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Role of Immunochromatographic Techniques in the Diagnosis of COVID-19

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ABSTRACT

The rapid and accurate diagnosis of SARS-CoV-2, the virus responsible for COVID-19, is crucial for controlling its spread and managing public health responses. SARS-CoV-2 is an airborne virus transmitted through direct contact or respiratory droplets from infected individuals. While some patients exhibit clear symptoms, others remain asymptomatic, making early detection essential to prevent further transmission. Diagnostic methods for SARS-CoV-2 primarily rely on two strategies: detecting viral RNA and identifying virus-specific antigens or antibodies. Viral RNA detection through reverse transcription polymerase chain reaction (RT-PCR) remains the gold standard for identifying active infections. In contrast, immunochromatographic techniques (ICT) offer a rapid and cost-effective alternative by detecting viral antigens or host antibodies. Immunological assays such as enzyme-linked immunosorbent assay (ELISA), chemiluminescent immunoassay (CLIA), and lateral flow immunoassay (LFIA) play a complementary role in identifying individuals with current or past infections. This review explores various molecular and serological methods for SARS-CoV-2 detection. RT-PCR offers high sensitivity and specificity, detecting viral RNA in respiratory samples, saliva, blood, urine, and stool within hours. However, it requires expensive equipment and trained personnel, with accuracy affected by sample quality and test kit efficiency. CRISPR-based detection provides high sensitivity and specificity, delivering results within an hour and integrating with lateral flow assays without the need for a thermocycler. Lateral flow assays enable rapid detection within 15 minutes by non-professionals but are limited by delayed antibody production. ELISA and CLIA are useful for immunity assessment, though ELISA lacks early detection capabilities, and CLIA is costly, limiting accessibility in economically challenged regions. Selecting an optimal method depends on available resources, accuracy, and the need for rapid diagnosis.

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 [1]. Coronavirinae are categorized into four genera. 1) Alpha coronaviruses which include HCoV-229 E and HCoV-NL63;2); Beta coronaviruses which involve HCoV-OC43, HCoV-HKU1, MERS - CoV, SARS-CoV, and SARS-CoV-2; 3) Gamma coronaviruses that infect whales and birds, and 4) Delta coronaviruses that cause sickness in pig and birds [2]. Coronavirus has a single-stranded positive sense RNA (+ssRNA) genome which is 30kb with a 5'cap structure and 3' poly-A tail. 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 [3]. Coronavirus is infectious to an extensive range of mammals including animals' humans' rodents and birds. SARS-CoV-2 is caused by COVID 19 which is an infectious disease.

Since CoVs contain +ssRNA genome which has greater mutation rate then other viruses, once transmitted from one host to another it can adapt to the environment of their host because of the higher mutation capability in their genome[3], [4]. 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 [5], [6]. The S protein, in particular, mediates the entrance of SARS-CoV-2 into host cells by binding to angiotensin-converting enzyme 2 (ACE2), a special viral receptor on host cells [7].

To develop antiviral vaccines and diagnose viral infections, the SARS-CoV-2 S glycoprotein is an essential target [7]. Recent investigations have shown that SARS-CoV-2 has a higher interpersonal transmission rate than other emerging coronaviruses because its protein binds with the viral receptor ACE2 more strongly than SARS-CoV's S protein does [8]. Antibodies against SARS-CoV-2 play a central role in clearing the virus from infected patients. To prevent COVID-19, antibodies should be able to engage the S1 subunit of the SARS-CoV-2 spike protein, which contains the receptor binding domain (RBD) to ACE2, and neutralize the virus [9].

Resemblance of SARS-CoV-2 with bat's genome

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 [10]. SARS-CoV-2 has 96% homology at the whole-genome level with bat coronavirus [11]. 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 [11]. 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 [12].

Variants of HCoVs

This virus has a virion diameter of 70–140 nm with recognizable spikes of 9–12 nm. 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.

Pandemic

As mentioned above, the recently identified SARS-CoV-2 which causes the COVID-19 pandemic in the world belongs to the beta CoVs [13]. 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 [14]. The epidemic of pneumonia caused by SARS-CoV-2 spread rapidly posing a serious threat to the life and health safety of people.

Immune system suppression

The people with compromised immunity such as in HIV had a greater risk of being infected by the virus and an even greater risk of dying from SARS-CoV-2. SARS-Cov-2 and SARS-CoV after entering the body showed pathogenic symptoms by suppressing the immune system of the host as they depleted the lymphocytes number especially T lymphocytes which resulted in depletion of CD4 and CD8 cells in the body[15].

Symptoms of COVID 19

The main clinical manifestations of COVID-19 are fever, fatigue and dry cough. Some patients have a sore throat, muscle pain or diarrhea, anosmia as well as ageusia. Acute respiratory distress syndrome, septic shock, multiple organ failure, ground glass opacities, and other symptoms can occur in severe cases [8], [16]. Patients also showed headaches, hemoptysis, abdominal pain, diarrhea, and the production of sputum less frequently [2]. 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 [1].

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 [2], [17], [18]. 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 [19].

Transmissibility

The disease spread rapidly among people through respiratory droplets during talking, coughing and sneezing [13]. It has an incubation period of 1–14 days (usually 3–10 days) with no noticeable symptoms which is longer than SARS CoV [20]. The rate of virus transfer is usually assessed based on the R_o parameter (basic reproduction number). R_o 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_o 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_o values of 1.4–2.5 for SRAS-CoV-2 while R_o 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_o of 2–5[20]. However, Liu et al. [21] found R_o ranging from 1.4 to 6.49 with an average R_o value of 3.28 and a median 2.79 by evaluating 12 studies. They concluded that SARS-CoV-2 is more transmissible than SARS-CoV [21]. 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 [22]. 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 [23]. 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 [24]. Another feature of SARS-CoV-2 is the higher viral load reported in elder people [25].

Early diagnosis

Moreover, symptoms of COVID-19 such as cold, fever, influenza are very similar to normal influenza and cold. Therefore, it is very important to diagnose and identify the infected patient in order to prevent further spreading of this disease. The early detection of this disease will also enable the physician to efficiently deal with the disease in order to treat the patient before developing serious complications. Besides fast and reliable and screening techniques can also help identify negative people and prevent the unnecessary quarantine of uninfected people

Detection method of SARS-CoV-2

Numerous studies have shown that the degree of antibody response is correlated with the severity of COVID-19 and that the quantity of neutralizing antibodies declines rather rapidly with time [26], [27]. The detection reagents of SARS-CoV2 can be divided into three categories according to their targets: nucleic acid detection, antigen detection and antibody detection. Nucleic acid detection is the "gold standard" for the diagnosis of SARS-CoV-2 infection [28]. However, nucleic acid detection based on PCR test largely depends on viral load. Low copy viruses will lead to false negative results, and it has been reported that the false negative rate is 2%–18 % [29]. At the same time, these tests have long turnaround times and are complex to operate, usually taking at least 2 hours on average to produce results. Serological assays for SARS-CoV-2 play a role in diagnosis of COVID-19, in understanding viral epidemiology and screening convalescent sera for therapeutic and prophylactic purposes, to better understand the immune response to the virus, and to assess the degree and duration of the response of specific antibodies [30].

Importance of antibodies

It is widely accepted that IgM provides the first line of defense during viral infections. Before the generation of adaptive, high-affinity IgG responses that are important for long term immunity and immunological memory. Furthermore, detection of IgM antibodies tends to indicate recent exposure to SARS-CoV-2, whereas the detection of COVID-19 IgG antibodies indicates virus exposure some time ago [28]. Thus, we believe that the detection of both IgM and IgG could provide information on the virus infection time course. The rapid detection of both IgM and IgG antibodies will add value to the diagnosis and treatment of COVID 19 disease [31].

Immunological assay

Immunoassays are greatly selective biochemical methods which are based on the specific antigen–antibody interaction [32]. Currently, immunoassays increase in the sensitivity by labelling one of the reagents: either the antigen or antibody. The labelling agents can be a radioisotope, enzyme, fluorescence, or chemical [33]. The reaction is catalyzed by labelling enzymes and substrate degradation to form the colored product for visual and spectrophotometric observation [34], [35]. Regarding the detection label, immunoassays applied to antibiotic determination have been classified. Numerous immunoassays have been developed for the screening of antibiotic residues [36], [37], [38]. Numerous screening assays generally take a pretty long reaction time. So, there is a growing need to introduce the immunoassays developed for the screening of antibiotic residues. Many of the immunochemical tests are commercially available in a kit format such as a card format and one step strip test for many drugs [39]

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 [40]. On the other hand, antibodies are more resistant than viral RNA and are less deteriorated by transportation, storage and collection [41]. 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 [42]. 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 [43], [44]. 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 [40]. 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], [30].

Enzyme linked immunosorbent assay (ELISA)

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 [42].

MATERIALS AND METHODS

The materials required for the performance of ELISA are antibodies, PVDF strips and a test kit which can be prepared easily.

Antibodies

SARS-CoV/SARS-CoV-2 (COVID-19) spike antibody (1A9); SARS-CoV/SARS-CoV-2 (COVID-19) nucleocapsid antibody (6H3) Cat; Mouse IgG (Fc fragment) antibody, F(ab') 2 fragment, pre-adsorbed (AP); Rabbit anti-SARS Virus Spike Protein; Rabbit anti-SARS Virus Nucleocapsid Protein; 2019-nCoV Spike Protein (S1+S2 ECD, His tag); 2019-nCoV Nucleocapsid Protein (His tag); Actin antibody and other antibodies can be obtained easily from the market[45].

PVDF Strip Preparation Protocol

The steps for preparing the PVDF strips armed with the primary antibodies of interest were as follows: Hydration of the PVDF strip with methanol for 5 min, followed by two washes with PBS for 5 min. Incubation with protein A 10 µg/mL for 1 h in PBS followed by two washes with PBS. After washing, the strips were blocked with 3% BSA solution in PBS for 1 h and washed three times with PBS for 5 min. The PVDF strips were then separately incubated with a solution containing rabbit antibody anti-SARS Virus Spike Protein or anti-SARS Virus Nucleocapsid Protein or anti-actin antibodies at a concentration of 3 µg/mL overnight at 4 ◦C via shaking. The strips were then washed three times with PBS for 5 min, followed by two washes with 0.2 M PBS TEA. The strips were further incubated with 25 mM DMP in 0.2 M TEA HCl, pH 8.2, followed by a solution containing 0.2 M TEA + 20 mM ethanolamine) and two washes with PBS for 5 min. The strips were then stored in 0.02% NaN3 in PBS. The strips were cut with a width of approximately 0.4 cm in order to fit on the device support to perform the ELISA analysis [45].

Device Design

The device uses dried or lyophilized antibodies, which are stable at room temperature; at the time of use, they are solubilized in a suitable buffer solution. The device can be built by assembling commercially available and custom-made semi-finished products. It included an instrument such as cytobrush and/or nasal swab and/or throat swab, which are useful for taking the biological sample at the level of the ororinopharyngeal mucosa; this is necessary to analyze it and verify the presence of viral biomarkers by using the ELISA method. The product has an extremely simple method of use [45].

Test Procedure

1) The reagents found in the wells/station in a dried and/or lyophilized state are dissolved into an appropriate buffer when opening the kit. 2) The cells from the ororhinopharyngeal mucosa and potentially infected sputum are collected with specific instruments. 3) The buffer is immersed with the biological sample for 8 min in the well/station 0 containing the lysis buffer. 4) The lysate is transferred into well/station 1. 5) The package of the support is opened to whichever PVDF membrane is adhered to, which is then immersedwell/station 1 for 8 min for the recognition of viral antigens by the primary antibodies immobilized on the PVDF strip. In this station, immune complexes were formed on the strip if there was the presence of viral proteins in the sample taken. 6) The PVDF strip with the immobilized immune complexes is immersed for 5 min in well/station 2, where there are primary monoclonal antibodies in the solution that bound specifically to the immunocomplex. 7) In wells/stations 3 and 4, the PVDF support holding the strip is washed in wells containing the T-PBS buffer to eliminate the proteins that are not attached to the immunocomplex. 8) The support was immersed with the PVDF strip for 5 min in well/station 5 containing the secondary antibodies conjugated to an enzymatic detection system (alkaline phosphatase). 9) In wells/stations 6 and 7, washings of the support with the PVDF strip takes place in wells containing the T-PBS buffer to eliminate any excess secondary antibody. 10) The support with the PVDF strip is immersed for 4 min in well/station 8 containing the substrate (BCIP/NBT, stable at room temperature) that is necessary for the colorimetric reaction. 11) The last phase consists of the interpretation of the result. 12) The colorimetric assay can be carried out in a temperature range between 20 and 30 ◦C. In fact, the alkaline phosphatase or peroxidase linked to secondary antibody possessed the maximum enzymatic activity in this temperature range, thus allowing for optimal signal amplification [45].

RESULTS

It is the qualitative detection of antigens associated with corona virus infection in the body. In ELISA a number of antibodies against specific proteins are adsorbed of the surface of membrane. The membrane is then immersed in a cell lysate. If the desired protein marker is present in the sample which has a concentration greater than threshold, then these proteins are captured on the membrane, which are then immersed in a solution containing secondary antibodies that are conjugated with alkaline phosphate, forming a sandwich that is detectable with the help of colorimetric technique. The membrane containing sandwiched complex is then immersed in colorimetric detection station where in the presence of the complex a pink or purple band will appear [45].

Figure 1: *The ELISA test strips that shows different colored bands at different concentration of antibodies [45]*

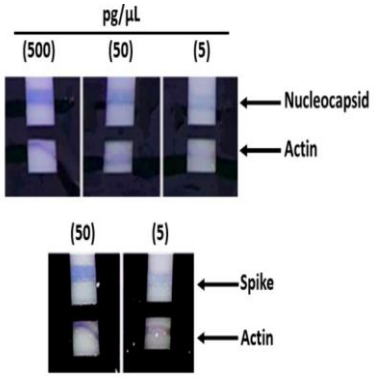


Figure 2: *The ELISA assay. The results are obtained by following the procedure discussed above [45].*

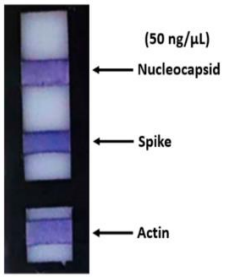
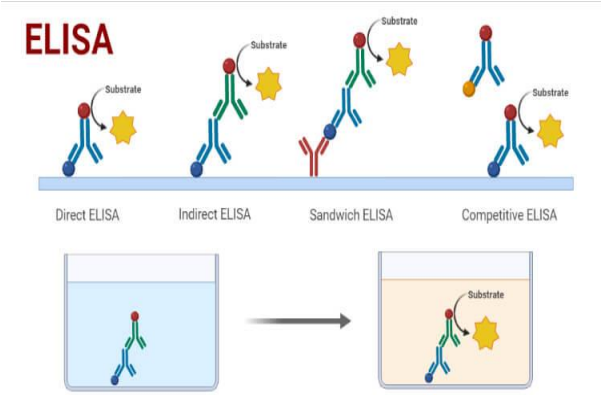


Figure 3: *Different methods to perform ELISA*



Chemiluminescence immunoassay (CLIA)

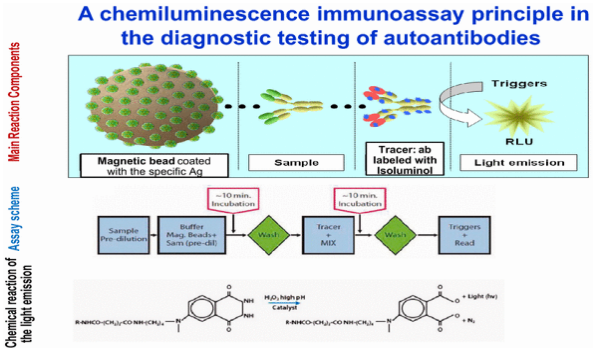


Figure 4

Introduction

Chemiluminescent immunoassay (CLIA) is an immunoassay technique where the label, i.e. the true "indicator" of the analytic reaction, is a luminescent molecule. In general, luminescence is the emission of visible or near-visible (λ = 300–800 nm) radiation which is generated when an electron transitions from an excited state to ground state. The resultant potential energy in the atom gets released in the form of light. In spectrophotometry, we refer to chemiluminescence, because the type of luminescence applied to immunoassay techniques generally identifies exergonic chemical reactions as the most suitable energy source for producing the electronic excited state. The heterogeneous method is the more widely used chemiluminescent assay. Chemiluminescent methods can be direct, using luminophore markers, or indirect, using enzyme markers [46]. In direct chemiluminescent methods, the luminophore markers used are acridinium and ruthenium esters, while the enzymatic markers used in indirect methods are alkaline phosphatase with adamantyl 1, 2-dioxetane aryl phosphate (AMPPD) substrate and horseradish peroxidase with luminol or its derivatives as substrate [46]. Synthesizing molecules such as AMPPD and isoluminol base molecule derivatives are more stable compared to other luminescent markers and result in light emission with a characteristically elevated quantum yield. Activation of these substrates requires chemical or enzymatic reactions associated with the immunological reaction. For example, the use of luminol and derivatives of isoluminol as chemiluminescent labels depends on the coupling of the immunoassay with enzymatic reactions catalyzed by peroxidase. The addition of an enhancer (e.g. ferrocyanide, metallic ions) further boosts the electronic activation, ultimately leading to extremely elevated analytic sensitivity (Mol−16 per litre), certainly superior to those attainable by other immunoassay methods such as Radio immunoassay (RIA), immunoenzymatic (ELISA) and fluoroimmunoenzymatic (FEIA) methods, etc. [46].

Methodology

Indirect CLIAs use recombinant antigen-coated magnetic beads as the solid phase, which are incubated with a liquid sample containing specific Abs to create immune-complexes. After formation of the immune-complex, an enzyme labeled anti-antibodies is added with the substrate to initiate a chemiluminescence reaction. The results are measured in relative light units and can quantify IgM, IgG, IgA, and total antibodies in samples. CLIA is similar in principle to ELISA [47]. With its superior sensitivity and specificity as compared to CGI and ELISA, as well as its shorter analysis time (which can be anywhere from 15 min to several hours), easy operation, and high automation [28].

Micro particle coated with SARS-CoV-2 antigen are combined with assay diluent followed by incubation. The antibodies present in the participant's serum binds with the antigen coated micro particle. Anti-human IgG labelled with acridinium conjugate is added followed by pre-trigger and trigger solution. The test reaction was measured by system optics and expressed as relative light unit (RLU). The level of RLU is directly proportional to the amount of IgG. It is then compared to the calibrator RLU to determine the presence and absence of IgG antibodies against SARS-CoV-2[48].

Advantages and Disadvantages

The key advantages of chemiluminescent analytical methods reside in the wide dynamic range, high signal intensity, absence of interfering emissions (i.e. high specificity), rapid acquisition of the analytical signal, high stability of reagents and their conjugates, low consumption of reagents, random access, reduced incubation time and full compatibility with immunology assay protocols (homogenous or non-homogenous)[49]. Some limitations are to be considered as well. The disadvantages of CLIA are represented by:

1. Limited Ag detection
2. High costs
3. Limited tests panel
4. Closed analytical systems.

Furthermore, for many chemiluminescent (CL) systems, there is a low background level of emission in the absence of analyte. Hence, CL signals in flow systems, increasing proportionally to the analyte concentration, appear as sharp peaks superimposed on a low constant blank signal, measured as viewed by the time window when the mixture of analyte and reagent(s) passes through the detector cell. Due to the small portion of CL emission that is only measured from this time profile, reactions with complex kinetics can give nonlinear plots of response versus analyte concentration [46].

Gold immunochromatography assay (GICA)

GICA with colloidal gold as a tracer in the alkaline environment with negative and positive charge groups and antibody protein molecules create a new immune detection method.

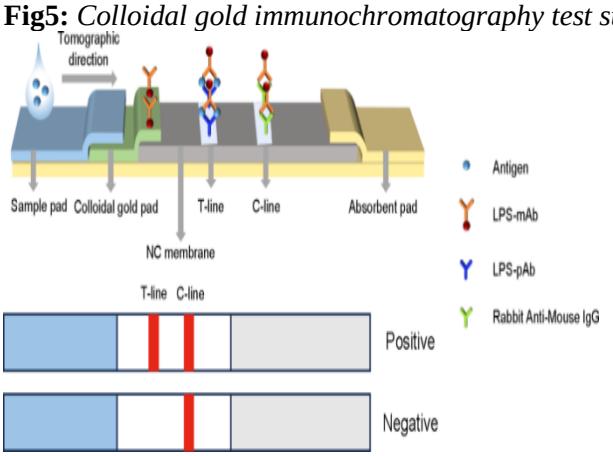
Among all the immunochromatography assay (ICA) the GICA provides rapid results and that is why it is often referred as dipstick method or rapid test paper method.

Principle

The principle of GICA is to utilize the high electron density property of gold particles, and employ colloidal gold as a tracer marker in the immunoanalysis, which is done by the encapsulation of proteins and other macromolecules that are adsorbed at the surface of colloidal gold particles [31]. For Instance, nitrate cord membrane was coated with mouse anti-human IgM (μ-chain) monoclonal antibody and sheep anti-mouse IgG (quality control) antibody, as well as colloidal gold labeled SARS-CoV-2

recombinant antigen and mouse IgG antibody can be used as tracers. If the sample contains SARS-COV-2 IgM antibody, it can bind to the colloidal gold-labeled SARS-CoV-2 antigen to form a complex, captured by coated mouse anti-human IgM antibodies. It then binds to the mouse anti-human IgM to form a complex that produces color Colloidal gold labeled mouse IgG antibody binds to the coated sheep anti-mouse IgG antibody for color development, which serves as the quality control line[28].

Fig5: *Colloidal gold immunochromatography test strip*



Benefits & Drawbacks

Out of all the ICA the GICA is simple to use and it does not require any additional equipment for the qualitative analysis of IgG and IgM antibodies and yields result that can be viewed with naked eye in less than 15 min. Although it is simple to use and does not require any extra machinery, its sensitivity and specificity in limited and it only gives us the qualitative analysis. Consequently, it can be used for the quick screening of clinically suspected cases, especially in situation when other ICAs or diagnostic tools are unavailable [31].

Lateral flow immunoassay (LFIA)

A classical LFIA is a strip of carrier substance that contains a dry reagent which was activated once put on to the fluid samples. LFIA was practical in a wide range of screening and diagnosis purposes [50]. Lateral flow

immunoassay test was developed for the detection of both IgM and IgG in blood samples. 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 finger stick 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 [43].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 [51].

Advantages and disadvantages

This assay has many advantages such as easily scalable to high-volume production, stable-shelf-lives of 12–24 months often without refrigeration, simple to use, no specific expert is be required: minimum operator-dependent steps and interpretation can handle small volumes of multiple sample types that can be integrated with on-board electronics, reader systems, highly sensitive information system, stable, specific, economical, and time-saving. However, LFIA also have also some of the limitations such as ambiguous results, miniaturization and in the simultaneous investigation of multiple markers are difficult to integrate on-board electronics and to built-in QC functions challenge, the sensitivity issues in some of the systems test-to-test reproducibility challenging [52].

Sensitivity and specificity of immunological assays

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) [53]. 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[40], [54]. 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 [54].

Reverse transcription polymerase chain reaction (RT-PCR)

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 [55]. Quantitative PCR determines a DNA molecule with the help of fluorescent dye or fluorophore-attached DNA probe such as TagMan [55]. 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. 4) Relative expression estimation based on Ct data of control and experimental samples [22].

The selection of a standard positive control influences the accuracy of RT-PCR [56]. PCR result is considered positive if the Ct value was less than 40[57]. 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 [57]. 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 [23].

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 [58]. 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 [1].

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 [58].

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 NH2-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 105 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 [59]. 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 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 [60].

Table 1

Comparison of different immunoassays

Methods	RT-qPCR	ELISA	CLIA
Category	Nucleic acid amplification test(NAAT)	Serological test	-
WHO's Remarks	Gold standard for covid-19 diagnosis	Acceptable specificity Can be set as	-
Targeted site	Nucleic acid	Viral protein	Nucleocapsid protein of SARS-CoV-2
Sensitivity	High	Limited	High
Specificity	High	Varies	High
Sample collection	Nasopharyngeal swab, oropharyngeal swab, sputum, saliva	Nasopharyngeal swab, oropharyngeal swab, sputum, saliva	-
Cost	High	Moderate	High
Result generation time	Varies	Varies	Quick
Trained operator	Yes	Yes	-
Effect of antigenically different variants	Not likely	Yes	-

Table 2

Various diagnostic assays for SARS-CoV-2

Method	Biomarker	Real sample	Remarks	No of samples	References
CI	Synthetic peptides sequence of ORF1a/b, S, N proteins	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	[61]
CI and RT-PCR	N-protein of SARS-CoV-2	Serum NP, OP	Correlation between time and speed of IgM production and severity of sickness	736, 228confirmed cases	[62]
ELISA	RBD rS, rN protein	Serum	The positive rate for N &S-based ELISAs for	214	[63]

			(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.		
ELISA	Mammalian cell expressed RBD of S protein SARS-CoV-2	Plasma	Antibodies were <40% 1-week after premorbid and rapidly 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	[64]
ELISA	SARS-CoV Rp3 NP	Oral and anal swabs, blood, serum	Shift from oral positive to anal swab during later day infection	178	[65]
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)	[66]
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)	[67]
ELISA	rS SARS-CoV-2 protein	Serum	Most PCR-confirmed SARS-CoV-2 were seroconverted. IgA had greater sensitivity in the Euro immune 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	[54]
ELISA and 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	[68]
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	[69]
LFI	Anti-human IgG, Anti-human IgM, Anti-rabbit IgG	Serum/blood	Fast (15 min), Portable, Able to detect finger stick blood sample, 88.66% sensitivity 90.63% Specificity	397	[43]
LFI	Anti-human IgG, Anti-human IgM, 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 cases and 124 negative control	[70]
LFI	Nucleoprotein antigen	NP	Specificity 100% and sensitivity of the30.2%. Higher viral loads better antigen detection rates. Not good for frontline detection	148	[71]
FLFI and RT-PCR	Mouse nucleocapsid protein of SARS-CoV-2	NP & urine	The sensitivity of 100%, Detection of nucleocapsid protein in the urine	239	[51]
FICA	ORF1ab,N gene	-	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	[72]
ELISA and 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 cases)	[73]

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. However, some kits showed lower sensitivity. It can be performed in 30 min using a crude sample that allows their possible integration into 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 an expensive thermocycler for 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. On the other hand, CLIA can also be used for quick identification of the presence of antibodies in the sample. However, the assay kit is very expensive and therefore it is not applicable in countries that are already going through an economic crisis.

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