enzyme, reverse transcriptase enzyme as well as incubation temperature were optimized. Final RT-LAMP assay was optimized using *in vitro* transcribed RNA from N gene of SARS-CoV-2. Amplification products were detected

visually with addition of hydroxynaphthol blue (HNB) dye, and an assembled lateral flow strip (LFS) that simultaneously detected SARS-CoV-2 N gene and RNAP IAC. Both methods detected up to 1 copy of IVT RNA within 40 minutes at 65^{o}C. Diagnostic performance was tested using 162 clinical samples (81 positive and 81 negative) confirmed with RT-PCR. Sensitivity and specificity were 97.53 % (95 % CI: 91.36 - 99.70 %) for the colorimetric RT-LAMP assay, while LFS detection was 97.53 % (95 % CI: 91.36 - 99.70 %) sensitive and 90.12 % (95 % CI: 81.46 - 95.64 %) specific. HNB-RT-LAMP and LFS-RT-LAMP were 97.53 % and 93.83 % accurate, respectively. Overall, 97.5 % of RT-PCR confirmed clinical samples having Ct value 12.87 – 41.10 were detected by both assays. Based on *in silico* analysis, the RT-LAMP primers could also detect Alpha, Beta, Delta, Gamma, Lambda and Mu SARS-CoV-2 variants from within and outside Malaysia. Although further work on the RT-LAMP assay is required, the assay can be used to augment RT-PCR in community surveillance testing especially in resource-limited settings as the COVID-19 pandemic continues.

CHAPTER 1

INTRODUCTION

1.1 Background of study

On 31st December 2019, Chinese authorities reported cases of pneumonia in Wuhan, Hubei Province in China, whose causal agent was unknown. On 7th January 2020, it was identified and confirmed to be a novel coronavirus. This made the outbreak the third human viral respiratory disease resulting from an animal coronavirus (Gorbalenya *et al.*, 2020). Within the space of four days (31st December 2019 to 3rd January 2020), 44 cases had been identified and between 13th to 20th January 2020, Thailand, Japan, and the Republic of Korea each reported first cases of the virus. The World Health Organization (WHO) declared the outbreak a public health emergency of international concern (PHEIC) on 30th January 2020 (WHO, 2020b) and renamed the disease from the interim name "2019-nCoV acute respiratory disease" (where 'n' is for novel and 'CoV' is for coronavirus), to COVID-19 (coronavirus disease 2019) on 11th February, 2020 (WHO, 2020c). Though reported by the WHO to have a low mortality rate, the pandemic has led to over 238 million infections and over 4.8 million deaths globally (Johns Hopkins Coronavirus Resource Center, 2021). The COVID-19 outbreak is the sixth known PHEIC after H1N1 influenza virus pandemic (2009), polio virus (2014), Zika virus (2016) and Ebola virus disease (2014 and 2018 in West Africa and the Democratic Republic of Congo respectively) (Lai *et al.*, 2020a). The first case of COVID-19 in Malaysia was reported on 25th January 2020 from three individuals who had contact with an infected person in Singapore enroute Kuala Lumpur. On 4th February, the first case involving a Malaysian who had also returned from Singapore, was reported (Elengoe, 2020). The

number of cases progressively increased within Malaysia after these cases were discovered.

COVID-19 is an acute respiratory viral infection of humans caused by a novel coronavirus believed to have originated from bats. Known initially as the "Wuhan coronavirus" or the "Chinese coronavirus", the International Committee on Taxonomy of Viruses (ICTV) later adopted the name severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) for the viral agent of COVID-19 (Gorbalenya *et al.*, 2020). SARS-CoV-2 is a single-stranded ribonucleic acid (RNA) virus with a positive sense and non-segmented RNA (Mautner *et al.*, 2020). It belongs to the genus beta-coronavirus (β-CoV) which also includes the severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) viruses. In 2003, SARS epidemic resulted in over 8,000 cases in 26 countries of the world. Similarly in 2012, a MERS outbreak affected 4 sub-continents leading to over 2,000 infections across 27 countries (Khailany *et al.*, 2020). The COVID-19 pandemic figures, however, far surpasses those of MERS-CoV and SARS-CoV, having spread to virtually every part of the world. SARS-CoV-2 and SARS-CoV both belong to the *Severe acute respiratory syndrome-related coronavirus* species, found in human and bats (Gorbalenya *et al.*, 2020). Other known human coronaviruses (hCoV) include hCoV-HKU1, hCoV-NL63, hCoV-OC43 and hCoV-229E. The first genome sequence of SARS-CoV-2 was published on January 10, 2020, with more sequence data released on 12th January 2020 (Hu *et al.*, 2021). Structurally, SARS-CoV-2 genome is covered by a capsid, which are together encased in an envelope having glycoprotein spikes protruding from its surface. The SARS-CoV-2 genome is made up of about 30,000 nucleotides which code for an RNA-dependent RNA polymerase (RdRp) and four structural proteins (Zhu *et al.*, 2020). The genome also

contains six accessory proteins which are encoded by open reading frame (ORF) 3a, 6, 7a, 7b, 8 and 10 genes (Khailany *et al.*, 2020).

COVID-19 has an average incubation period of 14 days, with symptoms resembling those of common flu and can include cough, headache, sore throat, and fever. Mild symptoms such as myalgia, fatigue, dyspnea, and anorexia may show up at the start and progress to atypical symptoms, such as diarrhoea and nausea, and complications such as acute respiratory distress syndrome, arrhythmia and shock which may appear between 2 to 14 days after infection. The disease is more severe in the elderly and people with underlying comorbidities such as hypertension, coronary heart disease, and diabetes (Wang *et al.*, 2020; Zhou *et al.*, 2020). A retrospective study of 1099 patients in China found that fever was the most common symptom in 88.7% of patients, while cough, the next most common symptom was experienced by 67.8% of cases and only about 3.8% of patients experienced diarrhoea. In patients with severe cases, 38.7% had other underlying disorders, and 70.5% of them experienced cough. Fatigue (39.9%) and breathlessness (37.6%) were also common in severe cases (Guan *et al.*, 2020). Patients with mild symptoms could recover within 14 days. Hence, the disease is a self-limiting infection (Jin *et al.*, 2020). Sometimes, SARS-CoV-2 infections may present without symptoms, in which case an asymptomatic infection results (Carlos *et al.*, 2020). Though previous cases were mostly reported in adults, the virus also infects people of all ages, races, regions of the world. Infections in neonates (Zeng *et al.*, 2020), a six months old infant (Kam *et al.*, 2020), children and teenagers (Chang *et al.*, 2020; Shen *et al.*, 2020) have been seen. Though the actual origin of SARS-CoV-2 is still a debate, Andersen *et al.* (2020) opines that it could have arisen from a natural selection process in an animal host before moving into a human host zoonotically, or through natural selection in a human host after zoonosis.

Based on virulence characteristics and amino acid changes, SARS-CoV-2 were classified into three broad variant forms or clades, namely S, L and V (Yap *et al.*, 2020). This is on the basis of the belief that the functional sites on the receptor binding domain (RBD) of SARS-CoV-2 spike protein may have undergone some changes to facilitate natural selection of the virus (Choudhary *et al.*, 2021). Using whole genome sequencing (WGS), viral determinants of virulence as well as viral evolution can be studied. Mutations in the SARS-CoV-2 has given rise to different variants of the virus, particularly arising from non-structural proteins (NSPs) such as the NSP2, NSP3, as well as the spike (S) protein and RdRp (Pachetti *et al.*, 2020). These changes, especially in the NSPs and S protein, impact how the virus evolves and spreads as well as its virulence (Angeletti *et al.*, 2020). The Centres for Disease Control and Prevention (CDC) have classed SARS-CoV-2 variants into three, namely: variants of concern, variants of interest and variants of high consequence. Three notable variants of SARS-CoV-2 include the Alpha variant (B.1.1.7), the Beta variant (B.1.351) and the Gamma variant also known as P.1 variant, which have been associated with a higher infectivity and morbidity (Abdool Karim and de Oliveira, 2021). These three variants of concern also emerged in India in March 2021, resulting in an unprecedented spike in the number of cases recorded prior to the time and have been associated with failure in diagnostic tests and antibody escape. The variant of interest B.1.681 and the variant of concern B.1.617 have also both been reported in India (Singh *et al.*, 2021). In Malaysia, the B.6 lineage has been reported to be more dominant among other nine lineage groups (Chong *et al.*, 2020).

COVID-19 infections occur through human to human transmission via droplets, fomites, aerosols and direct contact (Carlos *et al.*, 2020). Earlier reports also suggest transmission from bats to humans via an intermediate domesticated, domestic

or wild animal host (Triggle *et al.*, 2021). Respiratory droplets generated by humans during talking, singing, sneezing, or coughing are predominantly responsible for transmission of the virus between people. Droplets deposited on surfaces and picked up by the hands can also aide transmission through routes such as the conjunctiva cells of the eyes and physical contact such as handshakes and hugs. There has also been report of SARS-CoV-2 presence in human faeces, which may also suggest the possibility of transmission via the fecal-oral route (Hui *et al.*, 2020; Xu *et al.*, 2020; Wang *et al.*, 2021). Air-borne transmission of SARS-CoV-2 has also been hypothesized as a possible dominant community transmission route. In a study and analysis of mitigation measures in four different countries of the world, findings from Zhang *et al.* (2020) revealed that the mandatory use of face coverings significantly reduced infections and also blocked air-borne transmission. Long distance transmission of the viral particle is less likely than at a range less than 1 meter, considering that aerosol concentration is likely to decrease with distance, thereby reducing the concentration of particles that can be inhaled at a time (Tang *et al.*, 2021). SARS-CoV-2 has been reported to remain viable in aerosols for up to 3 hours and up to 72 hours on surfaces such as plastic and stainless steel. On copper, it remains stable for up to 4 hours and up to 24 hours on cardboard paper (van Doremalen *et al.*, 2020). Transmission control during the SARS-CoV-2 pandemic has shown to be more difficult, compared to SARS-CoV. This is because unlike in SARS-CoV where infectivity and viral shedding occurs late in the disease course (7-10 days post symptom onset), infectivity and viral shedding in SARS-CoV-2 can occur at or even before symptoms begin to manifest. This has made it possible for infected asymptomatic patients to transmit the virus to others (Bae *et al.*, 2021). A study of COVID-19 household transmission conducted in China for instance, revealed a 1.4-

fold higher chance of transmission before symptoms onset than after symptoms onset (Li *et al.*, 2021).

SARS-CoV-2 infection begins with the recognition of the human angiotensin-converting enzyme 2 (hACE2) by the spike protein. Besides hACE2, SARS-CoV-2 can also recognize angiotensin-converting enzyme 2 (ACE2) of dog, ferrets, dogs, civet, rhesus monkeys, cats, rabbits, and pangolins. The implication of this, is the possibility of SARS-CoV-2 having a wide range of hosts that can be infected (Hu *et al.*, 2021). The hACE2 is found expressed in cells of the lungs, endothelia, liver, renal system, heart and gastrointestinal cells, which may explain the incidence of pneumonia in some COVID-19 cases as well as SARS-CoV-2 detection in faeces (Di Gennaro *et al.*, 2020; Wang *et al.*, 2021). The affinity of SARS-CoV-2 spike to hACE2 is 10-20-fold higher than that of SARS-CoV, which may explain its higher infectivity as compared to SARS-CoV. The entry of SARS-CoV-2 into the epithelial cells of the respiratory tract triggers a group of disorders which result in the production of pro-inflammatory cytokines in an uncontrolled manner that results in organ failure and acute respiratory distress syndrome (ARDS) as seen in critical cases of the disease (H. Li *et al.*, 2020).

Diagnosis of active infections of SARS-CoV-2 can be achieved by means of tests that detect either the presence of viral nucleic acid, antigens or viral antibodies, in the patient's sample (Dao Thi *et al.*, 2020). Samples for testing can be oropharyngeal or nasopharyngeal swab, sputum, bronchoalveolar lavage (BAL) and tracheal (Wong, 2021), as well as blood and tears (Kevadiya *et al.*, 2021b). Real-time reverse-transcription polymerase chain reaction (RT-PCR) is the gold standard for diagnosis of COVID-19 (Corman *et al.*, 2020). The RT-PCR assay is based on the conversion of SARS-CoV-2 RNA extracted from a patient's sample first to complementary DNA,

which is then amplified and the amplicons detected based on fluorescence, using real-time PCR machines (Udugama *et al.*, 2020). Several commercial kits for the detection of SARS-CoV-2 genes are now available. Available RT-PCR assays target the RdRp, N, ORF, S protein or envelop (E) gene in patient samples (Wong, 2021). Antigen detection tests for SARS-CoV-2 are designed to directly detect proteins which are produced in respiratory fluids during viral replication. They are mostly in the form of rapid antigen diagnostic tests (Ag-RDTs) (WHO, 2021b) such as immunochromatographic assays on lateral flow strips (Chaimayo *et al.*, 2020). Antibody detection of SARS-CoV-2 involves the use of rapid serological tests that are based on chemiluminescence immunoassays (CLIA) or enzyme linked immunosorbent assays. Such tests detect either IgA, IgG, IgM or a combination of individual antibodies. The levels of these antibodies fluctuate based on the time of sample collection, relative to the age of the infection. Hence, it is prone to errors (Watson *et al.*, 2020; Lagerqvist *et al.*, 2021). Before alternative detection methods became available, imaging techniques such as computed tomography (CT) scan and X-rays were applied in Wuhan for the diagnosis of COVID-19. Features such as typical ground-glass opacities appearing as hazy opaque areas and the presence of fluid or solid material in compressible lung tissue (Bernheim *et al.*, 2020; Pan *et al.*, 2020). This method is however, weak in specificity because the features upon which diagnosis is based, overlaps with those exhibited by other viral pneumonia (Ai *et al.*, 2020). Alternative nucleic acid detection techniques such as isothermal amplification have found application in SARS-CoV-2 diagnosis. Some reverse-transcription loop-mediated isothermal amplification (RT-LAMP) (Dao Thi *et al.*, 2020; Anahtar *et al.*, 2021), reverse transcription–enzymatic recombinase amplification (RT-ERA) (Xia

and Chen, 2020) have also been developed for detecting COVID-19, as well as CRSIPR-Cas based diagnostic systems (Broughton *et al.*, 2020).

Management of transmission involves the implementation of measures that reduce contact between people (physical distancing), as well as the application of non-pharmaceutical interventions such as the use of hand sanitizers, frequent handwashing, and the use of facemask in public places. Isolation and quarantining of suspected cases also helped to break the chain of transmission. The imposition of movement restrictions through lockdowns amidst closure of airports, malls and other public places, and the suspension of public gatherings (Elengoe, 2020; Bae *et al.*, 2021). No effective drug has been found for treating COVID-19 yet, but some clinical trials have been conducted with already available drugs being used for the treatment of other viral diseases such as HIV, Ebola, influenza, and hepatitis. The anti-malarial and anti-parasitic drugs such as chloroquine, hydroxychloroquine and ivermectin have also been used in an attempt to find therapeutic interventions with efficacy against the virus (Triggle *et al.*, 2021). Remdesivir was granted an Emergency Use Authorization (EUA) by the US Food and Drug Administration (FDA) for hospitalized patients with severe illness, but this move was discouraged by the WHO later in November of 2020 (El-Elimat *et al.*, 2021). Attempts have also been made at using convalescent plasma and antibody therapy for the treatment of COVID-19 (Jones *et al.*, 2020; Weinreich *et al.*, 2021). For control and prevention of many diseases known to man, artificial immunity obtained through vaccination is a very important tool. As of December 2020, more than 80 candidate vaccines were at the preclinical development stage with both human and animal trials being conducted. The concerted efforts at vaccine development for COVID-19 resulted in the availability of some vaccines which have currently been approved for use, with more candidate vaccines still undergoing studies

and trials. Examples of these already approved COVID-19 vaccines include the Oxford/AstraZeneca, Pfizer-BioNTech, Moderna, Janssen (Johnson & Johnson), Sinopharm and Sinovac vaccines which are now in use in different countries of the world (Zimmer *et al.*, 2021). COVID-19 vaccines differ in their make-up and can be either a viral vector (Oxford/AstraZeneca), inactivated virus (Sinopharm), mRNA (Moderna, Pfizer-BioNTech) or recombinant protein particles (Novavax). The dosage and number of days between different doses varies with the type of vaccine (Triggle *et al.*, 2021).

1.2 Rationale of study

The rapid spread of COVID-19 to almost every country of the world was largely unexpected after the first cases were discovered in China and has affected several sectors of human life, including the global economic. A combination of continuous testing and isolation of infected people, coupled with vaccination of healthy populations and is key to restoring the world to the pre-COVID-19 era. While this is easier to achieve for wealthy countries, it might remain a herculean task for poorer ones who may not have enough financial might and yet are not spared from the virus. In the case of the SARS-CoV-2, monitoring of body temperature was initially adopted worldwide for screening of apparently healthy individuals in public places, a method which is neither sensitive nor specific (Österdahl *et al.*, 2020).

Among the currently available diagnostic assays for COVID-19, RT-PCR and rapid antibody detection kits are the most popular. RT-PCR has remained the gold standard for determining the COVID-19 status of patients. Although it is reported to have high specificity and sensitivity (Corman *et al.*, 2020), its use in rapid large scale community testing is not practical for some reasons. The workflow required for RT-

PCR assay is cumbersome, time-consuming, expensive and requires trained personnel for its application, thereby limiting its availability to specialized testing laboratories in most cases (Zhu *et al.*, 2020). In rural communities, stable power which is also required for running RT-PCR equipment may not be easy to come by. Averagely, the turn-around time can extend from 4 to 48 hours from sample collection to result especially when large sample size is to be tested, at the risk of reduced specificity (Nawattanapaiboon *et al.*, 2021; Udugama *et al.*, 2020). In addition, false positive swab tests have been reported with RT-PCR (Winichakoon *et al.*, 2020). It is, therefore, important to develop more rapid, simpler, cheaper and yet sensitive diagnostic tool for point-of-care testing, that is also suitable for resource constrained nations or communities.

Isothermal amplification techniques are fast and are conducted at a stable, single temperature throughout amplification. This removes the need for expensive thermal cycling equipment. Loop-mediated isothermal amplification (LAMP) first reported by Notomi *et al.* (2000) as an isothermal molecular diagnostic technique has found wide application in diagnosing infectious disease due to its cheaper cost, rapidity, sensitivity and its adaptability to a range of detection methods (Obande and Singh, 2020; Zhang and Tanner, 2021). LAMP can be coupled with a reverse transcriptase enzyme which can enable the detection of RNA targets, and thus can be used effectively for COVID-19 diagnosis. With the addition of intercalating dyes, results of RT-LAMP can be read with naked eye, thereby excluding the need for agarose gel electrophoresis. The assay can also be coupled with lateral flow strips and be performed using common laboratory heat block or water bath (Obande and Singh, 2020). These qualities make RT-LAMP ideal for large scale community testing and surveillance programs.

1.3 Objectives of the study

1.3.1 General objective:

To develop reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid and simple detection of SARS-CoV-2.

1.3.2 Specific objectives:

i. To identify a suitable conserved region from the nucleocapsid (N) gene of SARS-CoV-2

ii. To design and validate specific LAMP primers targeting N gene of SARS-CoV-2

iii. To develop and optimize colorimetric and lateral flow strip based RT-LAMP assay for specific detection of SARS-CoV-2 N gene with an internal amplification control (IAC)

iv. To evaluate the analytical and diagnostic performance of the developed colorimetric and lateral flow strip based RT-LAMP for SARS-CoV-2 detection

v. To compare the performance of the colorimetric and lateral flow strip based RT-LAMP with the RT-PCR gold standard for SARS-CoV-2 detection.

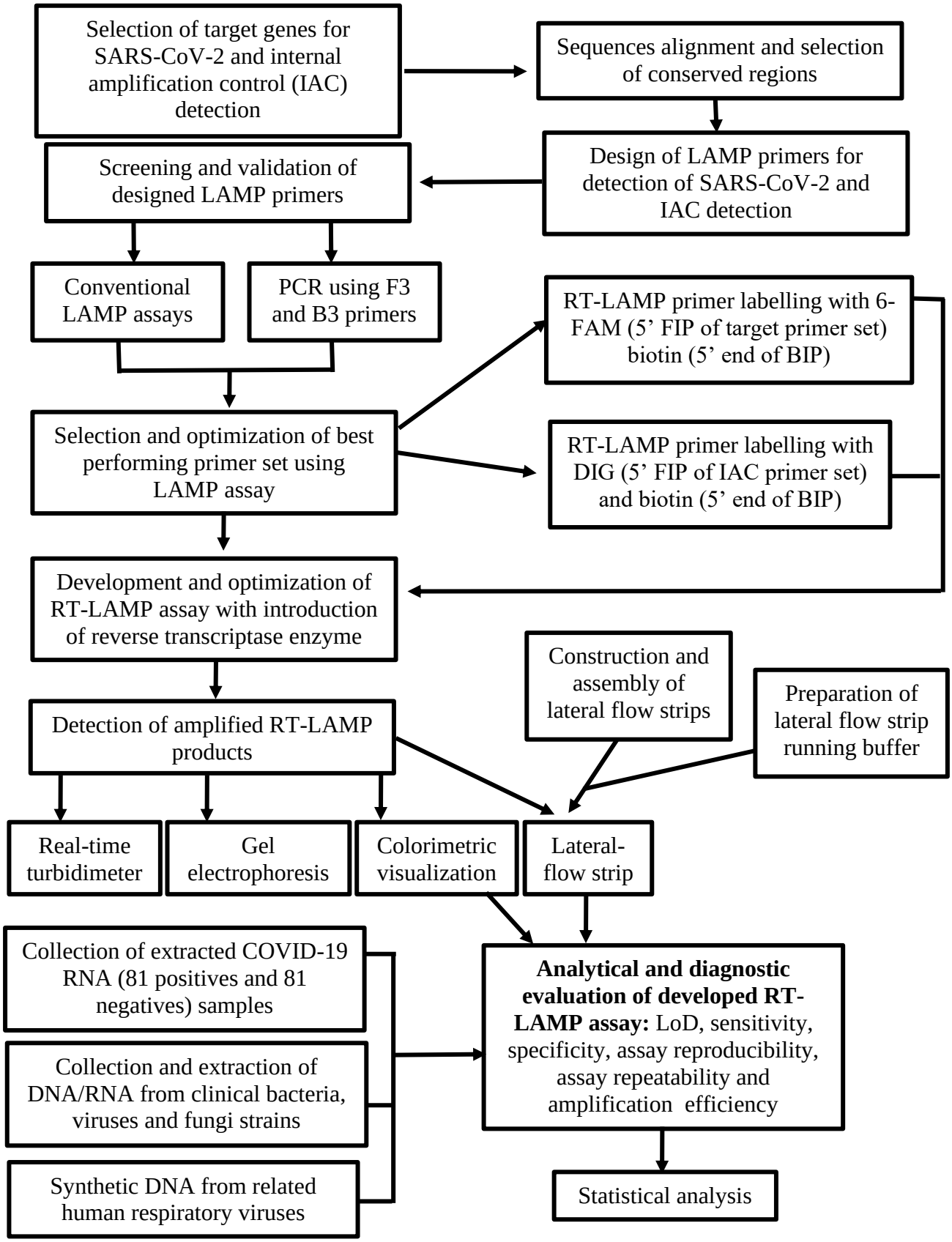


Figure 1.1 Flow chart for the study

CHAPTER 2

LITERATURE REVIEW

2.1 Coronaviruses

The name Coronavirus was derived from the word corona, which means “crown” or “wreath”. This Latin term was itself borrowed from the Greek word korṓnē which means “garland” or “wreath”. This term describes the crown-like appearance of the virus when viewed under the microscope (Woo *et al.*, 2010; Chathappady *et al.*, 2021). Coronaviruses (CoVs) are a family of single-stranded, positive-sense RNA viruses with an envelope covering. The coronaviruses belong to the Kingdom *Orthonavirae*, which consists of 5 phyla, 103 families and 895 genera and 3,577 species (International Committee on Taxonomy of Viruses, 2020). The *Coronaviridae* family to which coronaviruses belong, is made up of four genera which include the alpha (α-coronaviruses), beta (β-coronaviruses), gamma (γ-coronaviruses) and delta (δ-coronaviruses) coronaviruses with the first two being more predominant (Figure 2.1). Coronaviruses are single-stranded RNA viruses with positive-sense genome usually 27 to 32 kilobases (kb) in size. Their genome code for non-structural proteins which play vital roles in transcription and replication of the viruses. The structural proteins which include envelop spike glycoproteins (S), envelope (E), membrane (M) and nucleocapsid (N) proteins are located at the 3’ end of their genome and they also play important roles in the replication and transmission of the viruses. Their genome structure shares resemblances that confers some close genotypic relationships and similarities among members of the family. While the α-coronaviruses and β-coronaviruses infect only mammals, γ-coronaviruses and δ-coronaviruses can infect both birds and mammals.

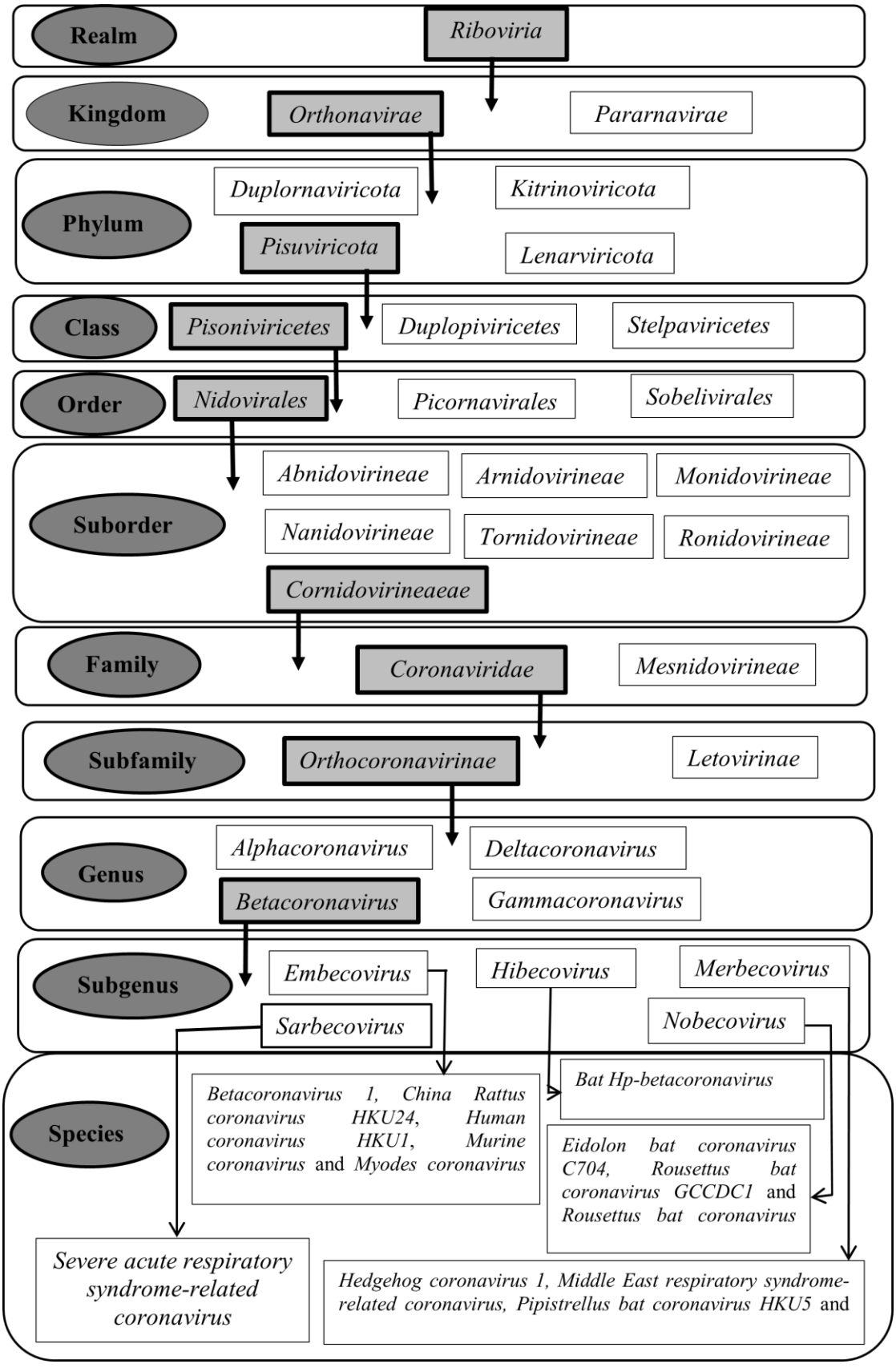


Figure 2.1 Taxonomy of coronaviruses according to the International Committee on Taxonomy of Viruses (ICTV) (Accessed on 27th October 2021)

While SARS-CoV and MERS-CoV cause severe respiratory diseases in humans, human coronavirus (HCoV) NL63, HCoV-HKU1, HCoV-OC43 and HCoV-229E which are also closely related with severe acute respiratory syndrome-related coronavirus, cause mild diseases which could be more severe in infants, children and the aged (Cui *et al.*, 2018). Based on available sequence information, all human coronaviruses are believed to originate from animals. For instance, MERS-CoV, SARS-CoV, HCoV-229E and HCoV-NL63 have their origin from bats, while HCoV-HKU1 and HCoV-OC43 are likely to have originated from rodents (Forni *et al.*, 2017; Su *et al.*, 2016). Bats are considered to be the main reservoir in nature for α-coronaviruses and β-coronaviruses (Woo *et al.*, 2012). Generally, 15% of common cold infections are cause by coronaviruses and influenza viruses, which are not treatable with any known antibiotics (Chathappady *et al.*, 2021). Notably, about 20, 000 deaths were reportedly associated with seasonal flus between 2019 and 2020 alone (Abdelrahman *et al.*, 2020). MERS-CoV and SARS-CoV are also two highly pathogenic coronaviruses in the family *Coronaviridae*.

2.2 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

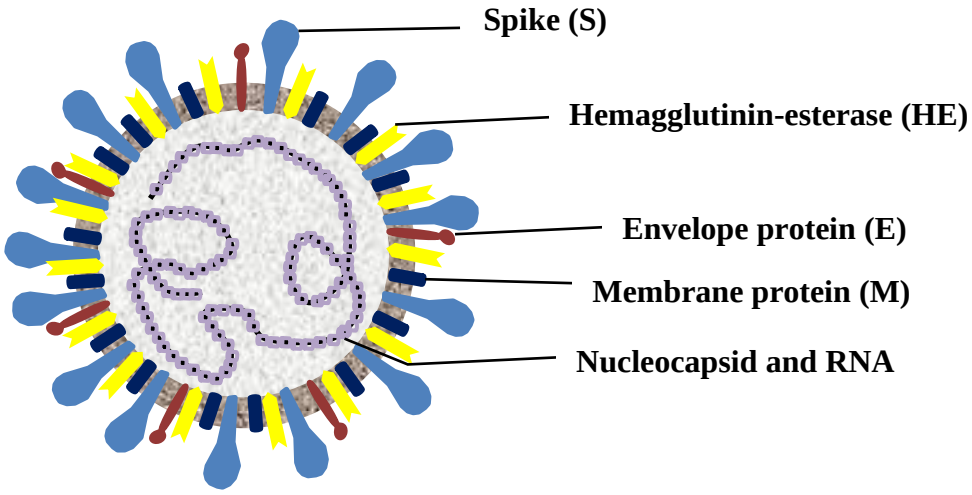
Since the 1918 Spanish flu outbreak, the on-going SARS-CoV-2 pandemic appears to have been a more infectious viral respiratory disease of humans, much more than SARS-CoV and MERS-CoV. Available data as of 12th October 2021 showed that over 238 million infections and over 4.8 mortalities have been recorded globally (Johns Hopkins Coronavirus Resource Center, 2021). SARS-CoV-2 virus belongs to the family *Coronaviridae* and genus *Betacoronavirus* just like SARS-CoV and MERS-CoV but is a member of the subgenus *Sarbecovirus* (International Committee on Taxonomy of Viruses, 2020).

2.2.1 Genomic structure of SARS-CoV-2

SARS-CoV-2 possesses a single-stranded, positive-sense RNA of approximately 30,000 nucleotide (Chan *et al.*, 2020; Zhou *et al.*, 2020; Zhu *et al.*, 2020). SARS-CoV-2 genome codes for an RNA-dependent RNA polymerase (RdRp) and four structural proteins namely nucleocapsid protein (N), a small envelope protein (E), spike surface glycoprotein (S) and matrix protein (M), along with 27 other proteins (Figure 2.2). The RNA is encased in the nucleocapsid and occurs as a large ribonucleoprotein (RNP) complex surrounded by an envelope. The genome contains 14 open reading frames (ORFs) which encode 27 proteins. The ORF1a/b which is located from the 5' end, is cleaved post-translationally to obtain non-structural proteins 1 to 16 that make up the replicase/transcription complex (RTC) that bear enzymes such as the papain-like protease and other enzymes which partake in the mechanism of replication (Alsobaie, 2021; Kim *et al.*, 2020).

E gene encodes the envelop of the virus and is involved in other replication processes such as viral assembly, budding and pathogeneses (Schoeman and Fielding, 2019).The viral M glycoprotein is also known as the matrix protein. It is reportedly the most abundant structural protein, providing the connection between the viral nucleic acid within the viral structure, and the envelope surrounding the structure (Kim *et al.*, 2020). The N protein encoded by the N gene is an important structural protein in CoVs because of its role in processes such as transcription and replication. The eventual shape of the viral nucleocapsid is a function of the N protein because it is involved in the formation of helical ribonucleoproteins during viral RNA packaging, regulation of metabolism in infected cells and regulation or viral RNA synthesis during replication (Kang *et al.*, 2020; Kim *et al.*, 2020). The spikes found in the viral particle are responsible for viral attachment to the potential host cell membrane, and generates

A.



B.

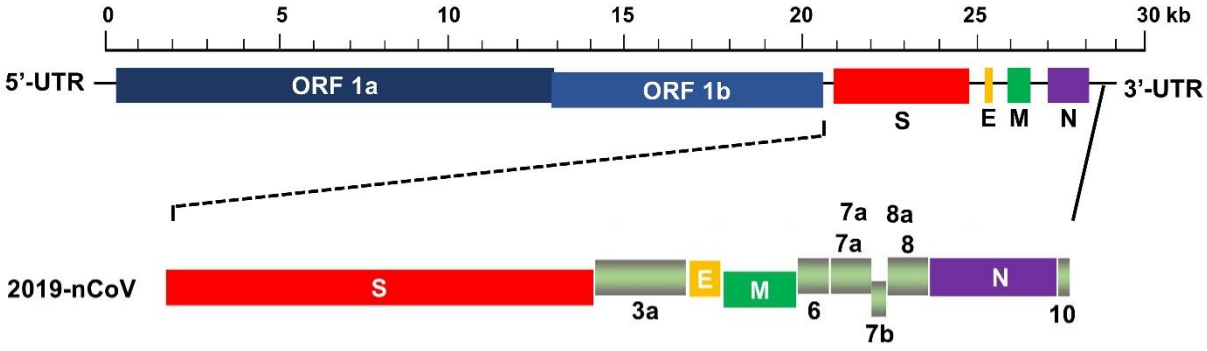


Figure 2.2 Structure of the SARS-CoV-2: (A). Structure of a mature SARS-CoV-2 virus particle (Adapted from Dube *et al.*, 2020); (B). An outline of the structural organization of the SARS-CoV-2 virus genome (Adopted from Wang *et al.*, 2020).

cell-mediated and humoral immune responses within the host cell upon infection (Belouzard *et al.*, 2012). This property makes the S protein a common target for vaccine design and therapeutics against viruses (Amanat and Krammer, 2020). The S protein is a large, functional trimeric structure formed by the aggregation of three individual spike molecules and there are approximately 26 spike trimers in a SARS-CoV-2 particle (Bamford, 2020). It is composed of 2 subunits namely the S1 (amino-terminal) and S2 (a carboxyl-terminal) subunits (Wang *et al.*, 2020). The S1 subunit is composed the N-terminal domain, and the receptor-binding domain (RBD). The S2 subunit is composed of the fusion peptide, heptapeptide repeat sequence 1 (HR1), HR2, TM domain, and cytoplasm domain. While the RBD of the virus is responsible for attachment to the host cell, the carboxy-terminus domain (CTD) of the S2 subunit is responsible for fusion with, and entry of the virus into the host membrane (Huang *et al.*, 2020; Alsobaie, 2021).

Phylogenetic analysis by Zhou *et al.* (2020) showed that the bat coronavirus BatCoV RaTG13 which was formerly detected in the horseshoe bat *Rhinolophus affinis*, shares an overall sequence similarity of 96.2% with SARS-CoV-2 (Zhou *et al.*, 2020), as well as very close sequence similarity between their RdRp and S genes. Using molecular analysis, a close relationship between the SAR-CoV-2 and two other coronaviruses found in bats, namely bat-SL-CoVZC45 and bat- SL-CoVZXC21, was also established. A distant genomic similarity also exists between the SARS-CoV-2, SARS-CoV and the MERS-CoV (Jiang *et al.*, 2020; Lu *et al.*, 2020; Ren *et al.*, 2020). For instance, report of a deep sequencing analysis of clinical samples obtained from suspected cases showed more 75 % homology between SARS-CoV-2 and SARS-CoV (Brant *et al.*, 2021). SARS-CoV-2 also shares up to 50 % homology with MERS-CoV (Lu *et al.*, 2020).

2.2.2 Variations and mutations in SARS-CoV-2

Like other viruses, the SARS-CoV-2 is prone to mutations that result in mutant strains arising during the pandemic. These variants possess characteristics that are dissimilar with those of the parent strains (Cascella *et al.*, 2021a). Variations in viral genome occurs via mutations in their genetic codes, as a natural consequence of viral replication (Grubaugh *et al.*, 2020). SARS-CoV-2 variants are defined by differences in properties such as transmissibility, antigenicity, or virulence (Lauring and Hodcroft, 2021). As of early May 2021, over 1 million SARS-CoV-2 sequences have been deposited in the database of the Global Initiative on Sharing Avian Influenza Data (GISAID), with 3,913 identified major representative variants (Khateeb *et al.*, 2021). In SARS-CoV-2, mutations in the spike protein are the most important. Among the non-structural proteins, mutations occur most in the nsp1 of ORF1a/ORF1b, and ORF8 (Khateeb *et al.*, 2021).

The S1 subunit of the S protein which holds the RBD of SARS-CoV-2, experiences the most mutations on the S protein. These mutations have led to an increase in the virus' binding affinity for ACE2 which is the host cell receptor for SARS-CoV-2, thereby increasing transmissibility and virulence in the resulting variants (Khateeb *et al.*, 2021) while reducing affinity to neutralizing antibodies (Piccoli *et al.*, 2020; Starr *et al.*, 2021). SARS-CoV-2 variants have been classified into 3 major categories by the WHO and the Centers for Disease Control and Prevention (CDC), namely Variants of Concern (VOC), Variants of Interest (VOI) and Variants of High Consequence (VHC) (Bollinger and Ray, 2021). VOCs are defined as variants that have evolved with higher transmissibility, virulence, and decreased responsiveness to infection control measures, including diagnostic tests, vaccines, and therapeutics. They include the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1) and Kappa (B.1.617.1) and Delta (B.1.617.2) variants. In

a recent reclassification however, the WHO now places the Kappa variant under a new group known as Variants Under Monitoring (VUM) (WHO, 2021b). The Alpha variant was first identified in the UK in 2020 with 2 times higher infectivity than the parent SARS-CoV-2 (Lauring and Malani, 2021). In the Alpha variant, the amino acid asparagine (N) at the 501 residue is replaced by tyrosine (Y) (N501Y), while lysine (K) is replaced by asparagine (N) at the 417 residue (K417N). The Beta variant has the E484K mutation just like the Alpha variant. It was first identified in South Africa. The Gamma variant has both the E484K and the K417T mutation in which lysine (K) is replaced by threonine (T). Gamma variant was first identified in Brazil. Both Beta and Gamma variants exhibit increased transmissibility, though not as much as Alpha variant and are reported to show reduced susceptibility to some currently available vaccines (Lauring and Malani, 2021). The Delta and Kappa variants share the mutations L452R (leucine L replaced by arginine R) and E484Q (substitution of glutamic acid E by glutamine Q). These two mutations were found in the second wave experienced in India. The Delta variant also harbors the T478K mutation in which threonine (T) replaced at the 478 residue by lysine (K) (Khateeb *et al.*, 2021). Delta variant remained very dominant in the USA as of August 2021, with double the transmissibility of the parent SARS-CoV-2 strain. The risk of infection with this variant is less in fully vaccinated individuals and highest in unvaccinated persons (Lauring and Malani, 2021). A VOI is one in which mutation influences disease severity, transmissibility, detection by diagnostic tests, susceptibility to host immune response or therapeutic interventions. These variants are also associated with multiple clusters or increased community transmission and increased prevalence in multiple countries. In addition, these variants cause increase in number of cases and pose emerging public health risk (WHO, 2021b). Examples of VOI are Lambda (C.37) and Mu (B.1.621) which were first found in Peru

(December 2020) and Columbia (January 2021) respectively (WHO, 2021b). VHCs are those against which no protection is provided by any available vaccine. Presently, there are no known variants in this category (Bollinger and Ray, 2021). The new category known as VUM, refers to variants with mutations that could affect virus characteristics with indications that could potentially pose risk in the future, though without any current evidence of phenotypic or epidemiological impact. This category of variants requires continuous monitoring and assessment, with the possibility of additions or subtraction from the group over time (WHO, 2021b). As of 4th November 2021, VUM include the former VOIs Iota (B.1.526), Kappa (B.1.617.1), Epsilon (B.1.427/B.1.429) and Eta (B.1.525). Others include R.1, B.1.466.2, B.1.1.318, C.36.3 and C.1.2 (WHO, 2021b).

2.3 Epidemiology of coronavirus disease 2019 (COVID-19)

2.3.1 Origin, nomenclature and spread of COVID-19 globally

Hubei Province of China reported the emergence of an unknown disease among its human population in late 2019. Initial suspicions linked the emergence of the disease to a wet market in Hubei, the Huanan Seafood Market where wild animals were sold for consumption (Brant *et al.*, 2021). Official reports of pneumonia-like symptoms arising from the infection was subsequently made on 31st December 2019, which was linked to an unknown causal agent. By 3rd January 2020, about 44 cases of the infection had been reported and by 7th January 2020, evidences of the emergence of a novel coronavirus were confirmed (WHO, 2020b). Eventually, cases were also discovered in patients who had not previous contact with the wet food market, suggesting that the market may not have been the actual source of the earlier recorded cases. Clusters of infections were observed in families and nosocomial spread were also observed in healthcare facilities, thereby confirming transmission between humans (Hu *et al.*, 2021). Rapid spread of the

disease to other countries outside China was reported between 13th to 20th January 2020 when cases were recorded in the Republic of Korea, Japan, and Thailand. The spread to other countries was facilitated by the movement of people between countries and cities as the period coincided with the Chinese lunar New Year festival (Hu *et al.*, 2021). This high infectivity of the virus led to it being declared by the WHO as a public health emergency of international concern (PHEIC) on 30th January 2020 The rapid spread of the virus from China to virtually every part of the world confirms its higher transmissibility and infectivity than SARS-CoV and MERS-CoV, though with a lower reported case fatality ratio of between 1-7.5% globally (Abdelrahman *et al.*, 2020; Hasan *et al.*, 2021). SARS-CoV-2 became the 6th known PHEIC after the H1N1 influenza of 2009, Ebola virus disease of 2014 and 2019 which ravaged parts of West Africa and Republic of Congo, the poliovirus epidemic of 2014 and Zika virus epidemic which surfaced in 2016 (Lai *et al.*, 2020a).

Initially, the virus was called the Chinese coronavirus or the Wuhan coronavirus but was eventually changed. Based on the agreed guidelines of the WHO, the World Organization for Animal Health (Office International des Epizooties; OIE) and the Food and Agricultural Organization (FAO), the name of the disease was changed by the WHO, from 2019 novel coronavirus (2019-nCoV) to COVID-19 (coronavirus disease 2019) on 11th February 2020. The name change was necessary to avoid the use of names with an inaccurate and stigmatizing tag (WHO, 2020c). This move was timely because reports of discrimination and stigmatization of nationals shortly after an outbreak had previously surfaced (Nassar *et al.*, 2018). The name currently adopted for the viral disease agent by the International Committee on Taxonomy of Viruses (ICTV), is severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The name came from observed relatedness of the novel virus with the severe acute respiratory syndrome

coronavirus (SARS-CoV) which also belong to the species *Severe acute respiratory syndrome-related coronavirus* that had caused severe respiratory disease in humans and was also found in bats (Gorbalenya *et al.*, 2020). SARS-CoV-2 is the first novel coronavirus to cause a pandemic, and the 3rd coronavirus (CoV) to cause a zoonotic disease in humans (WHO, 2020b). COVID-19 was declared a pandemic by the WHO on 11th March 2020, after it had spread to 144 countries with 118,000 infections and 4,291 deaths (WHO, 2020d).

2.3.2 COVID-19 in Malaysia

The first case of COVID-19 in Malaysia was recorded on 25th January 2020, less than 48 hours after neighbouring Singapore had reported its first case. Malaysia experienced the first wave of infections between 25th January and 16th February 2020, while the 2nd and 3rd waves ran from 27th February to 30th June 2020, and 8th September 2020 to March 2021 (Rampal and Seng, 2021; WHO, 2021a). In March 2020, a surge in cases arising from attendees at a religious event held from 27th February to 3rd March 2020 in Sri Petaling, Kuala Lumpur was witnessed, aggravating the 2nd wave. The 4-day event had over 10,000 attendees, out of which 1,500 were foreigners (Barker, 2020). This singular event led to a surge in daily cases from double digits to triple digits on 11th March 2020, and rapidly to 4 digits on 21st March 2020 (Ministry of Health Malaysia, 2021). The 3rd wave was triggered by the state elections which held in Sabah, spiking daily cases up to 4 figures daily. Cumulative cases in Sabah increased by 91.5 % from 808 on nomination day, to 1, 547 on election day and soared to 11,285 within 1 month (Yusof, 2021). What is believed to be another wave began in April 2021 with surging cases up till June 2021. The highest number of daily cases in Malaysia were experienced from July 2021 when cases rose to 5 digits daily. The highest number of

daily cases since the start of the pandemic was recorded on 26th August 2021 (24,602). However, daily cases have been reducing since September into November 2021 (Ministry of Health Malaysia, 2021). As of 9th November 2021, a total of 2,517,173 cases and 29,427 deaths had been reported in Malaysia, with 307 existing active clusters. As of the same period, the highest number of cases reported was among the age group 18 – 59 years (70.3 %) and the least were in the age group 12 – 17 years (7.0 %). Sporadic transmission was the most common source and 57 % of total cases as of 9th November 2021, 57 % of cases were males (Ministry of Health Malaysia, 2021). Deaths were also higher among males (57.0 %) than females (43.0 %), and highest in people aged 60 years and above (54.9 %). The least fatality was among the age group of 12 – 17 years (0.1 %), while the overall case fatality ratio was 1.2 % (Ministry of Health Malaysia, 2021).

Control strategies implemented by the Malaysian government included the imposition of strict movement control orders (MCO), vigorous contact tracing, testing and isolation, as well as a vigorous vaccination effort (Aw *et al.*, 2021; Hashim *et al.*, 2021). Four phases of MCO (18th – 31st March; 1st – 14th April; 15th – 28th April and 29th April – 3rd May 2020) were imposed, which eventually transitioned into 2 conditional MCOs (4th – 12th May, and 13th May – 9th June 2020) and 3 phases of recovery MCOs (10th June – 31st August; 1st September – 31st December 2020, and 1st January – 31st March 2021) (Crisis24, 2021; Hashim *et al.*, 2021). Another total lockdown was imposed from 1st – 28th June 2021, which has now transitioned to 4 phases of a National Recovery Plan (NRP) that will run till 31st December 2021 (Bernama, 2021).

Contact tracing was aided with the use of a national phone application known as MySejahtera which was required to access public places like malls, schools, restaurants, and offices. Daily tests to identify positive cases were also performed using