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The Review of SARS-CoV-2: Recent Perspective and Advances in Detection

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ABSTRACT

Background: The outbreak of novel coronavirus (2019-nCoV), which began in Wuhan, China, has rapidly spread in many countries and is currently considered a pandemic. The virus (SARS-CoV-2) causes severe acute respiratory syndrome and is related to SARS-CoV and MERS-CoV.

Methods: In this review, an introduction to SARS-CoV-2 was provided comprising the following items: general features; pathogenesis; the existing knowledge on immunological properties; transmission routs; diagnostic features, especially discussion about new approaches for treatment and prevention; and different diagnostic methods including nucleic acid based assays, serological testing, and MALDI TOF-MS and LC-MS technologies.

Findings and Conclusion: Introducing the different methods for SARS-CoV-2 detection may be useful to provide new insights into the development and improvement of detection primers, probes, methods/techniques, potential targets for drug designation, and therapeutic candidates against the virus.

Keywords: 2019-nCoV; Acute respiratory syndrome; Nucleic acid base assays; Serological testing; MALDI TOF-MS and LC-MS.

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Introduction

The major causative agents of viral respiratory diseases (RD) include coronavirus, influenza virus, rhinovirus, respiratory syncytial virus (RSV), and adenovirus [1].

Coronaviruses belong to the Nidovirales order, Coronaviridae family, and Coronavirinae subfamily. They contain single, large, plus-stranded RNA as their genome and may cause respiratory, hepatic, enteric, and neurological diseases [2-3]. These viruses could infect a wide range of animals including carnivores, rodents, birds, humans, and other mammals [2].

Until now, six human coronaviruses (HCoVs) have been identified, including HCoV-HKU1, HCoV-229E, HCoV-NL63, HCoV-OC43, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERSCoV) [4]. Some members of the coronaviruses, specifically SARS-CoV and MERS-CoV, are extremely pathogenic, evolving, and reemerging coronaviruses which may cause epidemics or pandemics [1]. In December 2019, the office of the World Health Organization (WHO) in China reported some cases of pneumonia in Wuhan, Hubei province in China; one week later, the causative agent was identified as a novel coronavirus (2019-nCoV) or SARS-CoV-2, and Chinese National Health Commission provided guidance to laboratory diagnoses [5-6]. The bioinformatics analysis revealed that the new virus has the typical structures of coronaviruses [5]. The disease outbreak began from a local seafood market, disseminated significantly, and infected Chines people, leading to 3,242 deaths in China and subsequently leading to the infection of 209, 839 people worldwide with 8,778 deaths as of March 19, 2020 [7]. WHO declared the disease as pandemic on March 12, 2020 [8].

Objectives: In this study, at first, an overview was provided about the virus structure, pathogenesis, treatment, and diagnosis, and then different detection methods were focused on.

Virus structure

Viral particles in infected cells display a typical coronavirus morphology with approximately 60–140 nm in diameter by electron microscopy [9-10] and a genomic structure of +ssRNA with approximately 29891 bp (30 kb) in length [11] (the largest known RNA viruses), containing a 5′-cap structure and 3′-poly-A tail [10,12]. It comprises of two flanking untranslated regions (UTRs) and a single long open reading frame (ORF) encoding a polyprotein including 9860 amino acids with 38% G + C content [11]. The sequence of viral genome was released immediately by public health support via the community online resource virological. org on January 10, 2020 (Wuhan-Hu-1, GenBank Accession Number MN908947); subsequently, other four genomes were deposited in the viral sequence databases on January 12 [13].

The proteome of virus contains envelope protein (E), spike protein (S), membrane protein (M), nucleoprotein (N), and non-structural proteins such as RNA polymerase, papain-like protease, 3-chymotrypsin-like protease, glycoprotein, helicase, and accessory proteins [6,9,14], arranged in the order of 5′ to 3′ [11].

Spike glycoprotein comprises of two subunits: S1 and S2 [11]. The S1 subunit contains three segments: a receptor-binding domain (RBD), an N-terminal domain (NTD), and a signal peptide (SP); the S2 subunit encompasses cytoplasmic domain (CP), transmembrane domain (TM), heptad repeat (HR) 1 and 2 and fusion peptide (FP) (22). It has been reported that the S2 subunit is very conserved and shows 99% identity with that of bat SARS-like CoVs (SL-CoVZXC21 and ZC45) and human SARS-CoV [11] and could be targeted by a broad spectrum of antiviral therapies [11]. RBD contains a highly-conserved core

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domain and an external subdomain on which most of the amino acid differences are located and is involved in direct interaction with the host receptor [11]. Angiotensin-converting enzyme 2 (ACE2) is probably a functional cell receptor for 2019-nCoV, mediating virus enterance into host cells [9]a large number of severe acute respiratory syndrome-related coronaviruses (SARSr-CoV).

Zhou et al. (2020) analyzed 5 samples of 2019-nCoV metagenomically using next generation sequencing (GenBank Accession Number AY278488.2, and GISAID Accession Numbers EPI_ISL_402127–402130), which were identical more than 99.9% [9]. They reported that the virus genome contained six main ORFs which were common to other coronaviruses and several other accessory genes. The direction of genes (5′ to 3′) was reported to be as follows: replicase ORF1ab, spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Figure 1) [6]. The ORF1ab, S, ORF3a, E, M, and N genes were determined to be proteins with 21,291 nucleotides (nt) [6]. Furthermore, the strain WHCV viral genome was determined to be similar to SARS-CoV, while harboring a predicted ORF8 gene with 366 nt in length, located between the N and M genes [6]. In another study, 94.4% similarity was found in amino acid sequences of seven conserved replicase domains of ORF1ab between 2019-nCoV and SARS-CoV, which were used for CoV species classification. However, nucleotide sequence similarity of other genes between 2019-nCoV and SARS-CoV was determined as 80% [9]. Also, 96.2% identity was found between 2019-nCoV and bat-derived coronavirus (BatCoV RaTG13) sequences; BatCoV RaTG13 seems to be closely related to 2019-nCoV. The S gene encoding receptor-binding spike protein of 2019-nCoV was shown to have less than 75% and nearby 93.1% nucleotide sequence identity to that of SARSr-CoVs and RaTG13, respectively [9]. The S gene sequence of 2019-nCoV in comparison to SARS-CoV contained three short insertions in the N-terminal domain, conferring sialic acid binding activity as well as changes in four out of five significant residues in the receptor-binding motif [9]. The phylogenetic analysis provided evidence for the origination of 2019-nCoV from RaTG13 [9].

In another study by Wu et al. (2020), the bronchoalveolar lavage fluid (BALF) was collected from symptomatic patients and analyzed by meta-transcriptomic sequencing, showing a close relatedness to the bat SARS-like coronavirus (CoV) with 89.1% nucleotide identity (GenBank Accession Number MN908947). Their phylogenetic relatedness analysis with respect to the S gene determined that clinical strains of WHCV were most closely correlated to the bat coronavirus SL-CoVZC45 with 82.3% identity in amino acid sequences [6]. According to ORF1b analysis, they proposed that the strains belonged to Sarbecovirus cluster, it may be due to the recombination among the bat sarbecoviruses [6]. It was also shown that spike protein of RBD was closely related to those of SARS-like CoVs and SARS-CoVs with about 75.9–76.9% and 73.8–74.9 amino acid identity, respectively, indicating the virus ability to enter cell using human ACE2 receptor [9].

Pathogenesis

The increase in mortality rate due to SARS-CoV-2 infection is concomitant with severe symptoms of the illness [15]. Infected people exhibit higher leukocytes number, lower lymphocytes number, rough breath sounds, increased amounts of pro-inflammatory cytokines in plasma, increased levels of C-reactive proteins in the blood, elevated erythrocyte sedimentation rate, and increased D dimer rate [16]. The complications such as acute respiratory distress syndrome (patchy consolidation in multiple regions and ground-glass opacities in both lungs),

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RNAaemia, acute cardiac injury, secondary infection, and high plasma levels of IL2, IL10, IL7, GSCF, IP10, MIP1A, MCP1, and TNFα are the main pathogenesis of COVID19 disease, especially in ICU-admitted patients [16-17]. The mechanism of virus entry and immunological responses would be described.

Virus entry to cell: It has been determined that SARS-CoV-2 S protein (forming trimers with two of RBDs facing one direction and the other one facing the opposite way) [18] has a great affinity to bind to human ACE2 according to crystal structure analysis and biochemical interaction [18], and that glutamine (residue 394) in the RBD of SARS-CoV-2 could be recognized by the critical residue on human ACE2 receptor (lysine 31). SARS-CoV-2 RBD identifies and binds to ACE2 more efficiently than SARS-CoV RBD, increasing SARS-CoV-2 ability to transmit from human to human [19]. Overall, ACE2 is required for SARS-CoV-2 entrance to host cell and following viral replication [20]. The overexpression of human receptors has been shown to enhance the severity of disease in mouse model, demonstrating that the step 1 (virus attachment and penetration) is a critical step in the virus pathogenesis. ACE2 has been shown to play a significant role not only in virus entrance but also in the lung injury so that blocking of the renin-angiotensin pathway could attenuate/decrease the lung injury [20]. High expression level of ACE2 has been detected in alveolar type II cells (AT2) of the lung [21], upper esophagus stratified epithelial cells [22], clonal and ileal absorptive enterocytes [22], bile duct choanocytes [23], myocardial cells [24], kidney proximal tubular cells [24], bladder urothelial cells [24], and mucus of oral cavity, especially in tongue epithelial cells [25]. These findings explain the susceptibility of the above organs to the virus entry [24-25].

Activation of Sprotein via cellular transmembrane protease serine 2 (TMPRSS2) has been shown to be essential for viral penetration and spread [26-29]. Proteolytic cleavage of SARS-CoV-2 S protein (at 797 position) has also been shown to mediate the fusion of virus into the membrane and virus entrance in the early step [30]. In the case of MERS-CoV, S protein has been shown to be activated by Furin, a broadly expressed protease in human cells, by a two-step cleavage occurring in distinct sites [31]. It has also been determined that SARS-CoV entry is mediated by a clathrin and caveolae-independent mechanism [32]. SARS-CoV and MERS-CoV RNA have distinct translation mechanisms [33].

Approximately two-thirds of the viral genome is translated to two large polyproteins, and the remains is transcribed into sub-genomic mRNAs [33] (Figure 1). The replicase polyproteins pp1a and pp1ab are cleaved into 16 putative non-structural proteins (nsps), including two viral cysteine proteases; papain-like (nsp3), chymotrypsin-like, 3C-like, or main proteases (nsp5); RNA-dependent RNA polymerase (RdRp)/nsp12; helicase (nsp13); and other nsps which are probably involved in the replication and transcription of the virus [11]. The orfs and nsps of 2019-nCoV and SARS-CoV are similar, and the major discrepancy is in orf3b, spike, and orf8 [11]. Orf3b encodes a fully novel short protein, new orf8 may encode a secreted protein containing an alpha-helix with a beta-sheet (s) [11].

The newly expressed E is combined with RNA and N proteins to form nucleocapsid and buddd ER–Golgi intermediate compartment; finally, virions are released from the cell by fusing the vesicles with cytoplasmic membrane [33].

Immunological processes: When the virus enters the cell and replicates, the antigen presentation is performed by the major histocompatibility complex (MHC) [34]. Unfortunately, there is still no report about

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antigen presentation of SARS-CoV-2, and our knowledge is according to previous research on MERS-CoV and SARS-CoV. The presentation of SARS-CoV antigens is principally based on MHC I molecules activity, although MHC II molecules also play a role [34]. It has been determined that the polymorphism of human leukocyte antigen (HLA) and human mannose-binding lectin (hMBL) is correlated with the susceptibility to SARS-CoV and MERS-CoV infections [34]. If this is also true about COVID 19 infection, it may explain the disease severity some human populations and specific races.

T cells play an essential role in virus eradication. CD8 cytotoxic T cells (CTLs) have the ability to secret a series of molecules, such as granzymes, perforin, and IFN-γ, to clear viruses from the host. Besides, $CD4^+$ helper T cells (Th) could enhance and maintain cytotoxic T cells responses and support B cells in antibody production [35-36]. The total number of T cells (CTLs and Th) has been determined to be intensely decreased in SARS-CoV-2 infection, especially in suspected and intensive care unit (ICU) patients [36]. T cell reduction is negatively linked to patient's survival. It has also been demonstrated that T cell count has a reverse effect on IL-10, IL-6, and TNF-α concentrations in the blood; on the other hand, cytokines IL-6, IL-10, and TNF-α might mediate T cell reduction [36]. Furthermore, CTLs and Th in infected patients with SARS-CoV-2 significantly possess higher amounts of Programmed cell death protein-1 (PD-1) and T-cell immunoglobulin mucin-3 (tim-3) as a marker of exhausted T cells, especially in the symptomatic stage. It has been indicated that in severe cases, the naïve Th cells percentage increases, while memory Th and regulatory T cells count decreases [37]. In severe cases, the count of natural killer cells and B lymphocytes decreases, while the serum level of TNF-α, IL-6, IL-1, and IL-8 increases compared to cases with mild infection [37]. The elevated pro-inflammatory cytokines may be correlated to the severity and progression of the disease and SARS-CoV-2 pathogenesis. The main leading cause of death in patients with COVID 19 is acute respiratory distress syndrome (ARDS) [17]. The cytokine storm is one of the main mechanisms of ARDS. The release of large amount of pro-inflammatory cytokines (IFN-γ, IFN-α, IL-6, IL-1β, IL-18, IL-12, IL-33, TGFβ, and TNF-α) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, and CXCL10) has been reported in SARC-CoV patients [4]. In the case of MERS-CoV, high levels of IL-6 and IFN-α as well as CCL5, CXCL8, and CXCL-10 have been found in the serum of severely ill patients compared to moderately ill patients [38].

The production of IgM and IgG (S and N specific antibodies) against SARS-CoV virus has been well established [18]. It has also been demonstrated that IgM antibodies are presented until the end of Week 12, but IgG antibodies remains for a long time [39]. COVID 19 is assumed to follow a similar pattern.

SARS-CoV and MERS-CoV have developed various strategies to evade immune responses. The viruses avoid the dsRNA detection by the host immune system through inducing double-membrane vesicles production with no pattern recognition receptors (PRRs) and hiding the viral replication complex [40]. Another mechanism established for immune-evasion in MERS-CoV is the downregulation of genes involved in antigen presentation via DNA methylation [4]. The immune-evasion mechanism of coronavirus could be considered as a new approach to COVID 19 therapy.

Transmission route: The common transmission routes of COVID19 include human to human contact (through coughing, sneezing, droplet inhalation, eye mucous, oral, nasal, and membranes) and direct contact with symptomatic and asymptomatic patients [10]. It seems that eye exposure may be

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an effective route for the virus transmission [41]. The ocular tropism of coronavirus (NL63, SARS) has been determined, and body fluids and infectious droplets could simply contaminate the conjunctival epithelium of human with 2019-nCoV [42]. Direct or indirect contact, inhalation of large or small droplets [43], saliva [44], and aerosols are also important routes for virus spread. 2019-nCoV has been detected in the some patients' feces [45], there is also evidence of SARS-CoV-2 gastrointestinal infection, shedding of live virus in feces, and possible fecal-oral transmission route [46-47]. According to CDC report, the transmission of the virus through sewerage systems is low [41], although transmission through this route may be possible, but there is no evidence to date [41]. Medical healthcare workers, including physicians, nurses, laboratory experts, ophthalmologists, and dentists may be particularly at higher risk of COVID-19 infection [43, 48].

Diagnosis is based on the findings of clinical history, chest radiography, and laboratory assays [49].

Clinical symptoms

The incubation time could be mostly 3-7 days and up to 2 weeks, and the longest time for the onset of infection symptoms is 12.5 days but usually appear 2 to 14 days after virus exposure [45, 50]. Clinical manifestations of the illness include dry cough, fever, dyspnoea, pain, weakness, and pneumonia. The disease may be the consequence of progressive respiratory failure due to alveolar injury which could be observed by CT scan (transverse chest computerized-tomography images), less common symptoms are headache, sputum production, sore throat, hemoptysis, and diarrhea [51]. The criteria for diagnosing the disease by clinician include virus-induced pneumonia; a rise in body temperature; dizzy; Lymphocytopenia; higher leukocytes counts (although levels are normal sometimes) and neutrophil-lymphocyte ratio (NLR); lower percentages of eosinophils, basophils, and monocytes [37]; hypoxemia (after onset of the disease); pulmonary infiltrates on chest radiography; and kidney failure [6, 9, 24].

Chest CT: CT scan is an important technique in the diagnosis of the lung disease. High-resolution CT (HRCT) of the chest is critical for early diagnosis and assessment of disease severity in patients with SARS-CoV-2 [52]. Different radiological alterations in the lungs of SARS-CoV-2 patients have been characterized at different times of the disease course. The abnormalities of CT imaging, in symptomatic and asymptomatic patients, happen rapidly and progress from focal unilateral to diffuse bilateral, subpleural, ground-glass opacities which evolve to or co-exist with consolidations peaking within 1–3 weeks or around 2 weeks after the disease onset [53]. The chest CT specificity is comparatively low and could not differentiate SARS-CoV-2 infection from other chest infection, alone [54]. It seems the combination of imaging features, laboratory and clinical results could facilitate early diagnosis of SARS-CoV-2 pneumonia [54]. Laboratory detection of COVID 19 would be described in detail in a separate section.

Detection

Laboratory detection: Exact and fast diagnosis of the etiological agent is important for determining the suitable treatment, saving people lives, epidemiologic monitoring, taking effective preventive steps, breaking epidemics, and decreasing unnecessary use of drugs. Poor clinical description and absence of special prevention methods and treatment approaches mean that early diagnosis is critical. The viral culture and indirect immunofluorescence assay (IFA) as conventional diagnostic methods are labor-intensive and time-consuming with limited sensitivity [1]. In a study, the isolation of SARS-CoV-2 virus was performed with several cell lines, such as Huh-7 from human

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airway epithelial cells and Vero E6 [9]. Clear cytopathic effects (CPE) were observed three days after inoculation. Distinctive crown-like particles were detected with negative staining by transmission electron microscope (TEM) [9].

Specimens: The sample collection, quality, transport and, storage play a significant role in screening and detecting SARS-CoV-2 [54]. SARS-CoV-2 as an RNA virus is prone to be destroy and degrade during the processes of sample collection and transportation to the laboratory for testing or due to some technical factors such as operator expertise and PCR inhibition [54]. The health worker should wear proper personal protective equipment (PPE) (gloves, gowns, eye protection) to collect specimens. Specimen processing should be performed in accordance with biosafety guideline level 2 or higher [55-56]. Specimens should be delivered to a special laboratory as soon as possible. Up to 72 hours after collection, specimens could be stored at 4°C, but for longer time, specimens should be store at -70°C or lower temperature [56].

The specimens required for isolation and detection of SARS-CoV-2 include: upper respiratory specimens such as oropharyngeal and nasopharyngeal swabs or wash fluid in ambulatory patients, as well as lower respiratory specimens such as endotracheal aspirate, sputum, bronchoalveolar lavage in severe form, and mucus of productive coughs. Additional specimens may be collected from the blood, tissue biopsy, stool, and urine [55-56].

The positive detection rate of SARS-CoV-2 in BAL, sputum, nasal swabs, fibro bronchoscope brush biopsy, pharyngeal swabs, feces, blood, and urine were reported 93%, 72%, 63%, 46%, 32%, 29%, 1% and 0%, respectively. different types of specimen has been reported as follows:, [47]. Evidence shows the shedding of live virus (14, 67), indicating the possibility of isolation and detection of virus from stool. Zhang et al. (2020) found that fecal samples were as suitable as pharyngeal swab for the detection of SARS-CoV-2, yielding similarly accurate results [57]. In another study, the virus detection in the stool swab of patients with negative pharyngeal and nasal swabs was reported to be positive [54]. It was reported that the use of a combination of nasal and stool swabs in SARS-CoV-2 patients screening was more sensitive than either alone [54].

Nucleic acid amplification tests (NAAT) for SARS-CoV-2:

Polymerase chain reaction (PCR) based methods: PCR and qRT-PCR: Nucleic acid-based detection methods have advanced quickly and become an innovative tool for virus detection due to the developments in molecular biology technology, especially PCR-based methods with high specificity, sensitivity, and rapidity of detection. These methods are regarded as the gold standard, routine, and reliable techniques for virus detection [58]. In these methods, coronavirus RNA is extracted and converted into cDNA by reverse transcription. The products are analyzed by gel documentation and sequencing, which are the conventional methods for coronaviruses (SARS, MERS viruses) detection [59-60] but are not generally used in clinical samples due to the time-consuming processes and high cost [58].

The detection based on real-time reverse transcriptase-PCR (RT-PCR) is presently preferred for the detection of coronaviruses owing to its advantages such as specificity, sensitivity, simplicity, and quantitative assay, which provide the possibility of early detection [76-77]. As a result, a real time RT-PCR based assay is still a primary method for detecting coronaviruses, including SARS-CoV-2 [61].

Extensive efforts are being made to improve and advance the real time RT-PCR assay due to the contamination risk, the need for post-

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PCR analysis, and being time-consuming for sample handling. A TaqMan-based real time RT-PCR has been designed with specific primers and probes targeting the N gene, which could straightforwardly be employed in the routine clinical and diagnostic settings for the detection of HCoVs (229E, OC43) in nasal wash (NW), combined nasal and throat swabs (NTSs), and bronchoalveolar lavage (BAL) specimens [62]. Yip et al. (2005) designed a 1-step real-time quantitative RT-PCR (qRT-PCR) assay for SARS-CoV detection using 2 TaqMan probes, instead of 1 probe, with an increase in reaction sensitivity and a detection limit of 1 RNA copy per reaction [63]. The fast-mutation nature of coronaviruses with great mutation rates compared to DNA viruses is considered as an adaptation mechanism for survival and as the intrinsic polymorphic nature of these viruses [48, 81], highlighting the necessity for precise detection of these genetically diverse coronaviruses [48, 58]. For this purpose, multi-target detection real-time RT-PCR methods with appropriate sensitivity have been established for coronavirus. In a study, a real-time RT-PCR test via mismatch-tolerant molecular beacons was developed to detect and distinguish SARS-CoV from pathogenic and non-pathogenic strains. This assay was executed by four beacons, specific for the E, S, N and M genes with an internal positive control (IPC) [64]. The assay exhibited high ability for target detection and specificity with a detection limit of 5 copies per reaction (100%) [64]. This assay provided a simple and fast approach to detect SARS-CoV and a pattern for molecular detection of emerging mutant pathogens [64].

Two RT-PCR assays were recommended by Corman et al. (2014) for human coronaviruses (hCoV-NL63, hCoV-229E, SARS-CoV, hCoV-OC43) detection, targeting upstream regions of the E gene (upE) or within ORF1b for screening and detection, respectively [61]. In another study, two real time RT-PCR assays were established, targeting MERS-CoV N gene and upstream regions of MERS-CoV E gene (upE) for the detection and confirmation of MERS-CoV infection in respiratory, serum, and stool specimens [13].

According to several real-time RT-PCR assays applied for coronaviruses, different assays have been announced for the detection of COVID19 causative agent.

A qPCR-based detection method based on the sequence of the RBD of the S gene was proposed by Zhou et al. (2020), but it was not reproducible. Other qPCR targets, including RdRp or E genes, were also recommended for the routine detection of 2019-nCoV [9].

Recently, the World Health Organization (WHO) has declared several primer and probe sets for SARS-CoV-2 detection, which were developed in China, Hong Kong, Japan, Germany, Thailand, Pasteur institute, and USA [36, 61-62, 65-69].

Corman et al. (2020) established and validated a workflow for 2019-nCoV screening and confirmation without virus isolates on Jan 13. They used synthetic nucleic acid technology for designing virus genome closely related to 2003 SARS-CoV [61] before public release of virus sequences from 2019-nCoV cases. They recommended the assay stand on E gene for the first-line screening tool, followed by RdRp/orf1 gene assay for confirmation. The assay was performed with dual-color technology for discriminating 2019-nCoV (both probes positive) from SARS-CoV RNA (Table 1, Figure 1) [61]. The two probes for the second assay included a broad-range reacting probe for SARS-CoV and 2019-nCoV and another probe for annealing only with 2019-nCoV (Table 1) [44]. They suggested the pick out of only 2019-nCoV-specific probe, alternatively [61]. All assays displayed detection limits of 5.2 and 3.8 copies per reaction for the E and RdRp genes, respectively, displaying high sensitivity; the N gene had slightly fewer

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sensitivity and was omitted in the second protocol uploaded on Jan 17 by Corman et al. (2020) [61]. No cross-reactivity with other coronaviruses or non-specific reactivity between oligonucleotides was detected in all assays [61].

The University of Hong Kong, School of Public Health reported the use of two monoplex assays: the N gene-targeted RT-PCR assay for screening, which overlaps with SARS-CoV and bat SARS-like coronaviruses, and the Orf1 (ORF1b-NSP14) based assay as a confirmatory assay (Table 1, Figure1). They recommended sequence analysis of products for confirmation and discrimination when the results were positive [65, 70]. The evaluation of confirmatory assay indicated a wide dynamic range (2^{-4}-2000 TCID50/reaction) and exclusivity to COVID19 [65, 70]. However, in-silico analysis indicated the overlapping of confirmatory assays with other coronaviruses genome.

Naganori et al. (2020) in Japan designed a new panel to detect 2019-nCoV: 1) nested RT-PCR and sequencing by 3 primer sets for the Orf1b and S genes, in which the second PCR assay (nested-PCR) was performed after the first PCR, the products were visualized by 2% agarose gel electrophoresis, and the rest of products were purified and subjected to sequencing with special primers (Table 1, Figure1) [66], and 2) real-time RT-PCR with specific primers and probes for the N gene [66] (Table 1, Figure 1). It seems the mentioned N gene specific primers and probes do not overlap with other coronaviruses according to our in-silico analysis.

Ministry of Public Health, Thailand released a protocol for RT-PCR assays based on the N gene detection by primers and probes [71], which was indicated to be specific just for COVID 19.

Accordingly, the Centers for Disease Control and Prevention (CDC) of US provided a RT-PCR based diagnostic panel for the novel coronavirus [67]; accordingly, it was designed with 4 primer pairs, 3 for the N gene (different region) and one for RNase (Table 1, Figure 1) [67].This panel was recommended for the detection as follows: by $N1^{+}$, $N2^{+}$, $N3^{+}$, and $RP^{\pm}$, the result is considered as COVID19 positive, when only one or two of three N targets are positive and $RP^{\pm}$, it is considered as a not conclusive result, and with $N1^{-}$, $N2^{-}$, $N3^{-}$, and RP^{+}, the result is considered as COVID19 negative, and negative results for all target genes is considered as invalid [56].

Institute Pasteur of France presented a protocol for SARS-CoV-2 detection, containing 2 series of assays (simplex and multiplex), targeting the E and RdRp genes (spanning 2 regions), respectively [69]. The limit of detection was reported to be about 100 copies of RNA genome per reaction for the E gene, and 10 copies could be detected with multiplex assay by CI 95% [69]. The specificity of primer and probe sets was evaluated with specimens which were positive for a panel of respiratory viruses; no cross-reactivity was detected [69].

China CDC released a protocol for the detection of SARS-CoV-2 from clinical specimens based on the N and ORF1ab genes amplification, in which ORF1ab specific primers and probe were used for confirmation [72] with good sensitivity and specificity. The N gene-targeted primers and probes were applied for screening (Table 1, Figure 1).

In another study, two monoplex real-time RT-PCR assays were performed based on the Hong kong procedure [65], targeting the N gene and RdRP with weak sensitivity.

The high rate of false-negative results is the most commonly reported problem associated with most of the primers and probes used for detection. Jung el al. (2020) performed the first analysis on Feb 25 and compared various primer-probe sets which provided the laboratory confirmation of SARS-CoV-2 [68]. They selected 10 primer-probe sets, 7 sets for the N gene and the other sets for various

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Table 1) Several primers and probes for real-time RT-PCR and nested-PCR detection of SARS-CoV-2

Target Gene/ Assay	Name	Sequence 5'---3'	Product Size (bp)	Reference
RdRP / real-time RT-PCR	RdRp-SARSr-F	GTGARATGGTCATGTGTGGCGG	100	61
	RdRp-SARSr-R	CARATGTTAAASACACTATTAGCATA		
	RdRP-SARSr-P1	AM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ		
	RdRp-SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ		
E / real-time RT-PCR	E-Sarbeco-F	ACAGGTACGTTAATAGTTAATAGCGT	113	
	E-Sarbeco-R	ATATTGCAGCAGTACGCACACA		
	E-Sarbeco-P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ		
N/ real-time RT-PCR	N-Sarbeco-F	CACATTGGCACCCGCAATC	128	
	N-Sarbeco-R	GAGGAACGAGAAGAGGCTTG		
	N-Sarbeco-P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ		
ORF1b-nsp14/ real-time RT-PCR	HKU-ORF1b-nsp14F	TGGGGYTTTACRGGTAACCT	132	65
	HKU- ORF1b-nsp14R	AACRCGCTTAACAAAGCACTC		
	HKU-ORF1b-nsp141P	FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA		
N/ real-time RT-PCR	HKU-NF	TAATCAGACAAGGAACTGATTA	109	
	HKU-NR	CGAAGGTGTGACTTCCATG		
	HKU-NP	FAM-GCAAATTGTGCAATTTGCGG-TAMRA		
N/ real-time RT-PCR	N (54)-F	CGTTTGGTGGACCCTCAGAT	57	61
	N (54)-R	CCCCACTGCGTTCTCCATT		
	N-P	FAM-CAACTGGCAGTAACCA- BQH1		
N / nested-PCR-sequencing	NIID-WH-1-F501	TTCGGATGCTCGAACTGCACC	413	66
	NIID-WH-1-R913	CTTTACCAGCACGTGCTAGAAGG		
	NIID-WH-1-F509	CTCGAACTGCACCTCATGG	346	
	NIID-WH-1-R854	CAGAAGTTGTTATCGACATAGC		
	NIID-WH-1_Seq-F519	ACCTCATGGTCATGTTATGG	336	
	NIID-WH-1-Seq-R840	GACATAGCGAGTGTATGCC		
	WuhanCoV-spk1-F	TTGGCAAAATTCAAGACTCACTTT	547	
	WuhanCoV-spk2-R	TGTGGTTCATAAAAATTCCTTTGTG		
S / nested-PCR-sequencing	NIID-WH-1-F24381	TCAAGACTCACTTTCTTCCAC	493	
	NIID-WH-1-R24873	ATTTGAAACAAAGACACCTTCAC		
	NIID-WH-1-Seq-F24 383	AAGACTCACTTTCTTCCACAG	482	
	NIID-WH-1-Seq-R24 865	CAAAGACACCTTCACGAGG		
N/ real-time RT-PCR	NIID-2019-nCOV-N-F2	AAATTTTGGGGACCAGGAAC	57	
	NIID-2019-nCOV-N-R2	TGGCAGCTGTGTAGGTCAAC		
	NIID-2019-nCOV–N-P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ		
N/ real-time RT-PCR	2019-nCoV-N1-F	GACCCCAAAATCAGCGAAAT	72	67
	2019-nCoV-N1-R	TCTGGTTACTGCCAGTTGAATCTG		
	2019-nCoV-N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
	2019-nCoV-N2-F	TTACAAACATTGGCCGCAAA		
	2019-nCoV-N2 -R	GCGCGACATTCCGAAGAA	67	
	2019-nCoV-N2 -P	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1		

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Table 1) continued

Target Gene/ Assay	Name	Sequence 5'---3'	Product Size (bp)	Reference
N/ real-time RT-PCR	2019-nCoV-N3-F	GGGAGCCTTGAATACACCAAAA	72	67
	2019-nCoV-N3-R	TGTAGCACGATTGCAGCATTG		
	2019-nCoV-N3 -P	FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1		
RNAse/ real-time RT-PCR	RNAse P-F	AGATTTGGACCTGCGAGCG		
	RNAse P-R	GAGCGGCTGTCTCCACAAGT		
	RNAse P -P	FAM-TTCTGACCTGAAGGCTCTGCGCG- BHQ		
RdRp/Orf1/ real-time RT-PCR	ORF1ab-F	CCC TGTGGGTTTTACACTTAA	119	70
	ORF1ab-R	ACGATTGTGCATCAGCTG A		
	ORF1ab-P	FAM-CCGTCTGCGGTATGTGGAAAGGTTATG G-BHQ1		
N/ real-time RT-PCR	N (60)-F	GGGGAACTTCTCCTGCTAGAAT	99	
	N (60)-R	CAGACATTTTGCTCTCAAGCTG		
	N-P	FAM-TTGCTGCTGCTTGACAGATT-BHQ1		
RdRp/Helicase/ real-time RT-PCR	RdRp/Helicase-F	CGCATACAGTCTTRCAGGCT	134	73
	RdRp/Helicase-R	GTGTGATGTTGAWATGACATGGTC		
	RdRp/Helicase-P	FAM-TTAAGATGTGGTGCTTGCATACGTAGAC-lABkFQ		
S/ real-time RT-PCR	S-F	CCTACTAAATTAAATGATCTCTGCTTTACT	158	
	S-R	CAAGCTATAACGCAGCCTGTA		
	S-P	HEX-CGCTCCAGGGCAAACTGGAAAG-IABkFQ		
N/ real-time RT-PCR	N (122)-F	GCGTTCTTCGGAATGTCG	97	
	N (122)-R	TTGGATCTTTGTCATCCAATTTG		
	N-P	FAM-AACGTGGTTGACCTACACAGST-lABkFQ		
RdRp/ real-time RT-PCR	nCoV_IP2-12669-F	ATGAGCTTAGTCCTGTTG	108	69
	nCoV_IP2-12759-R	CTCCCTTTGTTGTGTTGT		
	nCoV_IP2-12696bProbe (+)	Hex-AGATGTCTTGTGCTGCCGGTA-BHQ-1		
	nCoV_IP4-14059-F	GGTAACTGGTATGATTTCG	108	
	nCoV_IP4-14146-R	CTGGTCAAGGTTAATATAGG		
	nCoV_IP4-14084Probe(+)	FAM-TCATACAAACCACGCCAGG-BHQ-1		

regions of the ORF1 gene, including RdRp, ORF1b-Nsp14, and ORF1-Nsp10 according to sequence data from different national institutes [56, 61, 65-68, 71]. Among which ORF1ab set was provided by China, targeting the RdRp/Orf1 region; in the case of targeting the N gene, USA recommended N2 and N3; and Japanese provided NIID-2019-nCOV-N sets which displayed the highest sensitivity compared to other sets and was suggested for reliable and sensitive laboratory confirmation of COVID19 [68]. According to the results of mentioned studies and our In-silico analysis, it seems the use of a combination of ORF1ab (China CDC); USA-provided 2019-nCoV-N2 and N3; and NIID-2019-nCOV-N by Japan provides appropriate primers and probes to decrease false-negative confirmation of SARS-CoV-2.

Very recently, Yuen et al. (2020) [73] designed a new platform to detect SARS-CoV-2 by 3 novel real time RT-PCR assays targeting the

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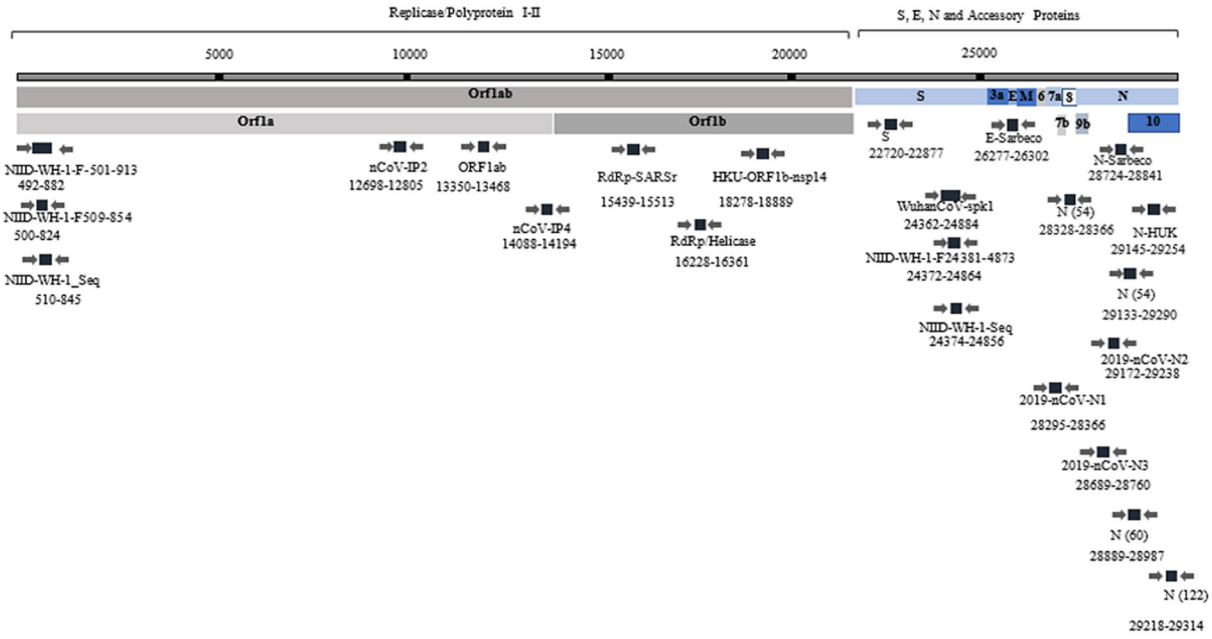

Figure 1: Schematic representation of COVID19 genome and relative position of primers and probes.

RdRp/ Hel, N and S genes of SARS-CoV-2 [73] and compared the results with PdRp-P2 assay from Berlin protocol [61]. RdRp/Hel assay had the least limit of detection in vitro and was positive in specimens diagnosed as negative by RdRd-P2 [73]. Furthermore, RdRp/Hel assay, unlike RdRp-P2 assay, did not show cross-reactivity with other HCoV in clinical specimens and cell culture [73]. The high sensitivity and specificity of the new RdRp/Hel assay was determined [73], and it was suitable for reducing false negative cases.

Another approach recommended for reducing the false negative detection is the use of a combination of RT-PCR and CT scan [54-55]. The accomplishment of both RT-PCR and CT has been determined to have a higher sensitivity (91.9%) compared to CT (66.7%) or RT-PCR alone (78.2%) [54]; indeed, RT-PCR compared to CT is superior in diagnosing mild infections [54]. While qRT-PCR method is a gold-standard assay to detect coronaviruses such as MERS and SARS [68, 74], the recommended qRT-PCR assays for SARS-CoV-2 have some alarms: i) cross-reactivity of primers-probes regarding the high similarity of SARS-CoV-2 to SARS-CoV, ii) the assays sensitivity in the detection of the disease in the early stage, RT-PCR assay was reported to be positive in 46.7% of patients with slight infection [62], iii) false-negative results in patients with positive CT scan results [75-76], IV) the efficiency of molecular diagnostic assay might be correlated to primers, probes, and reagents [68], and false negative results could be caused by poor specimen quality, such as respiratory samples collected from the oropharynx instead of the nasopharynx or sampling in the early or late stage of the disease [54], V) the RT-PCR assays have long turnaround times, are complicated, and usually take an average of more than 2 to 3 hours to generate results; hence, the development of new assays seems to be essential.

Isothermal nucleic acid amplification-based methods: Numerous molecular assays based on non-PCR methods have been

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established to detect the new coronavirus, such as loop mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification [58]. The LAMP assay requires only a single temperature for amplification, is a quick test, and does not require expensive instruments and reagents. At the end of the reaction, gel electrophoresis is generally used for the analysis of endpoint products. A simple LAMP assay was previously reported for SARS-CoV detection, targeting the orf1b region in the presence of 6 primers [77]. The sensitivity of LAMP assay for SARS-CoV detection was similar to those of PCR-based methods [77]. In another study, the LAMP assay was applied for the detection of HCoV-NL63 with specific primers designed based on conserved fragment of the N gene [78]. The assay had high specificity and sensitivity with a detection limit of 1 copy of RNA template per reaction [78].

The LAMP assay could also be applied for RNA detection in combination with reverse transcription (RT-LAMP). Shirato et al. (2014) [74] developed a RT-LAMP assay for the detection and epidemiological surveillance of human MERS-CoV with primers targeting conserved region of the N gene [78]. The assay was very specific and did not show cross-reactivity with other respiratory viruses [78]. The LAMP assay product could be detected by fluorescence dye or the precipitation of magnesium pyrophosphate in real time, resolving the end point detection limit. In a study, a one-step real-time quantitative RT-LAMP assay was developed for the early and rapid diagnosis of SARS-CoV [79]. It had a detection limit of 0.01 plaque forming units (PFU) and provided a sensitivity 100-fold higher than that of RT-PCR [79].

In a study, sequence-specific LAMP-based methods were developed for improving the specificity of assays and separating true signal from nonspecific one [58]. In another study, the RT-LAMP assay was improved using a quenching probe (QProbe) for monitoring signal in addition to two primer sets (targeting N and Orf1a sequences) for the detection of MERS-CoV [80].

Isothermal nucleic acid-based amplification methods could be developed and applied for the detection and confirmation of SARS-CoV-2 due to the similarity of the new virus to SARS-CoV and MERS-COV and the success of the LAMP technique in their detection.

Microarray-based methods: Oligonucleotide microarrays have been used for monitoring, detecting, and analyzing virus in clinical samples. In this method, for RNA viruses such as coronavirus, RNA is converted to cDNA, labeled by a specific probe, and fixed on the microarray; thus, the RNA of coronavirus could be detected by a specific probe [58]. Shie et al. (2003) designed and synthesized thirty 60mer specific oligonucleotides according to the sequence of TOR2 (SARS-CoV), which covered the whole genome, [81] and printed them into a microarray. The results indicated that the SARS-CoV genome could be detected separately by the 60mer oligonucleotide microarray, thereby improving the positive ratio of the diagnosis [81].

Guo et al. (2014) developed a single nucleotide polymorphism (SNP) DNA microarray to detect and genotype the S gene of SARS-CoV with 100% accuracy [82]. Although microarray is a specific and sensitive method, its high cost limits its further application in the detection of coronavirus; therefore, it could be used in SARS-CoV-2 research.

CRISPR diagnostics: Microbial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (CRISPR-Cas) adaptive immune systems have programmable endonucleases that could provide approaches for CRISPR-based diagnostics (CRISPR-Dx) [83]. On March 6, 2020, Broughton et al. released a protocol for

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detecting SARS-CoV-2 based on the extracted sample RNA in approximately 30 minutes by CRISPR- Cas12, called SARS-CoV-2 DETECTR [84]. The nasopharyngeal or oropharyngeal extracted sample RNA was converted to DNA and amplified simultaneously by reverse transcription and loop-mediated amplification (RT-LAMP) with primers targeting the E and N genes of SARS-CoV-2; afterwards Cas12-guide RNAs in order to detect three SARS-like coronaviruses (SARS-CoV-2, bat SARS-like coronavirus, and SARS-CoV) in the E gene and specifically to detect SARS-CoV-2 only in the N gene, detected coronavirus sequences, cleaved reporter molecule, and confirmed virus detection [84]. SARS-CoV-2 DETECTR was evaluated by reference and clinical samples, and the results were determined to be comparable with RT-PCR assay [84].

Zhang et al. (2020) described a protocol for detecting COVID-19 using a CRISPR-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) technique [85-86]. At first, extracted/synthetic RNA was amplified by isothermal method with specific primer for the S and Orf1ab genes, followed by the addition of LwaCas13a crRNA for detecting the S and Orf1ab genes[85]. The authors declared that they continue to optimize this protocol [85].

Serological testing: There is no data about the time of antibody production against SARS-CoV-2, since this virus belongs to the viral family causing the MERS and SARS outbreak, the antibody generation time and process is thought to be the same [87]. It has been reported that IgM and IgG antibodies could be detected in the blood of SARS patients 3 and 5 days after the onset of fever, respectively [88]. Rapid detection of IgM and IgG antibodies would be valuable in the diagnosis and treatment of SARS-CoV-2 disease.

Serology testing is a substantial assay for the detection of SARS-CoV-2 due to the comparatively short diagnosis time and the possibility of examining an active immune response against pathogen [89]. Currently, the standard method to diagnosis disease is PCR based on viral RNA, and no serological test is available [89]. Theoretically, the combination of PCR and serological assay seems to increase the detection sensitivity [79]. Two possible serological assays would be as follows: i) detection of viral proteins (S, M, E or N) with specific antibodies produced in animal, and ii) ELISA assay which detects patient's antibodies against virus using serum [89].

It has been reported that N protein of bat SARSr-CoV Rp3 shares 92% amino acid identity to that of 2019-nCoV and could be used as a target for IgG and IgM enzymes-related immunosorbent assays (ELISAs) with no cross-reactivity against other human coronaviruses except SARSr-CoV [9].

Tian et al. (2020) reported that human monoclonal antibody CR3022 (specific for S protein RBD domain in SARS-CoV) could bind effectively to 2019-nCoV RBD [90]. It could be related to the identity (73%) between RBDs in 2019-nCoV and SARS-CoV [90]. It was determined that the CR3022 epitope did not overlap with the binding site of RBD to ACE2 and had the potential to be used as a therapeutic candidate and in vaccine development for SARS-CoV-2 infection [90]. CR3022 has cross-reactivity with SARS-CoV and could not be applied to specifically detect the new virus but could be effective in the development of monoclonal antibodies specific for SARS-CoV-2 S protein.

Xiang et al. (2020) evaluated Enzyme-Linked Immunoassay and Colloidal Gold-Immuno-Chromatographic Assay Kits (GICA) for SARS-CoV-2 IgM and IgG detection in serum samples [49]. The novel coronavirus IgG/IgM antibody ELISA and GICA kits, manufactured by Zhu Hai Liv Zon Diagnostics Inc, China,

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were used to capture and detect viral IgM and IgG according to the manufacturer protocol, and the results were compared by qRT-PCR (81). The sensitivity of the combined ELISA and GICA was reported as 87.3 and 82.4%, respectively [49]. They concluded the used assays were fast, simple, and safe for COVID-19 diagnosis [49].

Li et al. (2020) developed a rapid and simple immunoassay to simultaneously detect IgM and IgG antibodies against a recombinant RBD of SARS-CoV-2 in the blood within 15 minutes at different infection stages [87]. The kit sensitivity and specificity was determined as 88.66 and 90.63%, respectively. This simultaneous antibody detection kit has numerous advantages compared to RT-PCR, does not require equipment, is fast and simple, and could be used in any clinic and laboratory, train stations, or airports to screen people, especially asymptomatic people [87]. It is more convenient and appropriate to use the blood of finger or heel instead of vein. In a specimen prepared during the early stage of SARS-CoV-2 from throat swab or sputum, the virus may not be detectable, since the combined kit detects IgM and IgG simultaneously, it could be applied for both early stage diagnosis (IgM) as well as for illness monitoring during other stages [87]. Nevertheless, there were still false negative and false positive cases due to the low antibody concentrations or cross-reactivity with other viruses.

Many antibody detection or virus antigen detection kits are under development.

MALDI TOF-MS and LC-MS technologies

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography–mass spectrometry (LC-MS) are analytical chemistry methods which have been highly successful in identifying all classes of biomolecules: proteins, lipids, and nucleic acid. These techniques provide accurate mass information on intact protein/DNA, proteolytic digests or products of mass probe [91-92].

Xue el al. (2017) established a universal screening method for coronavirus using MALDI-TOF Mass Spectrometry that comprised of a 17-plex analysis to detect six HCoVs in Panel A which included primers and nucleotide extension probes specific to the Orf1 and N genes of six coronaviruses and primers and probes targeting the Orf1b and upE genes of SARS-CoV and MERS-CoV with ribonuclease P as internal control, as well as another 17-plex analysis to detect Alphacoronavirus and Betacoronavirus in Panel B which included primers and probes for the RdRp gene in Alphacoronavirus and Betacoronavirus [91, 93]. They reported that the mCoV-MS method is a sensitive and relatively simple assay but may not be successful in detection, especially when the virus load in sample is very low [91]. It seems the method could be improved by increasing the number of primers and probes.

Sampath et al. (2005) presented an approach for rapid identification of known and emerging coronaviruses [94]. They designed a broad-range PCR targeting regions of Orf1b and NSP14 to amplify nucleic acids of large coronavirus groups; they used electrospray ionization mass spectrometry (EIS) to accurately measure PCR products as well as nucleotide composition analysis to identify viruses. This method was able to detect and distinguish SARS-CoV from others with 1 PFU/mL sensitivity [94].

Very recently, Jenkins et al. (2020) developed methods for in-silico detection of SARS-CoV-2 in clinical specimens by LC-MS [95]. At first, they created protein FASTA files and digest map (by shotgun proteomics and trypsin proteolysis); afterward spectral libraries were generated using FASTA files, transition lists were derived from

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libraries (special software), and post translational modifications were identified [96]. They provided a procedure for SARS-CoV-2 detection by LC-MS, which continues to be refined.

Conclusion

In conclusion, the development of new approaches for the treatment and detection of COVID19 could be effective in preventing and decreasing the progression of the disease. In this study, different approaches to treatment were introduced, and different primers and probes for detection were compared. According to our literature review and analysis, the use of a combination of Orf1ab (China CDC), RdRp/Hel, USA provided 2019-nCoV-N2 and N3, and NIID-2019-nCOV-N by Japan provides appropriate primers and probes to decrease false negative confirmation of SARS-CoV-2. Also, the use of both qRT-PCR and CT-scan is recommend to decrease false negative cases.

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