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Review

Diagnostic techniques for COVID-19 and new developments

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ABSTRACT

COVID-19 pandemic is a serious global health issue today due to the rapid human to human transmission of SARS-CoV-2, a new type of coronavirus that causes fatal pneumonia. SARS -CoV-2 has a faster rate of transmission than other coronaviruses such as SARS and MERS and until now there are no approved specific drugs or vaccines for treatment. Thus, early diagnosis is crucial to prevent the extensive spread of the disease. The reverse transcription-polymerase chain reaction (RT-PCR) is the most routinely used method until now to detect SARS-CoV-2 infections. However, several other faster and accurate assays are being developed for the diagnosis of COVID-19 aiming to control the spread of infection through the identification of patients and immediate isolation. In this review, we will discuss the various detection methods of the SARS-CoV-2 virus including the recent developments in immunological assays, amplification techniques as well as biosensors.

1. Introduction

Coronaviruses (CoVs) which are responsible for respiratory, enteric, hepatic and neurological diseases belong to *coronaviridae* family and order *Nidovirales*. This family has two *Coronavirinae* and *Torovirinae* subfamilies.

Coronavirinae are categorized into four genera. 1) Alphacoronaviruses which include HCoV-229 E and HCoV-NL63; 2); Betacoronaviruses which involve HCoV-OC43, HCoV-HKU1, MERS - CoV, SARS-CoV, and SARS-CoV-2; 3) Gammacoronaviruses that infect whales and birds, and 4) Deltacoronaviruses that cause sickness in pig and birds [1]. Their name is derived from coronam, which is a Latin word of a crown because these viruses have a crown-like image on the electron microscope due to club-like spikes projections of protein on their surface [2].

Coronaviruses are infectious for an extensive range of mammals such as animals, humans, birds and rodents. Once transmitted, CoVs can adjust to the new host because of their high frequency of recombination and mutation rate [2,3]. The genome structure of the single-stranded non-segmented positive-sense RNA of the CoVs includes two-thirds of RNA which are responsible for encoding viral polymerase RNA-dependent RNA polymerase(*RdRp*), RNA synthesis materials, and two large nonstructural polyproteins that are not involved in host response modulation, open reading frames (*ORF1a-ORF1b*). The other one-third of the genome encodes four structural proteins; *spike (S)*, *envelope (E)*, *membrane (M)*, *nucleocapsid (N)*, and other helper proteins [4,5]. The nucleotides of SARS-CoV-2 have 84%, 79.6% and almost 50% similarity with bat SARS-like coronavirus, SARS-CoV and MERS-CoV, respectively [6]. SARS-CoV-2 has 96% homology at the whole-genome level with bat coronavirus [7]. There are seven conserved replicate domains in the *ORF1ab* SARS-CoV-2 gene that share a 94.4% sequence identity with SARS-CoV. Besides, a short RdRp region from a bat coronavirus called BatCoVRaTG13 had demonstrated high sequence identity to SARS-CoV-2. The full-length sequencing of this RNA resulted in 96.2% complete genome sequence equality. The receptor-binding protein spike *S* gene in SARS-CoV-2 that showed high diversity in other CoVs was 93.1% identical to the RaTG13 *S* gene except for three short insertions in the N-terminal and four out of five key residue changes in the receptor-binding motif [7]. Moreover, investigation of the coding region of the SARS-CoV-2 genome has shown that the nucleotides and amino acids in this region have 92.67% and 96.92% resemblance at the nucleotide level and 97.82% and 98.67% at amino acid level with pangolin and bat CoV genome [8]. This phylogenetic data is supporting the theory of bat origin of SARS-CoV-2. However, more investigations are still needed to clarify the presence of intermediate host which promoted the transmission of the virus as there are pieces of evidence that the virus was not transferred from bat to human [1]. SARS-CoV-2 and SARS-CoV utilize Angiotensin-converting enzyme II (ACE2) as a cell

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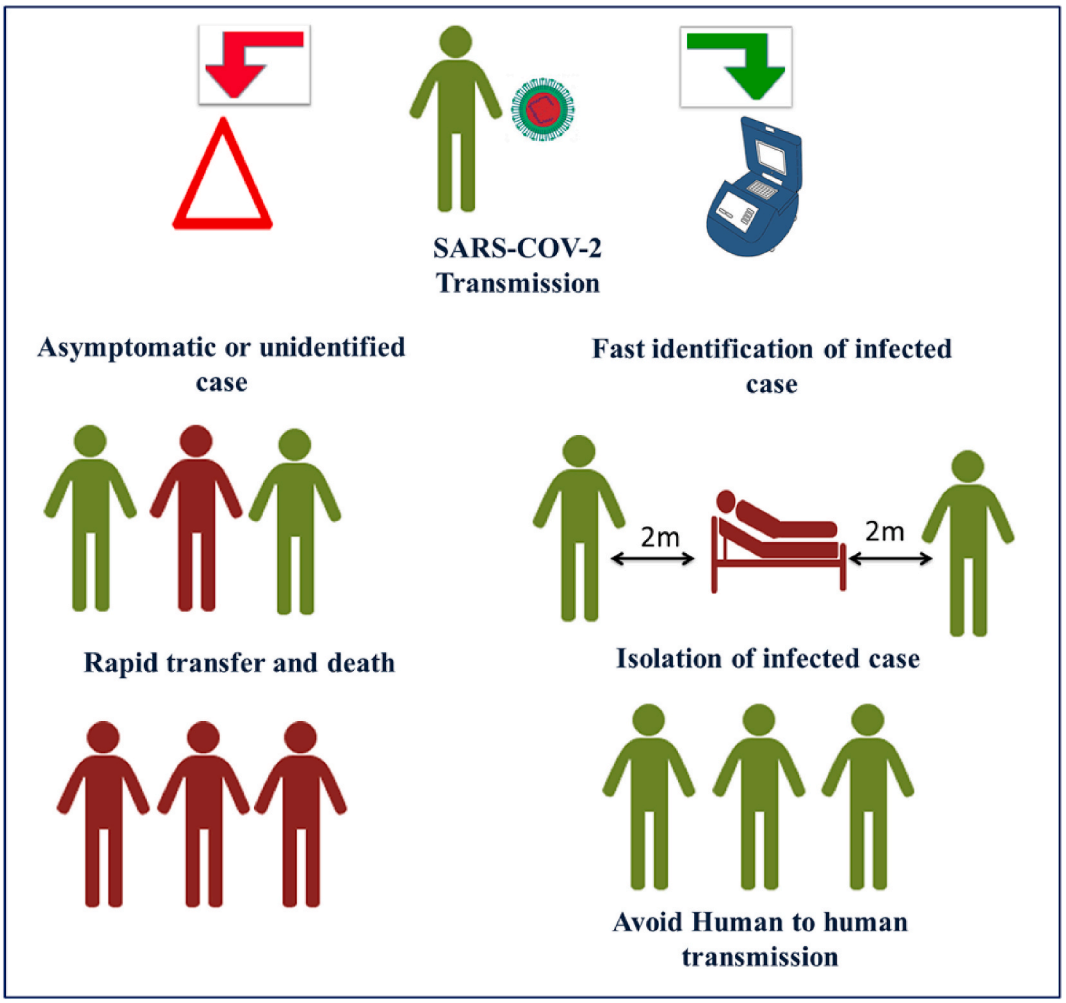

Fig. 1. Schematic of COVID-19 transmission and the importance of isolation of infected individuals.

entry receptor [7,9]. This virus has a virion diameter of 70–140 nm with recognizable spikes of 9–12 nm [10]. Until now seven human coronaviruses (HCoVs) which cause respiratory difficulties have been discovered including HCoV-229 E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, severe acute respiratory syndrome (SARS)-CoV, Middle East respiratory syndrome (MERS) -CoV, and (SARS)-CoV-2 [11].

As mentioned above, the recently identified SARS-CoV-2 which causes the COVID-19 pandemic in the world belongs to the beta CoVs [12]. SARS-CoV-2 is a new zoonotic coronavirus that was discovered in Hubei Province, China in December 2019. The genetic sequence of the virus was then announced by the Chinese Center for Disease Control and Prevention on January 9, 2020. Due to the global outbreak of COVID-19, the World Health Organization (WHO) declared the virus as a pandemic and a public health emergency of international concern [13].

Fever, nonproductive cough, shortness of breath, myalgia, fatigue, anosmia, ageusia, normal or decreased leukocyte count and ground-glass opacities are the most common symptoms of COVID-19 [14,15]. Patients also showed headaches, hemoptysis, abdominal pain, diarrhea, and the production of sputum less frequently [1]. A systematic literature review with meta-analysis showed that fever, cough and dyspnea were the most reported symptoms. Fever and cough were seen in adults more than children. The frequency of fever was similar in SARS-CoV and MERS-CoV. However, cough was reported with higher frequency in SARS and SARS-CoV-2 than MERS. Diarrhea was reported in 20–25% of SARS and MERS patients. Abnormalities like Lymphopenia, hypoalbuminemia, elevated inflammatory markers, such as C-reactive protein, LDH, and ESR, were reported. SARS-CoV-2 and SARS-CoV have shown an effect on lymphocytes particularly T lymphocytes which resulted in depletion of CD4 and CD8 cells [16]. SARS-CoV-2 and MERS-CoV impede the interferon signaling pathways, which lead to higher respiratory virus load, positive viremia, and eventually, poor prognosis [17]. Acute respiratory problems and kidney failure which resulted in acute renal tubular injury were observed in severe cases. Hypoxemia, organ damage, acute respiratory distress syndrome (ARDS), arrhythmia, shock, acute cardiac injury, and cytokine storm have been also detected as reasons for most death cases among patients [1,18,19]. Besides, it is believed that males are more susceptible to SARS-CoV-2 than females because of the female sex hormones that have an important role in innate and adaptive immunity [20].

The disease spread rapidly among people through respiratory droplets during talking, coughing and sneezing [12]. It has an incubation period of 1–14 days (usually 3–10 days) with no noticeable symptoms which is longer than SARS CoV [21].

The rate of virus transfer is usually assessed based on the R_0 parameter (basic reproduction number). R_0 is a key threshold quantity that is related to viral transmissibility. It is defined as the average number of people who were infected due to contact with a sick person in an entirely exposed population. The values are ranging from ≤ 1 to 1. When R_0 value exceeds 1, it means that the infected cases rise exponentially which leads to the epidemic. WHO initial estimation on Jan 23, 2020 showed R_0 values of 1.4–2.5 for SRAS-CoV-2 while R_0 of 3.3–5.5 was reported in the early phase of the outbreak. This value is a little higher than SARS-CoV which showed R_0 of 2–5 [21]. However, Liu et al. [22] found R_0 ranging from 1.4 to 6.49 with an average R_0 value of 3.28

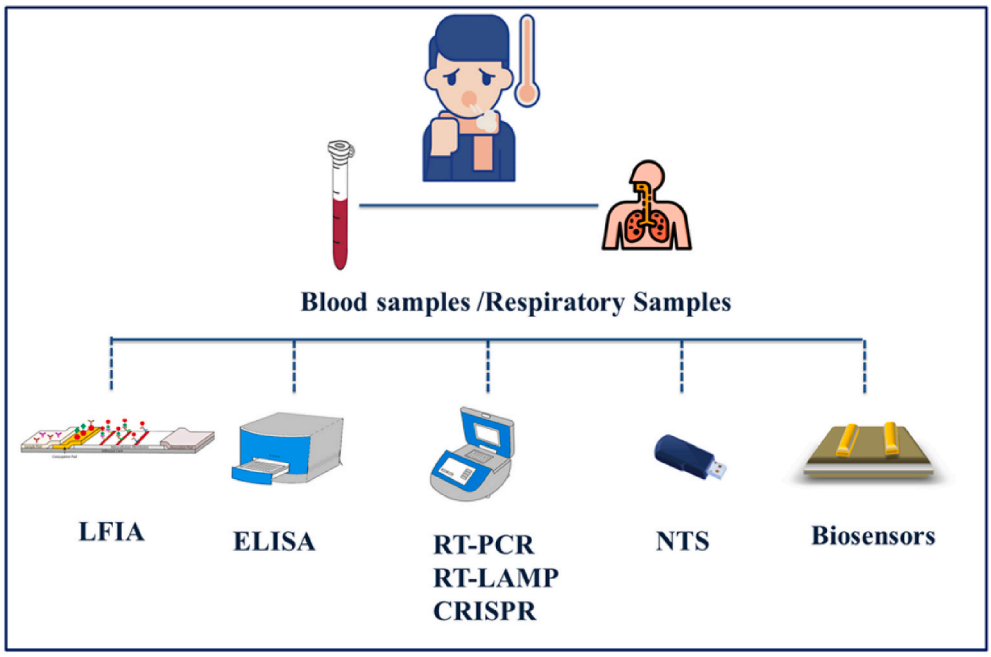

Fig. 2. Schematic of diagnostic methods for SARS-CoV-2 detection from respiratory and serum samples.

and a median 2.79 by evaluating 12 studies. They concluded that SARS-CoV-2 is more transmissible than SARS-CoV [22].

Moreover, mutations lead to faster transport of viruses from animals to humans and humans to humans. Mutations in the ORF8 region at 28,144 and the ORF1B region at 8872 were reported in the early phase of the SRAS-CoV-2 epidemic [23].

Patients with COVID-19 showed a similar pattern of viral load change to those with influenza, and different from SARS and MERS. In SARS and MERS viral load reached the maximum value about 10 days after the beginning of symptoms [24] While in SARS CoV-2, high viral loads in the upper respiratory tract and as a consequence high risk of transmission were reported in the early days from the onset of symptom. Moreover, the RT-PCR test revealed low levels of virus in the upper respiratory tract even after the disappearance of symptoms [17]. Another feature of SARS-CoV-2 is the higher viral load reported in elder people [25] As of June 29, 2020, the disease has infected over 10.1 million people worldwide leading to around 502 K deaths [26]. Because of the many asymptomatic cases and poor testing, it is expected that the total number of identified COVID-19 infections worldwide is underestimated. These asymptomatic individuals pose a serious risk because they are capable of further spreading of the disease [25]. Moreover, most of the symptoms of COVID-19 are similar to those of normal influenza and cold. Therefore, it is highly important to early and accurate diagnosis the infected individuals to prevent the extensive spread of this fatal disease. Particularly, the identification of the COVID-19 patients in early stages will allow the physicians to help them before developing serious complications. Developing fast and reliable screening tools for COVID-19 will also help to identify negative people and avoid unnecessary quarantine that negatively impacted social life and caused a serious economic crisis. A schematic diagram that showed the importance of fast detection and isolation of infected cases is shown in Fig. 1.

In this report, we discuss various existing diagnostic methods for COVID-19 as well as ongoing developments and innovations such as point-of-care (POC) diagnostic tests and biosensors. Different diagnostic methods for detection SARS-CoV-2 is shown in Fig. 2.

2. Current detection methods for SARS-CoV-2

Two main detection strategies are currently available for the diagnosis of SARS-CoV-2 either via the detection of the viral RNA or the antibody produced upon exposure to the infection. The SARS-CoV-2 viral RNA is usually detected by polymerase chain reaction (PCR) or nucleic acid hybridization techniques. The virus antibody or antigens can be detected using immunological and serological assays such as ELISA. It should be noted that both detection categories are important and complement each other. The determination of the RNA of the virus leads to the detection of the virus in its active stage, whereas the serological assays help to identify people whose immune system has already developed antibodies to fight the infection.

2.1. *Immunological assays*

Immunoassays are methods that rely on the detection or quantitation of antigen/antibody interactions. They can produce valuable data about the dynamic of virus infections and earlier exposures [27]. On the other hand, antibodies are more resistant than viral RNA and are less deteriorated by transportation, storage and collection [28].

Antibodies or immunoglobulins are produced by an immune system to defend the host against foreign agents like bacteria or viruses. IgG is the most applicable antibody among IgA, IgD, IgE, IgG and IgM in immunoassay techniques [29]. Microbial infections usually result in the production of IgM at the first line of defense and IgG is generated in the next stage as long term immunity and immunological memory. During SARS infection, IgM and IgG were detectable in the patient blood after 3–6 days and 8 days, respectively [30,31]. So, the detection of both antibodies could help to determine the date of infection. For SARS-CoV-2, IgM and IgG can be detected 3–4 days after premorbid, respectively [27]. However, some studies have shown that the number of positive tests for IgG was higher than IgM after symptom onset and three types of SARS-COV-2 seroconversion were shown: simultaneous seroconversion of both antibodies and IgM earlier and later than IgG [25,32].

Some immunological assays have been developed to detect the COVID-19 virus. Peptide-based luminescent immunoassay has been developed to detect IgG and IgM antibodies of SARS-CoV-2 [33]. Twenty

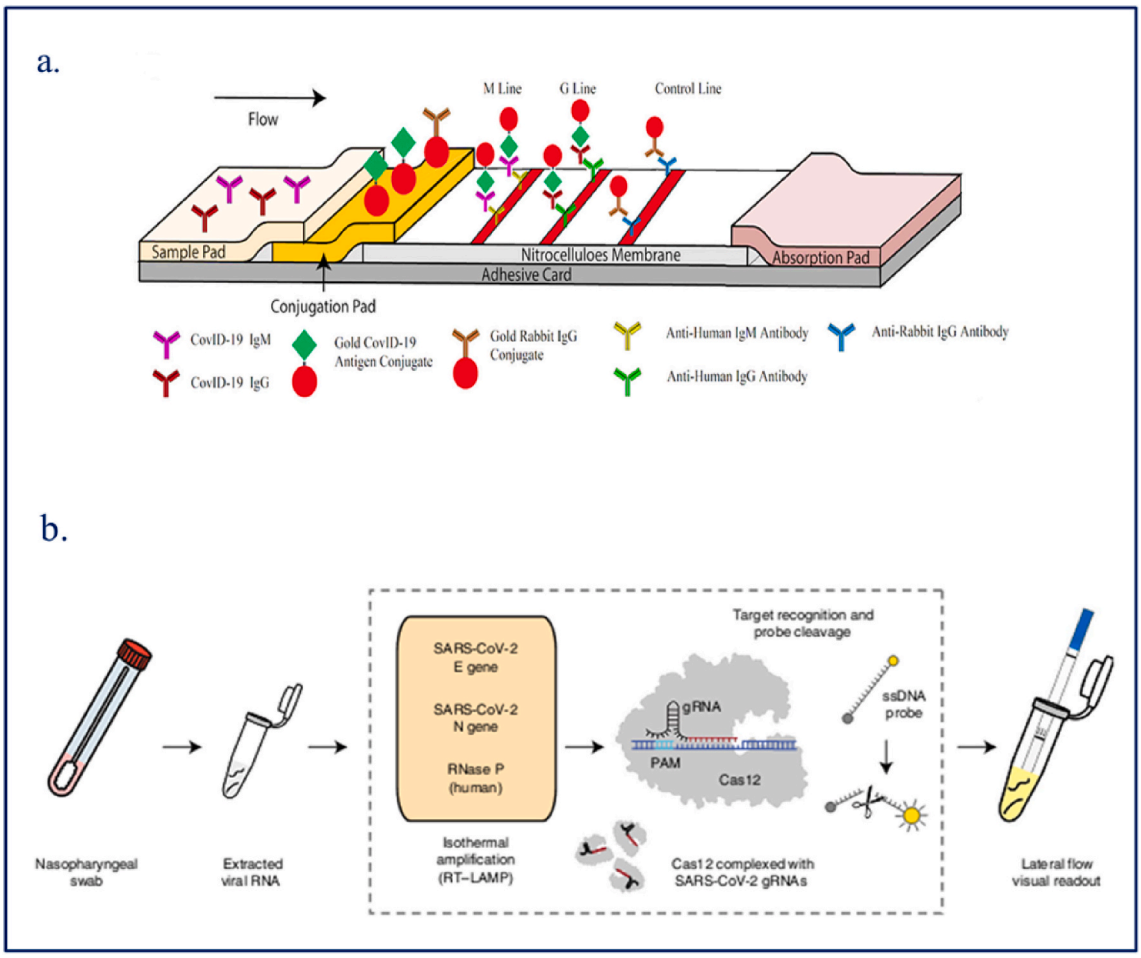

Fig. 3. a) Lateral flow immunoassay for the detection of SARS-CoV-2 IgM and IgG with permission ref [30], b) CRISPR-Cas12DETECTR lateral flow assay for SARS-CoV-2 with permission ref [85].

synthetic peptides from *ORF1ab*, spike (*S*) and nucleocapsid (*N*) proteins were chosen as antigens. Purified peptides labeled with biotin were captured by streptavidin-coated magnetic beads. Then the serum samples containing the antibodies were mixed with the modified beads and the reactivity was measured by a luminescence reader. One peptide from *S* protein has shown the best results. The Cut-off value and specificity of the assay were evaluated by the detection of 200 healthy sera and 167 sera from patients infected with other respiratory-related pathogens, respectively. Moreover, a stability study was performed by repeating the measurement of serum samples with different concentrations for 10 times. The positive rates of IgG and IgM antibodies were 71.4% and 57.2% and for both antibodies were 81.5% [33]. An automated chemiluminescent immunoassay was developed to evaluate serum IgM and IgG. The magnetic particles were modified with SARS-CoV-2 antigens; *S* and *N*. Both antibodies were detected 7–12 days after the onset of infection. The study indicated that there was probably an association between the time and speed of IgM production and the severity of sickness. The person who had weaker symptoms was the first patient to show specific antibodies on day 7 after the onset of disease and the patients with higher clinical signs showed the antibodies on the 12th day. The method was specific and the results of control samples were negative [34].

Enzyme-linked immunosorbent assay (ELISA) is another developed method for the detection of COVID-19. ELISA is a sensitive method to detect the antigen or antibody of interest in the samples which can be performed using direct or indirect formats. In the direct method, an enzyme-linked antibody directly determines the antigen in the sample. In the indirect method, a primary antibody is used to bind to an antigen which was coated on a microplate. Then secondary enzyme-labeled antibody is applied to detect the primary antibody [29].

Immunochromatographic assay (ICA), namely lateral flow immunoassay, is a qualitative method to detect analyte by the naked eye. This method can produce semi-quantitative data by coupling it with a simple reader [35]. Jie Xiang et al. [36] have investigated the application of two new kits based on ELISA and colloidal gold-ICA assay for the detection of COVID-19. The ELISA kit was designed to detect IgM and IgG antibodies. IgM was measured by direct ELISA. For this purpose, microplate modified with mouse anti-human IgM monoclonal antibody and enzyme-labeled antibody was used to detect IgM of SARS-CoV-2 in serum samples. For the indirect measurements of IgG, the microplate was coated with the recombinant antigen of SARS-CoV-2. In the next step, the HRP-conjugated monoclonal mouse antibody for SARS-CoV-2 was used to detect IgG by competitive binding. Then, tetramethylbenzidine was utilized as an enzyme-substrate to produce the color. For the ICA assay, diluted serum samples were added to the sample pad of the test strip. If the color of both the test and control lines changed to red, the test result considered positive. Otherwise, if just the control line turned red, the result is negative and two colorless lines meant the test did not work. The sensitivity of the ELISA and ICA was 87.3% and 82.4%, respectively and both methods showed negative results for healthy controls. Both methods are relatively simple and fast and their answers can be used as reference results. The qRT-PCR test was also carried out for the studied group and the sensitivity was 51.9% [36]. Liu et al. have compared the sensitivity of recombinant SARS-CoV-2 nucleocapsid protein (*rN*) and spike protein (*rS*) for SARS-CoV-2 IgM/IgG detection via ELISA method in 214 serum samples from

confirmed cases. Their results concluded that *rS*-based ELISA had a superior sensitivity in the detection of IgM. Both kits had no positive results for healthy serum samples. They also investigated the dynamic trend of the positive rate at different stages of sickness. IgM and IgG antibodies detection based on *rN* and *rS* tests have shown 30–50% positive rates in (0–5) and (6–10) days post disease onset, respectively. They reached 88.9 and 90.7% in (11–15) days and IgM has shown a decreased positive rate in 35th day [37].

Zhao et al. introduced their patented technology which used two mammalian cell expression vectors to generate two recombinant SARS-CoV-2 proteins. By applying this technique, CHO-expressed SARS-CoV-2 *S1* was used to fabricate the ELISA kit. The proposed kit showed high specificity, sensitivity and an accuracy rate of 97.3% [38]. Another sandwich ELISA method using recombinant nucleocapsid protein of SARS-CoV-2 was applied to detect IgM and IgG of 216 samples from 85 confirmed COVID-19 cases. Both antibodies were detected after the fourth day of premorbid and were seropositive with the illness course for more than 30 days. Three cases in the control group showed positive results for IgG while all cases were negative for IgM. Both antibodies showed good results for the serodiagnosis of SARS-CoV-2 [39]. Combining the detection of both antibody and RNA using ELISA and RT-PCR methods, respectively has led to the sensitivity of 99.4% which compared to 67.1% in the absence of ELISA test even in 1 week after starting the infection [40]. Proteome microarray was applied to profile the IgG/IgM in the healing phase. Microarray slides were modified with 38 proteins plus positive and negative controls. It was shown that *N* and *S* proteins were capable of distinguishing COVID-19 patients from control groups for both IgG and IgM effectively. Moreover, the comprehensive analysis demonstrated that the level of *S1* IgG is related to age and positively to the level of lactate dehydrogenase (LDH), whereas the level of *S1* IgG was negatively correlated to Lymphocyte percentage especially in women [41].

Lateral flow immunoassay test was developed for the detection of both IgM and IgG in blood samples by Li et al. [30]. The test strip had two test lines for IgM and IgG and a control line which were modified with mouse anti-human IgM, mouse anti-human IgG and anti-rabbit IgG, respectively. The test results of 352 from 397 patients were positive (88.66% sensitivity) and 12 blood samples of 128 people without SARS-CoV-2 infection showed positive results which give a 90.63% specificity. Analyzing 58 patient samples from day 8–32 after infection showed that 94.83% of positive patients have both IgM and IgG and 1.72% and 3.45% of patients had only IgM and IgG, respectively. The strip test was checked with patient fingerstick blood, vein blood and plasma and it showed 100% uniformity and demonstrated the applicability of test for POC measurements. The test produced false-negative results likely due to the low amount of IgM and IgG or variation in the immune response of different people. Moreover, the IgM antibody level reduces after two weeks of infection [30]. The schematic of these methods were illustrated in Fig. 3A.IgG/IgM Rapid Test Cassette for COVID-19 which is available on the market from Zhejiang Orient Gene Biotech Co Ltd was developed for the detection of SARS-CoV-2 specific antibodies. Control experiments of 80 samples were negative for IgM but only one case provided false-positive result. There were no positive results for IgM and IgG for 6–12 months babies. When people were divided into two groups (9–17 days) and (18–29 days) after the beginning of infection, both groups showed more positive IgG results. The assay showed a sensitivity of 69% and 93.1% for IgM and IgG, respectively as well as 100% specificity and 99.2% sensitivity for both antibodies [15]. A fluorescence immunochromatographic assay was also applied in the detection of SARS-CoV-2 in which control and test lines were modified with goat anti-rabbit IgG antibody and mouse anti-nucleocapsid protein, respectively. Carboxylate-modified polystyrene Europium (III) chelate microparticles with the anti-nucleocapsid protein of SARS-CoV-2 monoclonal antibody M4 or rabbit IgG were added to conjugation pad. Capturing nucleocapsid protein by the antibody in the test and control lines caused the appearance of the fluorescent band which was measured by fluorescence analyzer. 100 nasal swab samples of healthy individuals were used to evaluate the Cut-off value of the assay. Diluted nasopharyngeal swab or urine samples were poured into the sample well and the strips were read after 10 min. The positive results were obtained by analyzing the value of the sample against the cut-off value. The samples were also tested with RT-PCR. There were 208 positive results from 239 patients. Among 208 people with positive RT-PCR results, 141 cases have shown antigen positive results (68%). All the negative samples with RT-PCR were also negative with the ICA test. 14/19 patients with positive results had antigen in their urine samples. One person showed antigen after 3 days of fever. The authors proposed determining *N* antigen in urine samples to check for the kidney failure of patients [42]. The efficiency of Coris COVID-19 Ag Respi-Strip as a frontline test for SARS-CoV2 has been investigated in nasopharyngeal samples. The assay showed 30.2% (32/106) sensitivity and 100% specificity among positive RT-PCR samples. Viral load of around 1.7×10^5 copies mL^{-1} caused a higher detection rate while 9.4×10^3 copies mL^{-1} showed a great decrease in sensitivity of the test [43].

Sensitivity and specificity of the immunological assays are considered imperative factors in the practical application of these methods. For the detection of SARS-CoV-2, immunological assays mostly utilized *S, N* and *receptor-binding domain (RBD)* proteins as targets. *S* protein is essential for the attachment of the virus to host cells while RBD of *S protein* plays the role of mediator with angiotensin-converting enzyme 2 (ACE2) [44]. The *S* protein antigen showed higher interference with the *S* protein SARS-CoV than MERS-CoV. But *S1* subunit protein has only shown cross-reactivity with SARS-CoV. The presence of a highly conserved *S2* subunit domain in coronavirus is probably the cause of this effect. Developed methods were more specific with the *S1* subunit. RBD region inside the S protein has also shown cross-reactivity between SARS-CoV and SARS-CoV-2 [27,45]. The *N* based –ELISA method has shown good specificity and sensitivity to detect SARS-CoV-2. Three ELISA methods that utilized *RDB, N, or S1* protein were compared. Among them, *RDB and N-protein based methods* showed more sensitivity than *S1* in patients with mild sickness. Comparison between IgA and IgM ELISA demonstrated that the former was more sensitive and the later was more specific [45].

2.2. Amplification techniques

2.2.1. Reverse transcription polymerase chain reaction

PCR methods are based on the amplification of genes and their RNA transcripts isolated from biological samples. DNA polymerase enzyme, extracted DNA Samples, primers and deoxynucleoside triphosphates are the essential components of a PCR test kit. Reverse transcription PCR (RT-PCR) is a type of PCR methods that uses reverse transcriptase enzyme to convert RNA molecules to cDNA molecules. Then cDNA works as a template sequence for the PCR reaction [46]. Quantitative PCR determines a DNA molecule with the help of fluorescent dye or fluorophore-attached DNA probe such as TagMan [46]. A typical RT-PCR method includes four steps. 1- RNA isolation followed by cDNA synthesis with reverse transcription kit. 2- Mixing buffer, DNA polymerase enzyme, primers of a target gene, deoxynucleoside triphosphate, cDNA template and fluorescent dye, 3- Incubation of the mixture at different temperatures to perform thermal cycling in PCR instrument and fluorescence measurements for calculating Cycle threshold (Ct) data, and 4- Relative expression estimation based on Ct data of control and experimental samples [23]. The selection of a standard positive control influences the accuracy of RT-PCR [47]. PCR result is considered positive if the Ct value was less than 40 [48]. This value is usually decreased in the third week of infection and may not be detected later. Ct values of extremely sick patients who were hospitalized are lower than patients with mild symptoms and may remain positive after 3 weeks of sickness. PCR positivity decreases more slowly in sputum and can be positive while nasopharyngeal swab is negative. Positive RT-PCR results were observed in stool (55 of 96) patients beyond

nasopharyngeal swab during 4–11days and was not correlated to the severity of the disease [48]. RT-PCR test of COVID 19 can give positive results one day before starting symptoms but in most cases, patients were not identified before the onset of symptoms due to low viral load [24].

RT-PCR methods are generally designed to amplify *S, E, N, RdRp and ORF1a/b* genes while *ORF1a/b* and *E* genes were used more frequently [44]. *Orf1ab* and *N* genes are regularly utilized for SRAS-CoV-2 in China while *N1, N2 and N3* genes and *E, N* and *RdRp* genes are mostly applied in US CDC and Europe, respectively [47].

There is still a lack of information about the variety of genetic SARS-CoV-2 in humans and animals. Therefore, two RT-PCR assays that can detect multiple coronaviruses in the subgenus of Sarbecovirus were developed [49]. These 1-step qRT- PCR assays have identified two different regions of the viral genome; *ORF1ab* and *N*. The study was applied to SARS-CoV-2 and SARS coronaviruses while RNA of SARS coronavirus was used as a positive control. Moreover, the RT-PCR products of SARS coronavirus produced by the *ORF1b* and *N* gene assays were cloned into plasmids. Because of the application of DNA plasmids as positive standards, the assay has realized a limit of detection of 10 copies per reaction. Control samples were completely negative and real samples from two infected patients had shown positive results. The authors have recommended the *N* gene for screening and the *ORF1b* gene for confirming the results. These assays were capable of achieving a wide dynamic range [49]. Spin column-and poly amino ester magnetic nanoparticle (pcMNPs) extraction method was utilized in the conventional RT-PCR and direct RT-PCR amplification of the SARS-CoV-2 virus. Direct RT-PCR was applied with magnetic nanoparticles coated with poly amino ester. The magnetic nanoparticles were synthesized with co-precipitation reaction and hydrolysis of TEOS/APTES. Then NH_2-MNP reacted with the prepared polymer to form poly amino ester through a Michael addition reaction. Direct RNA extraction protocol has shown nearly 100% RNA extraction efficiency in serum samples and provided high-purity products without interference with the PCR reagents. Using this method, lysis and binding steps were combined and the pcMNP was applied in the RT-PCR system directly. The pcMNPs had superb viral RNA binding ability that provided high sensitivity (10 copies) and a wide linear range (up to 10^5 copies). This method can be coupled with automated nucleic acid extraction systems. It is also adaptable to isothermal amplification methods and can be used in POC devices [50]. Detection of SARS-CoV-2 in saliva samples was investigated with both RT-PCR and viral culturing methods. 91.7% (11 of the 12) patients have shown positive results. However, salivary RNA levels were then decreased after hospitalization. The viral culturing method has shown the presence of live viruses in the saliva of 3 patients. The collection of saliva samples from patients has advantages in diagnosis. It is a non-invasive method and samples can be collected outside the hospital by non-experts [51]. A comparative RT-PCR test was performed with the nasopharyngeal swab and saliva samples (n = 53) to detect *RdRp*, *E* and *N* genes of SARS-CoV-2. The method showed 89 and 77% sensitivity for the nasopharyngeal swabs and saliva samples, respectively. There was no significant variation between nasopharyngeal swabs and saliva specimens but nasopharyngeal swabs were nearly 10% more sensitive than saliva. When specimens were collected in later times of illness, there was a greater difference in sensitivity between nasopharyngeal swabs and saliva samples likely due to the lower load of virus in this stage. Saliva can be replaced with nasopharyngeal swabs when a person cannot bear collecting nasopharyngeal swabs especially when viral concentration is higher in the upper respiratory tract. The nasopharyngeal swab should be checked as a second specimen in patients with a high index of clinical suspicion and their saliva is negative [52]. Three new real-time RT-PCR assays for *RdRp/helicase* (*Hel*), *S* and *N* genes of SARS-CoV-2 have been developed. Compared with the reported *RdRp-P2* assay which is used in more than 30 European laboratories, the lowest detection limit was achieved by the *RdRp/Hel* assay which was 1.8 TCID50 mL^{-1} and 11.2 RNA copies/reaction with genomic RNA and in vitro RNA transcripts, respectively. 28.2% test results from people confirmed with COVID-19 were positive by both the *RdRp/Hel* and *RdRp-P2* assays. The SARS-CoV-2 *RdRp/Hel* assay was positive for people whose *RdRd-P2* test results were negative. The SARS-CoV-2-*RdRp/Hel* assay was specific and there was no interference with HCoVs and other respiratory pathogens in cell culture and clinical samples [53]. Corman et al. [54] have designed a workflow for the detection of SARS-CoV-2 with the help of synthetic nucleic acid technology in the case that the virus isolates or real patient samples are not available. They proposed using *E* gene assay as the first-line screening tool and confirming the test results with the *RdRp* gene assay. *RdRp* gene test with dual-color detection was capable to distinguish SARS-CoV-2 from SARS-CoV. They obtained the best results with the *E* gene and *RdRp* gene (LOD of 3.2 and 3.7 copies/reaction, respectively), while the *N* gene was less sensitive. They also evaluated the LOD for in-vitro transcribed RNA that was identical to the sequence of SARS-CoV-2. The obtained LODs were 3.9 and 3.6 copies/reaction for *E* gene and *RdRp* assays, respectively. They have also designed a specific probe (*RdRp-SARSr-P_2*) for SARS-CoV-2RNA that did not respond to SARS-CoV RNA. Endemic human coronaviruses (HCoV); 229 E, NL63, OC43 and HKU1, as well as MERS-CoV had no interference with their results [54]. RT-PCR tests were performed to detect the presence of the virus in anal swabs and blood samples in the cases where they were not identified in oral swabs. It should be noted that patients may still carry the virus despite that their swab results were negative. The researchers also investigated the presence of IgM and IgG in patients by ELISA methods after 10 days of medical treatment. Both antibodies were low or unnoticeable on the first day of sampling. On day 5 both antibodies were detected in all patients [55]. RT-PCR was used to test samples collected from four medical staff that two of them were already recovered from sickness and another two who showed negative results at the beginning. In all cases, positive results were obtained after 5–13 days. This study suggested revising the guidelines for the discharge of infected people from hospitals or home lockdown [56]. Wang et al. [57] have investigated 205 patients with 1070 various samples including pharyngeal swabs, blood, sputum, feces, urine, and nasal samples. The positive rate was 14 of 15 (93%) in bronchoalveolar lavage fluids, 72 of 104 (72%) for sputum, 5of 8 (63%) for nasal swabs, 6 of 13 (46%) for fibrobronchoscope brush biopsy, 126 of 398 (32%) in pharyngeal swabs, 44 of 153 (29%) in feces, and 3 of 307 (1%) in blood. All urine samples were negative. Nasal swabs showed the highest mean cycle threshold 24.3 (1.4×10^6 copies mL^{-1}) while other samples had 30 ($<2.6 \times 10^4$ copies mL^{-1}) [57]. Tear and conjunctival secretions were studied with RT-PCR and viral culture methods to trace the existence of the SARS-CoV- 2 virus. Viral RNA was only found in one person who had conjunctivitis symptoms [58]. In another study, throat swab or sputum samples were investigated with RT-PCR method and the results demonstrated that sputum samples produced more positive results. Thus, they concluded that sputum can be used instead of throat swab in patients who produced sputum [59]. Zhou et al. [24] have evaluated five various RT-PCR kits with three different primers and probes for the detection of SARS-CoV-2. Nasopharyngeal and pharyngeal swab samples from confirmed patients were used. *ORF1ab* and *N* genes of Kit 1 showed the highest sensitivity among positive samples and showed positive results at less dilution ratio [24]. Just et al. [60] have performed SARS-CoV-2 PCR swab testing for 374 patients who had the predictive symptoms of the COVID-19. Patients with anosmia and who had the first-grade contact with an infected person showed more positive results. However, patients with sore throat had less positive results [60]. Ren et al. [61] have investigated RT-PCR tests and chest CT scans of 87 confirmed COVID-19 cases and 481 cases without COVID-19 infection. They found that both methods showed superior sensitivity (91.9%) in contrast to 78.2% for RT-PCR and 66.7% for CT scans alone. They reported that RT-PCR was more important in the detection of mild infection. They have also emphasized the application of stool samples for RT-PCR as an indicator to enhance the diagnosis rate and the discharge from hospitals [61]. *N*-gene-specific

qRT-PCR was applied to evaluate the viral load of SARS-CoV-2 in another study [62]. It was reported that the viral loads in throat swabs and sputum have reached the maximum level (10^4 to 10^7 copies mL^{-1}) after 5–6 days from the beginning of the infection, while for SARS the peak was reached after 10 days. The viral loads were between 641 and 1.34×10^{11} copies $mL^{-1}$with a median value of $7{\cdot}99 \times 10^4$ in throat samples and $7{\cdot}52 \times 10^5$ in sputum samples. However, stool samples showed less amount of viral load (550–1.21×10^5 copies mL^{-1}). Wikramaratna et al. [63] have investigated the public data from patients who had RT-PCR positive results at least one time. They concluded that the probability of positive test reduced if tests were performed at later date after symptom appearance and the nasal samples had more positive results than throat samples [63].

Some German researchers reported that the Real Star kit had better sensitivity and higher efficiency [64]. J. LeBlanc et al. have evaluated RT-PCR tests in Canadian Laboratories. Their LODs were ranging from 3.4 to 4.5 log $_{10}$ copies mL^{-1}) with was consistent with other reports. They also suggested that the detection of more than one target has improved the diagnosis of the virus in low viral load [65]. Diagnostic methods that are applied in China for SARS-CoV-2 detection were summarized in Ref. [66].

AusDiagnostics Multiplex-tandem PCR (MT-PCR) assay which includes two tandem amplification steps were also applied for the detection of SARS-CoV-2. The first amplification step (enrichment) utilized a specific outer primer with fewer numbers of PCR cycles. In the second amplification step, the target region within the product from the first step was amplified by inner primers. 7839 samples were analyzed with this method and 127 samples were detected positive. Comparative analysis with State Reference Laboratory showed 118/127 (92.9%) consistency. After investigation of discrepancies, 125/127 (98.4%) positive results were obtained and this method has demonstrated reliable diagnosis for SARS-CoV-2 [67]. Protocols established in various countries for RT-PCR available on the WHO website. CDC website also reported protocols for the United States. The WHO protocols, available PCR commercial kits and serological test kits were summarized in Refs. [28].

Real-time nanopore target sequencing (NTS) and amplification methods were employed for the simultaneous detection of SARS-CoV-2 and 10 other respiratory viruses in 6–10 h with LOD of 10 copies $mL^{-1}$with at least 1 h sequencing data. Oxford nanopore sequencer is a small device that can be coupled with a personal computer for data processing. In this method, 11 virulence-related and specific gene fragments of *ORF1ab* of SARS-CoV-2 were amplified with an in-house primer panel. Then, the amplified fragments were sequenced on a nanopore platform. A comparative study of approved qPCR kits and NTS method with samples from patients have shown that NTS provided more positive results. The system has shown two orders of magnitudes more sensitivity than qPCR as well as specificity against other mutated nucleic acid sequences or various respiratory virus infections in the samples [68]. Total RNA sequencing was carried out by the Shotgun metatranscriptomics method. The obtained data were utilized for phylogenetic analysis and were assigned to subclade in New York subway samples [69].

2.2.2. Isothermal nucleic acid amplification

Isothermal nucleic acid amplification is a technique that is used to amplify nucleic acids at constant temperature avoiding the complex requirement of the regular PCR that needs changing multiple temperatures in each cycle [70,71].

Several isothermal nucleic acid amplification techniques have been previously developed for the detection of SARS-CoV such as transcription-mediated amplification (TMA), Loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA) and clustered regularly interspaced short palindromic repeats (CRISPR). In the reverse transcription LAMP method for SARS-CoV detection [72], four primers were used to enhance the sensitivity of the assay. The amplification product in the LAMP method can be detected by measuring the turbidity of the solution or the fluorescence of an intercalating dye. Moreover, unpurified samples can be applied in LAMP directly [73]. This method is a rapid and cost-effective way for virus detection but is limited only to one sample per run.

RT-LAMP was carried out in one step at 63 °C within 30 min to detect SARS-CoV-2. The optical density at 400 nm and color change from orange to green were used to detect amplification. The assay was capable to identify *ORF1ab* gene, *E* gene and *N* gene simultaneously with accuracy rates of 99%, 98.5%, and 92.3%, respectively. *ORF1ab* and *N* genes showed higher specificity and sensitivity. Both RT-LAMP and RT-PCR had similar specificity of 99% for evaluating 208 clinical samples and sensitivity in 20 fold diluted samples with LOD of 1000 copies mL^{-1}. In this method, three gene amplifications were combined to prove the presence of SARS-CoV-2. The technique was specific because of using six to eight primers to distinguish eight different regions on the target DNA [6]. SARS-CoV-2 virus from purified RNA or cell lysis was visually detected with the LAMP method. The LAMP method was performed with 5 full primers sets targeting SARS-CoV-2 RNA with amplicon regions designed to the 5′ region of the *ORF1a* gene and *N* gene. The test showed identical results with the RT-qPCR test for RNA samples of respiratory swabs [74]. *ORF1ab* region and online software Primer Explorer V5 were applied to design RT-LAMP primers. By specificity analysis, one primer set with several pairs of loop primers was selected. These six primers were distinguished by 8 distinct regions of the *ORF1ab* gene. The primer sequence was checked against similar 11 related viruses by the BLAST method and there was no similarity with selected viruses. Amplification was detected by the change of the color from pink to yellow. The method illustrated a similar sensitivity with the RT-qPCR method and was capable to detect 10 copies of SARS-CoV-2 [75]. Another RT-LAMP method [76] that could distinguish SRAS-CoV-2 in simulated patient samples in less than 30 min was developed. Six RT-LAMP primers were arranged with a sequence from GenBank MN908947 which had great similarity with other SRAS-CoV-2 strains while it was different from Bat SARS-like coronavirus. Moreover, the primers were designed without four guanines in a row that can cause tetraplex structures formation which could interfere in the RT-LAMP procedure. Positive amplifications were detected by changing the color from orange to yellow as well as fluorescent and gel electrophoresis analysis. According to gel electrophoresis data, the best result was achieved at 63 °C for 30 min and all 6 primers were necessary to obtain positive results. The LOD of the method was around 1.02 f g and was in agreement with qRT-PCR which used primers with the same area of SRAS-CoV-2 genome. Different spiked samples like serum, saliva, urine, oropharyngeal swabs and nasopharyngeal swabs were utilized to determine the interference of the RT-LAMP method with other viruses and no cross-reactivity was observed. Urine and plasma samples were used without any treatment. A comparison between urine and serum samples with an equal amount of virus illustrated that urine samples could show greater signals [76]. Three different colorimetric isothermal amplification methods including LAMP, cross-priming amplification (CPA), and polymerase spiral reaction (PSR) were investigated and compared. Phenol red was used to detect the amplification products by color change. Among them, LAMP showed better results for the genomic RNA of SARS-CoV-2. The lyophilized LAMP kit has shown advantages such as fast detection of SARS-CoV-2 in spiked nasopharyngeal and oropharyngeal samples, high sensitivity (43 copies), early detection of virus, portability and capability of being used by untrained staff [77]. The performance of a laboratory-developed RT-PCR EUA from Stanford Health Care, Atila iAMP, Altona Diagnostics and *N1*and *N2* CDC kits for the detection of SRAS-CoV-2 in 80 nasopharyngeal swab specimens were evaluated. Samples with low viral load showed a discrepancy in the results. Atila iAMP assay showed a little decrease in sensitivity and needed the highest amount of nucleic acid (18 μL). This LAMP kit could provide faster results (1 h) than RT-PCR methods [78]. Loopamp kit based on the LAMP method was compared to the RT-PCR method for the detection SARS-CoV-2 in 76 nasopharyngeal swab samples. The method had

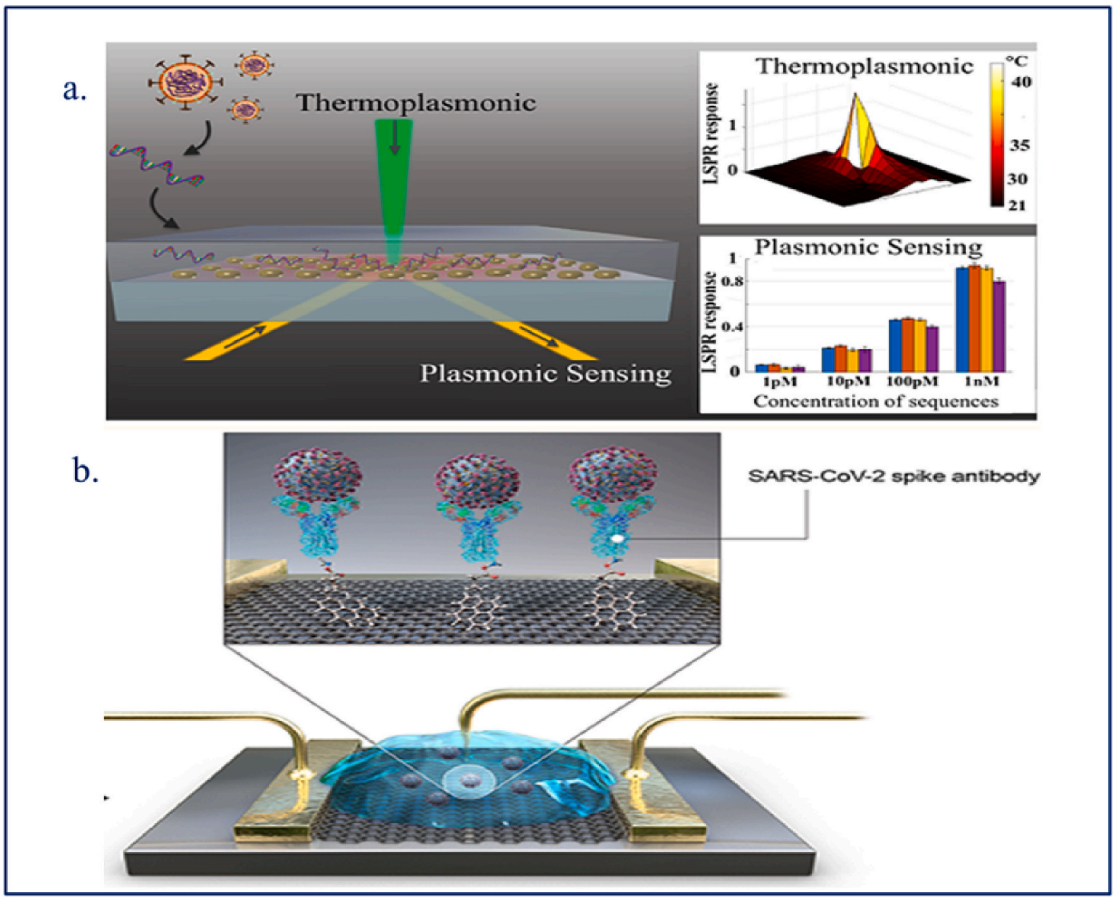

Fig. 4. a) Plasmonic photothermal biosensor for SARS-CoV-2 with permission ref [90]. b) FET biosensor for detection SARS-CoV-2 with permission ref [93].

demonstrated 100% sensitivity, 97.6% specificity with a LOD of 1.0×10^1 copies μL^{-1}. The high specificity of the method was originated from the use of four primers that could detect six different regions in the RNA [79].

TMA assay is an amplification technique that uses a retroviral reverse transcriptase and T7 RNA polymerase for detection [80]. This technique has the capability of high throughput screening of multiple samples as well as multiple pathogens simultaneously. A Hologic APTIMA transcription TMA assay was compared to the RT-PCR method in 116 nasopharyngeal swabs. Superior sensitivity (98.15%) of TMA assay (52/53) compared to 96.25% of RT-PCR (51/53) with a detection limit of 5.5×10^2 copies in 1 out of 5 samples were reported [81]. RCA is another promising isothermal amplification technique that received considerable attention in recent years because of its sensitivity and capability to amplify up to 10^9 fold within 90 min [82]. RCA has been previously used for the detection of SARS-CoV showing promising results in respiratory samples [83].

Circle-to-circle amplification which was a homogeneous and isothermal nucleic acid quantification method coupled with opto-magnetic chip for sensitive (0.4 f M) detection of SARS-CoV-2 was reported [84]. The method utilized the conserved region of the SARS-CoV-2 *RdRp* gene. It provided a wide linear range of 3 orders of magnitude and was able to distinguish SARS-CoV RdRp cDNA from SARS-CoV-2 RdRp cDNA.

CRISPR is also another promising isothermal amplification method for the detection of viruses. In this method, some bacterial enzymes such as Cas12 and Cas13 are programmed to cut certain viral RNA sequences followed by isothermal amplification and a visual readout with a fluorophore on paper strips. Broughton et al. [85] have reported a CRISPR–Cas12-based detection method for SARS-CoV-2 in respiratory swabs. This method showed high sensitivity and selectivity in RNA extracts compared to RT-PCR. In this method, CRISPR-Cas12 and lateral flow technology were coupled for the rapid detection of SARS-CoV-2 from nasopharyngeal or oropharyngeal swabs [85]. This assay is low-cost, relatively rapid and thus, has great potential for POC diagnosis of COVID 19. The procedure of this assay is illustrated in Fig. 3B. All in one Dual CRISPR-Cas12 assay was also developed for SARS-CoV-2 and HIV with a high sensitivity of few copies. The one-pot reaction system was applied in this method and all materials which were needed for amplification and CRISPR detection were mixed in a single step and incubated at 37 °C. This fast and robust method can be further developed to form a POC test [86].

3. Novel developed technologies for SARS-CoV-2 detection

As described above, the RT-PCR and immunological assays are currently the most widely used methods for the diagnosis of COVID 19. However, these methods require trained personnel to perform. Moreover, PCR takes up to few days to obtain the results. Immunological assays require complex production of antibodies and recombinant proteins. Therefore, there is a trend to produce novel faster, lower cost, more reliable diagnostic methods for the detection of SARS-CoV-2.

3.1. Biosensors

Biosensors are bioanalytical devices that combine the selectivity features of a biomolecule with the sensitivity of a physicochemical transducer [87]. They can be a fast and reliable alternative for clinical diagnosis, real-time detection and routine measurements [68]. There are various types of biosensors that have been previously applied for the

Table 1
Various diagnostic assays for SARS-CoV-2.

Method	Biomarker	LOD	Real sample	Remarks	No of samples	Ref
CI	Synthetic peptides sequence of *ORF1a/b, S, N* Protein	–	Serum	The positive rate of IgG and IgM were 71.4% (197/276) and 57.2% (158/276), Specific, CV of IgG and IgM detection in different concentrations were less than 6%. The best results were obtained by a peptide from S protein.	276	[33]
CI & RT-PCR	*N* protein *of* SARS-CoV-2 *ORF1ab, N* gene		Serum NP, OP	Correlation between time and speed of IgM production and severity of sickness.	736,228 confirmed case	[34]
ELISA	*RBD rS, rN* protein		Serum	The positive rate for *N* &S-based ELISAs for (IgM and/or IgG) detection were 80.4% (172/214) and 82.2% (176/214).S-based ELISA for IgM (28%) detection had significantly higher results than N-based ELISA, Positive rate for both antibodies increased in the later days after premorbid while IgM showed a decrease in positive rate after 35th.	214	[37]
ELISA	Mammalian cell-expressed RBD of *S* protein SARS-CoV-2		Plasma	Antibodies were <40% 1-week after premorbid and fastly rised to 100.0% (Ab), 94.3% (IgM) and 79.8% (IgG) at 15th. Seroconversion rate for Ab, IgM and IgG 93.1%, 82.7% and 64.7%.	535	[40]
ELISA	SARS-CoV *Rp3 NP*	–	Oral & Anal swabs, Blood, Serum	Shift from oral positive to anal swab during later day infection	178	[55]
ELISA	CHO-expressed recombinant full-length SARS-CoV-2-*S1* protein with 6*His tag		Serum	Specificity & Sensitivity 97.5%, Accuracy rate 97.3%. Positive ELISA test in person after 14 days lockdown with twice Negative PCR test.	412 healthy 69(Hospitalized/recovered)	[38]
ELISA	*r S1* domain of the SARS-CoV-2 protein		Blood samples	Good sensitivity for the detection of IgA and excellent sensitivity for the detection of IgG antibodies from samples collected ≥4 days after diagnosis by PCR. Good specificity for IgA and excellent specificity for IgG with human coronaviruses.	86 (-PCR) 84(+PCR)	[95]
ELISA	*rS* SARS-CoV-2 protein		Serum	Most PCR-confirmed SARS-CoV-2 were seroconverted. IgA had greater sensitivity in the Euroimmun ELISA *S1* kit. Cross-reactivity with the SARS-CoV *S* and *S1* proteins, and to a lower extent with MERS-CoV *S* protein, but not with the MERS-CoV *S1* protein. For three in-house ELISAs, the *RBD* and *N* were more sensitive than *S1* ELISA in detecting antibodies in mildly infected patients.	259	[45]
ELISA& ICA	anti-human IgM monoclonal antibody recombinant antigen	–	Serum	Simple, Fast, Safe, Portable, The sensitivity of ELISA for IgM and IgG 55/63 (87.3%), sensitivity GICA for IgM and IgG 75/91 (82.4%)	63	[36]
Proteome microarray	*ORF1ab, N* gene,*S, S1, S2* *RBD*		Serum	100% of patients had IgG/IgM responses to protein *N* and *S1,* Substantial antibody responses have shown against ORF9b and NSP5, Protein *S1* specific IgG positively correlates to age and LDH, and negatively to Lymphocyte percentage.	29	[41]
LFI	anti-human -Ig M, anti-human -IgG, anti-rabbit -IgG	–	Serum/Blood	Fast (15 min), Portable, Able to detect fingerstick blood sample, 88.66% sensitivity 90.63% specificity.	397	[30]
LFI	anti-human -Ig M, anti-human -IgG, anti-rabbit -IgG	–	Capillary blood sample, Serum	Fast, Sensitivity 69% and 93.1% for IgM and IgG & 99.2% for both antibodies, 100% specificity.	29 confirmed case & 124 negative control	[15]
LFI	Nucleoprotein antigen		NP	Specificity 100% and sensitivity of the30.2%. Higher viral loads better antigen detection rates. Not good for frontline detection	148	[43]

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Table 1 (*continued*)

Method	Biomarker	LOD	Real sample	Remarks	No of samples	Ref
FLFI RT-PCR	Mouse nucleocapsid protein of SARS-CoV-2 *ORF1ab*, *N* gene	–	NP & Urine	The sensitivity of 100%, Detection of nucleocapsid protein in the urine.	239	[42]
FICA			–	The positive detection rate of both antibodies for the negative and positive nucleic acid tests was 72.73% and 87.50%.	57 24(+) 33(−)PCR	[96]
ELISA & RT-PCR	*rN*protein of SARS-CoV-2 *ORF1ab*, *N* gene	–	Serum NP/OP	Investigations carried out during 3–40 days after symptom onset. Specific IgM and IgG seroconverted at 4th.	216,85 (confirmed case)	[39]
RT-PCR	*ORF1b*, *N gene*	10copies/reaction 2×10^{-4} - 2×10^{3} TCID50/reaction	Sputum, Throat swab	Using the *N* gene for screening and the *ORF1b* gene for confirmation.*N* gene assay was more sensitive than the *ORF-1b*gene.	2	[49]
RT-PCR	*ORFlab*, *N* gene	10 copies linear range 10 -10^{5}	Serum	Lysis and binding steps into one step Simple, Compatible with isothermal amplification methods	12	[50]
RT-qPCR	*S* gene of SARS-CoV-2	–	Saliva	Sensitivity 91.7% (11/12), Non-invasive	12	[51]
RT-PCR	*RdRp, E, N* genes	–	NP, Saliva	Sensitivity was 89% for NP and 77% for saliva	53	[52]
RT-PCR	*RdRp/Hel, S, N gene*	1.8 TCID50 ml $^{-1}$genomic RNA, 11.2 RNA copies/reaction in vitro RNA transcripts	NP aspirate/Swab, Throat swab, sputum	Best results obtained with *RdRp/Hel* RNA, Specific	273	[53]
RT-PCR	*E, RdRp, N* gene	3.2 & 3.7 copies/ reaction for *E* & *RdRp*	Sputum/Nose and Throat swabs	*E* gene assay as the first-line screening tool and confirming the test results with *RdRp* gene assay.	75	[54]
RT-PCR	*S* gene	–	Oral & Anal swabs and Blood	Presence of virus in anal swabs and blood as well, and more anal swab positives than oral swab positives in a later stage of infection	139	[55]
RT-PCR	-	–	Throat swab	Positive RT-PCR results after 5–13 days for medical professionals while they discharge from the hospital or have a first negative test.	4	[56]
RT-PCR	*ORFlab* gene	–	NP, Blood, Sputum, Feces, Urine, Nasal samples.	Nasal swabs had the highest mean cycle threshold 24.3 (1.4×10^{6}copies mL^{-1} while other samples had 30(<2.6×10^{4}copies mL^{-1}	1070	[57]
RT-PCR	-	–	Tear and conjunctival secretions	The only person who had conjunctivitis symptoms had positive RT-PCR result in conjunctival secretions	21	[58]
RT-PCR	*ORFlab*, *N gene*	–	Throat swab, sputum	Sputum samples were produced more positive results	54	[59]
RT-PCR	*ORFlab, E, N gene*	–	NP/OP	*ORF1ab* gene and *N* gene of Kit 1has the highest sensitivity	110	[24]
RT-PCR	-	–	Swab	Anosmia and first-grade contact with an infected person result in more positive results and patients with sore throat had less positive results	374	[60]
RT-PCR	*RdRp, E, N RNA*	–	Mix NP & nasal swabsa, stool	A combination of RT-PCR and CT has the superior sensitivity, Priority of RT-PCR in identifying mild infections.	584	[61]
RT-PCR	*N* gene	–	Throat swabs, sputum, urine, &stool	641–1.34×10^{11} copies mL^{-1} with a median value of $7{\cdot}99 \times 10^{4}$ in throat samples and $7{\cdot}52 \times 10^{5}$ in sputum samples. Stool samples viral load (550–1.21×10^{5} copies mL^{-1})	80	[62]
T-PCR	*ORF1ab*, *N* genes		Throat swabs& sputum	Positive rates of sputum sample and throat swabs were 76.9% and 44.2%.	104	[97]
RT-PCR			NP	Children less susceptible to SARS-CoV-2 infection, children are of virus transmitters.	811	[98]
RT-PCR	*N1* & *RdRP* (modified) genes *E* gene	*N1* (21), *E* (141) modified*E*(457) *RdRP*, (350) Modified *RdRP*(39Copies/ reaction	NP	*N, N2, N3* provide false-positive results, Amplifications efficiencies of *N1*, *RdRP*, *RdRP* (modified), *E*, and *E* (modified) 93.4%, 116.5%, 110%, 86% and 119.6%, respectively. *N1* and *RdRP* (modified) highest analytical sensitivity and *E* assay, in its original concentration, was a	60	[99]

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Table 1 (*continued*)

Method	Biomarker	LOD	Real sample	Remarks	No of samples	Ref
				tertiary confirmatory assay. Importance of validation of in-house assays before its availability to the population		
RT-PCR	*RdRp* gene		NP/OP	The virus replicated in Vero cells and cytopathic effects observed. Full genome sequencing showed sequence homology Korea patients from the patient from another country of more than 99.9%.		[100]
RT-PCR	LightMix ® E-gene kit (*E gene*) In-house assays *RdRp/Hel &N,*	1.8×10^{-1} TCID50 mL^{-1}	NP aspirate	LOD one log 10 lower than in- house RT-PCR. Sensitivity 51.9 (149/289). Specific among 17 respiratory viruses, except SARS-CoV. Similar sensitivity with in-house assays (144/28949.8% (144/289) for *RdRp/Hel* & 50.5 (146/289) for *N,* In-house *RdRp/Hel and -N* assays were specific without the interference of SARS-CoV	289	[101]
RT-PCR	Cepheids Xpert Xpress kit (*N2,E)* In house *RdRp, N1, E*	Cepheids Xpert Xpress 8.26 cp mL^{-1}	NP, Nasal wash	Specific, three samples containing various concentrations of heat-inactivated SARS-CoV-2 virus tested positive at three laboratories in both the in-house RT-PCR and the GeneXpert. LOD was lower than the company claim (250 cp mL^{-1}). Cepheids Xpert Xpress run time 45–50 min	88	[102]
RT-PCR	*RdRp-IP1,RdRp,E*		NP aspirates or NP swabs, BAL, Urine, Stool	Five cases at different stages of infection, High viral loads in the upper respiratory tract, and high risk of transmissibility, 2 positive stool samples. Virus identified at low levels in the upper respiratory tract when no symptoms remained	5	[17]
RT-qPCR	GeneSoC kitN gene *(N*gene*)*	*1.0 × 1[1] Copies/reaction*	NP	Compact, reciprocal flow PCR system, very short time (within 15 min), Single disposable tip per analysis.	78	[103]
qPCR **ELISA**	*RBD* of the *S* gene *N protein from bat SARSr-CoV Rp3* as antigen		Oral swabs, BAL, Serum	Primers could distinguish SARS-CoV-2 from all other human coronaviruses and bat SARSr-CoVWIV1, with 95% identity with SARS-CoV, For ELISA *N* protein showed no interference with other human coronaviruses except SARSr-CoV, Virus specific nucleotide-positive and viral protein seroconversion in all patients	6	[7]
RT-PCR	EUA *CDC* *N1 & N2* MiCo BioMedkit *ORF3a & N probes* (		NP	Validation studies are important for SARS-CoV-2 RT-qPCR commercial kits to prevent unreliable results.	54	[104]
RT-PCR	RealStar ® Altona *(E and S gene)* CDC *N &human RNase P gene (RP)* ePlex® *(N gene)*	1200 cp mL^{-1} 1200 cp mL^{-1} 600 cp mL^{-1}	NP/BAL/, Archived frozen specimens	There was 100% agreement between the three assays for both negative and positive clinical specimens. RealStar ® higher throughput than the CDC assay, CDC assay needs three separate wells per specimen. The GenMark ePlex, a relatively short turn-around-time, open access, easier workflow.		[105]
RT-PCR	*N1 & N2 (CDC*		NP,BAL, sputum, plasma, CSF, stool, (VTM),	CDC LDT equally well in various sample matrices, High sensitivity of the N2 primer set, CSF with a LOD of 1 copy/reaction. PBS and HBSS had equivalent LODs to VTM at 10 copies, No difference in analytical sensitivity between NP swabs and BAL, with a LOD of 6 copies/reaction using the CDC assay		[106]
RT-PCR	*RNA-dependent-RNA-polymerase-helicase* gene *rNP &RBD of* sSARS-CoV-2		Posterior Oropharyngeal (deep throat) saliva	Highest salivary viral load in the first week, Application of Posterior oropharyngeal saliva, Positive test even after 25 days of premorbid,	173	[25]

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Table 1 (*continued*)

Method	Biomarker	LOD	Real sample	Remarks	No of samples	Ref
				More patients had earlier seropositivity for anti-RBD than anti-*NP* for both IgG and IgM, more patients had earlier seroconversion for IgG than IgM for anti-NP anti-RBD, Patients with comorbidities had a lower anti-*RBD* IgG OD than did those without comorbidities, Peak viral load correlated positively with age, Serum antibody levels were not correlated with clinical severity.		
RT-PCR CRISPR Metagenomic next-generation sequencing (mNGS)	*N gene*		NP	Three methods consist of an identical RNA extraction procedure, which takes about an hour. Turn-around time of RT–PCR, CRISPR & mNGS is about 3, 2 and 24 h, no obvious difference in safety among these approaches		[107]
MT-PCR	*ORF1* gene		NP/OP	Tandem PCR, High specificity (98.4%)	7839	[67]
dPCR	*ORFAlab &N*		NP, Stool, Blood	LOD dPCR is at least 10-fold lower than that of RT-PCRAccuracy 96.3%.	108	[108]
RT-dPCR	*ORF 1* ab, *N & E* genes	2copies/reaction	NP	For fever suspected patients, the sensitivity was greatly enhanced from 28.2% by RT-qPCR to 87.4% by RT-dPCR. Total sensitivity, specificity & accuracy of RT-dPCR were 90%, 100% & 93%, respectively.	194	[109]
Real-time nanopore target sequencing	*ORFlab RNA*	10 copies mL^{-1}	Throat swab	Fast, Portable	61	[68]
RT-LAMP	*ORF1ab* gene, *E* gene *and N* gene	1000 copies mL^{-1}	NP	Simultaneous detection *ORF1ab gene, E gene and N gene* with accuracy rates 99%, 98.5%, and 92.3%, *ORF1ab* gene & N gene showed high specificity and sensitivity, Fast, One-step	208	[6]
RT-LAMP	*ORF1a, N* gene	4.8 copies μL^{-1}	Swab	Simple, Fast, direct tissue or cell lysate can be used without an RNA purification step	7	[74]
RT-LAMP	-	1.02 fg 0.204 fg - 10 ng	OP/NP, Swabs, Saliva, Urine, Serum	Simple, Specific, Fast (30 min)		[76]
RT-LAMP	*ORF1ab* gene	10 copies	–	Fast, Six primers were distinguished 8 distinct regions of the *ORF1ab* region.	43	[75]
RT-LAMP	*N* gene	118.6copies25μL^{-1} reaction.		Specific, high consistency (92.9%) with a commercial RT-qPCR	56	[110]
RT-LAMP	*N* gene	10^2RNA copies	Nasal swabs	30 min with colorimetric, Specific	154	[73]
RT-LAMP	*ORF1ab* gene	10 copies	Simulated NP& op	89.9% (223/248), Detection threshold concentration below 60 copies mL^{-1}		[111]
RT-LAMP	*N, S &RdRp* genes	30copies/reaction		*RdRp* primers showed higher amplification efficiency. Good specificity among 17 respiratory viruses.		[112]
RT-LAMP	*ORF1ab4& S123* gene	2×10^1 *ORF1ab4* 2×10^2 *S123* copies/ reaction	Swabs and bronchoalveolar lavage fluid	sensitivity was 100% (95% CI 92.3%- 10 0%), specificity 100% (95% CI 93.7%–100%)	130	[113]
RT-LAMP shotgun metatranscriptomics platform (total -RNA-seq	*N & E gene*	5–25 viral total	NP/OP lysate	LAMP (1 h) sensitivity of 95.6% and specificity of 99.2%, higher LAMP sensitivity at higher viral load. The highest viral load Ct < 20 showed 100.0% sensitivity and 97.4% specificity. LAMP applicable for environmental sampling.	857 87 subway sample	[69]
RT-LAMP	*ORF1ab, N & S*gene, *O117, S17, N1&N15*	80 copies mL^{-1}	Throat swab	Consistent with the conventional RT-qPCR. 30 min reaction	16	[114]
RT-LAMP PCR	LAMP Atila iAMP *ORF1ab & (N)* US CDC *N1& N2* Altona *E &S* EUA *E*		NP	Samples with low viral load showed a discrepancy in their results. Atila iAMP showed a little decrease in sensitivity and needed the most amount of nucleic acid (18 μL). 1 h faster results than RT-PCR	80	[78]
RT-LAMP CPA PSR	*N & Orf1ab*	10 DNAcopiesμl^{-1}	Simulated OP/NP	LAMP had better LOD result for SARS-CoV-2 extracted RNA, Specific, Primer pairs designed for LAMP, CPA		[77]

(*continued on next page*)

Table 1 (*continued*)

Method	Biomarker	LOD	Real sample	Remarks	No of samples	Ref
				and PSR targeting *N*-sequence Excellent for a real diagnosis.LOD Lyophilized LAMP kit for*Orf1ab* 21.57 & *N* 43.14 copies/reaction 431.47 & 862.9 of CPA and PSR copies/reaction.		
Hologic transcription TMA		5.5 × 10^2 copies in 1 out of 5	NP	Sensitivity 98.1% (52/53) for TMA and 96.2% (51/53) for RT-PCR	116	[81]
RT-RAA	*ORF1ab*gene	2 copies/reaction	NP/OP sputum, Nasal swab, BAL, Stool, Whole blood	Sensitivity and specificity of RT-RAA was 97.63% (330/338) and 97.87% (596/609), Ultrafast speed of detection, 16 samples per run, A single person can finish 16 samples in 40 min by working with automatic DNA extraction.	947	[115]
CRISPR-Cas12DETETER lateral flow	*E &N genes*	1 copies μL^{-1}	NP/OP	Low-cost, Relatively rapid positive predictive agreement and negative predictive agreement of SARS-CoV-2 DETECTR relative to the CDC qRT–PCR assay were 95% and 100%, respectively	83	[85]
Dual CRISPR -Cas12a	*N gene*	1.2 copies of DNA targets HIV 4.6 copies RNA targets SARS-CoV-2		40 min incubation time, Fast, One-pot, Robust		[86]
C2CA	*RdRp*	0.4 F M	FBS	Differentiate SARS-CoV & SARS-CoV-2 sequence.		[84]
LSPR	*RdRp-COVID*	0.22pM 0.1 pM to 1 μM	–	Specific, discriminate between Rd-Rp-COVID and Rd-Rp SARS, Wide dynamic range	–	[90]
FET	SARS-COV *spike S1* subunit protein antibody	0.2pM	–	Sensitive, SARS-CoV-2 *spike S1* had better sensitivity than angiotensin-converting enzyme 2 (ACE2)	–	[92]
FET	SARS-CoV-2 *spike* antibody	1 fg mL^{-1} & 100 fg mL^{-1}in PBS and clinical transport medium 1.6 × 10^1 pfu mL^{-1} culture medium 2.42 × 10^2 pfu mL^{-1} clinical sample	NP	Excellent sensitivity, Good liner rang, No pretreatment for clinical samples		[93]
Cell-based potentiometric biosensor	SARS-CoV-2 *Spike S1* antibody	1 fg mL^{-1} 10 fg -1 μg mL^{-1}		Selective, Excellent sensitivity, Good linear range	–	[94]

List of abbreviation: Chemiluminescent immunoassay (CI), Enzyme linked immunosorbent assay (ELISA), Immunochromatographic assay (ICA), Lateral flow immunoassay (LFI), Fluorescence Lateral flow immunoassay assay (FLFI), Fluorescence Immunochromatographic assay (FICA), Reverse transcription polymerase chain reaction, (RT-PCR), Transcription-mediated amplification (TMA), Loop mediated isothermal amplification (LAMP), Rolling circle amplification (RCA), Clustered regularly interspaced short palindromic repeats (CRISPR), Circle-to-circle amplification (C2CA) Field effect transistor(FET), recombinant (r),receptor-binding domain (RBD), *Open reading frames*(*ORFs*), *Spike* (*S*), *Membrane* (*M*), *Envelope* (*E*), and *Nucleocapsid* (*N*), Nasopharyngeal Swab (NP), Oropharyngeal (OP), Cerebral Spinal Fluid (CSF), Bronchoalveolar Lavage (BAL), Fetal bovine serum (FBS), Viral Transport Medium(VTM).

diagnosis of infectious diseases [88].

3.1.1. Localized surface plasmon resonance (LSPR) sensor

LSPR is an optical phenomenon produced when light waves are trapped in conductive nanoparticles which are smaller than the wavelength of light. The incident light and surface electrons in the conduction band interact to produce coherent localized plasmon oscillation. The resonance frequency is sensitive to local changes like the variation in refractive index and molecular binding [89].

Dual-functional plasmonic biosensor utilizing plasmonic photothermal (PPT) effect and localized surface plasmon resonance (LSPR) sensing transduction were applied for the detection of various viral sequences including RdRp-COVID, ORF1ab COVID, and E genes from SARS-CoV-2. The converted PPT heat energy, in the proximity of gold nanoislands, provided a stable heat source to enhance the in situ hybridization of *RdRp* of SAR S–CoV-2 and its complementary DNA. The slope of the photothermal enhanced LSPR curve was higher than the system without the photothermal effect. The proposed sensor was capable to discriminate between SARS-CoV and SARS- CoV-2 viruses. Without the assistance of the photothermal unit, a false positive response signal was obtained for the RdRp-SARS sequence. The sensor has shown a LOD of 0.22 pM [90]. The schematic procedure for this biosensor is depicted in Fig. 4a.

3.1.2. Field effect transistor (FET)

The FET transducer is based on modulation of carrier mobility across a biased semiconductor due to the electrostatic field. The gate surface of FET is covered with a layer that can be modified with biomolecules for selective detection of targets [91].

Graphene FET was decorated with an antibody of SARS-CoV-2 spike S1 subunit protein (CSAb) or angiotensin-converting enzyme 2 (ACE2) to detect SARS-CoV-2 spike protein *S1*. The binding of the *S1* protein that possesses a slightly positive charge with the CSAb/ACE2 receptors on the graphene surface changed the conductance/resistance in graphene-FET which was considered the basis of the detection. CSAb modified graphene-FET exhibited better sensitivity due to the higher affinity of this antibody. The proposed sensor showed a LOD of 0.2 pM [92]. The FET system has detected SARS-CoV-2 based on the changes in channel surface potential and its effect on the electrical response. As discussed previously, the *S* protein is an excellent antigen because it is a

major transmembrane protein of the virus and it shows amino acid sequence diversity among coronaviruses. The FET sensor was capable to detect *S1* protein down to 1 fg $mL^{-1}$and 100 fg mL^{-1}in PBS and clinical samples, respectively. Moreover, the FET sensor determined SARS-CoV-2 with LOD of 1.6×10^1 pfu $mL^{-1}$and 2.42×10^2 copies mL^{-1}in culture medium and clinical samples, respectively. The biosensor was also able to discriminate the SARS-CoV-2 antigen protein from the MERS-CoV protein which indicated good selectivity of this platform [93]. The schematic procedure for FET biosensor was illustrated in Fig. 4b.

3.1.3. Cell-based potentiometric biosensor

A membrane-engineered kidney cell modified with the SARS-CoV-2 Spike*S1* antibody via electro-insertion was applied to detect the SARS-CoV-2 *S1* antigen. The potential of the membrane is changed by the interaction of the antibody with the target protein. The device was fabricated on 8 gold screen printed electrodes which were covered by polydimethylsiloxane (PDMS) layer with eight wells. Suspension of the modified membrane was added to PDMS well, followed by the addition of protein solution and signal measurement with a potentiometer. The sensor has achieved an excellent detection limit of 1 fg $mL^{-1}$with a wide linear range of 10 fg to 1 μg mL^{-1} [94]. Table 1 summarizes different methods for SARS-CoV-2 detection.

Fig 4

4. Conclusion

We discussed different molecular and serological methods for the detection of SARS-CoV-2. RT-PCR can provide good sensitivity and specificity and the results can be obtained in a few hours. It can detect viral DNA in respiratory samples, saliva, blood, urine and stool. However, RT-PCR has some drawbacks including the need for expensive thermocycler and professional staff to perform the assay and interpret results. Moreover, the standard control has an important role in the accuracy of the results and false-negative results can be obtained due to sample degradation, time and quality of sample collection and the low efficiency of some test kits. LAMP methods have comparable sensitivity to RT-PCR and high specificity. However, some kits showed lower sensitivity. It can be performed in 30 min using a crude sample that allows their possible integration in POC tests. CRISPR method has been also developed for SARS CoV-2 detection showing high sensitivity and specificity. It can be performed in 1 h and can be coupled with Lateral flow assay. There is no need for expensive thermocycler for LAMP and CRISPR. Lateral flow assay is an easy method to apply with the ability to obtain results in 15 min by non-professional personnel in blood or serum samples. Moreover, antibodies are less affected by storage, transport and sample collection. It has the disadvantage of prolonged time of antibody production. ELISA is easy to perform but, like Lateral flow assay, cannot be used for early detection. However, it can be used to check the immunity of healthcare staff and for the investigation of herd immunity.

In the future, we expect that other easier and more mature molecular systems like LAMP and CRISPR as well as biosensor platforms will replace RT-PCR. More studies for the comparison between newly developed methods in terms of sensitivity, reproducibility, reliability, and robustness are still needed. Moreover, developed techniques that can analyze samples from different routes should be combined with oral swabs detection to validate the results for making an informed decision to discharge people from hospitals or home quarantine. Saliva, sputum, posterior Oropharyngeal can replace the nasopharyngeal samples that can be less invasive and less dangerous for healthcare staff. As current tests are not sufficient to distinguish infected people in public areas, there is still an urgent need to produce POC devices that can detect infections on-site without the need for professionally trained personnel.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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