

REVIEW

The next-generation coronavirus diagnostic techniques with particular emphasis on the SARS-CoV-2

Maged G. Hemida PhD^{1,2} 

¹Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al Ahsa, Saudi Arabia

²Department of Virology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr Elsheikh, Egypt

Correspondence

Maged G. Hemida, PhD, Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Hofuf, Al-Hasa, Saudi Arabia.
Email: mhemida@kfu.edu.sa

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Abstract

The potential zoonotic coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2) are of global health concerns. Early diagnosis is the milestone in their mitigation, control, and eradication. Many diagnostic techniques are showing great success and have many advantages, such as the rapid turnover of the results, high accuracy, and high specificity and sensitivity. However, some of these techniques have several pitfalls if samples were not collected, processed, and transported in the standard ways and if these techniques were not practiced with extreme caution and precision. This may lead to false-negative/positive results. This may affect the downstream management of the affected cases. These techniques require regular fine-tuning, upgrading, and optimization. The continuous evolution of new strains and viruses belong to the coronaviruses is hampering the success of many classical techniques. There are urgent needs for next generations of coronaviruses diagnostic assays that overcome these pitfalls. This new generation of diagnostic tests should be able to do simultaneous, multiplex, and high-throughput detection of various coronavirus in one reaction. Furthermore, the development of novel assays and techniques that enable the in situ detection of the virus on the environmental samples, especially air, water, and surfaces, should be given considerable attention in the future. These approaches will have a substantial positive impact on the mitigation and eradication of coronaviruses, including the current SARS-CoV-2 pandemic.

KEYWORDS

coronaviruses, diagnostic assays, DIVA, DIVI, high-throughput, multiplex, pitfalls, simultaneous detection

1 | INTRODUCTION

The emergence of SARS-CoV-2 in late 2019 is raising the total number of human coronaviruses into seven candidates.¹ Three of them cause serious illness in humans across the globe, which are SARS-CoV, MERS-CoV, and SARS-CoV-2. Identification of the SARS-CoV-2 patients and tracing their close contacts at a very early stage of infection should be considered the milestone in control and minimizing/stopping the spread of these viruses. Coronaviruses are among the largest RNA viruses with single-strand positive-sense

RNA genomes. The genomes of coronaviruses are ranging from 27 to 32 kilo-bases in size. The viral genome act as a messenger RNA (mRNA). Usually, coronavirus genomes are organized in a fixed pattern in which the 5' two-thirds part of the genome is occupied by the nonstructural proteins (NSPs).²⁻⁸ These proteins encode some important enzymes, particularly the viral polymerase called RNA-dependent RNA polymerase. Meanwhile, the 3' one-third of the genome is occupied with the major structural proteins (S, E, M, and N) interspersed with some nonstructural or accessory proteins.⁷ The spike glycoprotein (S) is the key protein in all coronaviruses.

This protein plays an important role in virus replication, pathogenesis, and immune response. The coronaviruses-S protein is one of the most important targets for vaccine development, therapy as well as in the development of various diagnostic assays.⁹⁻¹⁶ The S protein is composed of two subunits, S1 and S2. The S1 is much more variable while the S2 is conserved.¹⁷ The S protein is usually inert until it infects its host then utilizes the host cell's enzymes to cleave the S into S1 and S2. This step is the most crucial in the coronavirus replication cycle. The S1 gene contains two hypervariable regions (HVR) as well as the receptor-binding domains.^{18,19} Some sequences within the HVRs are the primary determinants of the tropism and immunogenicity of many coronaviruses.²⁰ The coronavirus genomes are prone to changes at short intervals due to different factors. These changes may result in the emergence of new viruses, new virus strains, and clades or subclades of the same virus. The main reasons behind these changes are the accumulated mutations because of the poor proofing capabilities of their viral polymerases. This is in addition to the possibility of viral genome recombination and the fast adaptation to a new host, particularly animals.²¹ In addition to the ongoing changes in the genetic makeup of coronaviruses, many factors hampered the development of diagnostic assays and vaccines and antiviral therapy, particularly for the SARS-CoV-2. Among these factors, the erratic immune response, the kinetics of the pathophysiology, the existence of asymptomatic patients.²²⁻²⁵ There are many laboratory techniques for the diagnosis of coronaviruses in general, including the SARS-CoV-2. There are several approaches to the laboratory diagnosis of coronaviruses. The direct approaches, which mainly based on the detection of the virus particles, isolation of the virus, antigens, or viral nucleic acids (NAT). The indirect techniques, which mainly depend on the detection of viral-specific antibodies in sera of infected/recovered patients. Another approach

is the identification of some specific immunological markers for SARS-CoV-2 in the sera and blood of infected/recovered patients. These markers include some differentially displayed immunology markers, such as CD4+T, CD8+T cells, and interleukin 6 (IL6).^{26,27} This article will discuss the urgent need for the development of novel next-generation laboratory diagnostic assays for coronaviruses with special emphasis on the currently available assays, their advantages, and pitfalls. Figure 1 is showing a summary of various coronavirus diagnostic approaches based on the clinical diagnosis, antigen-based detection, antibody-based detection, and NAT-based detection techniques. Sampling is the first and more crucial step in the diagnosis of most pathogens, including coronaviruses. Based on the WHO and the Centers for Disease Control and Prevention (CDC) recommendations, several clinical samples can be collected and of high values during the SARS-CoV-2 infection in affected patients.²⁸ Samples can be classified into several categories based on the site of collection. The upper respiratory tract samples (URT) include the nasopharyngeal swabs (NPS) and the mid-turbinate nasal swabs (NTS). The lower respiratory tract samples (LRT) include the bronchoalveolar lavage and sputum (Figure 2). The digestive system samples include the saliva, oropharyngeal swabs (OPS), stool, and anal and rectal swabs (Figure 2). Some generalized samples can be considered, such as the whole blood, the sera, the conjunctival (ocular) swabs, tears, and urine (Figure 2). This is in addition to some autopsy or biopsy specimens from some tissues (Figure 2). The selection of the right sample at the right time is the milestone in the identification of cases and for the follow-up during viral infection. In some COVID-19 cases, the URT showing negative reverse-transcriptase polymerase chain reaction (RT-PCR) testing at the beginning of the infection. In that case, it is recommended to repeat the test for both the URT and LRT samples to monitor the progress of the diseases.²⁸ Since the

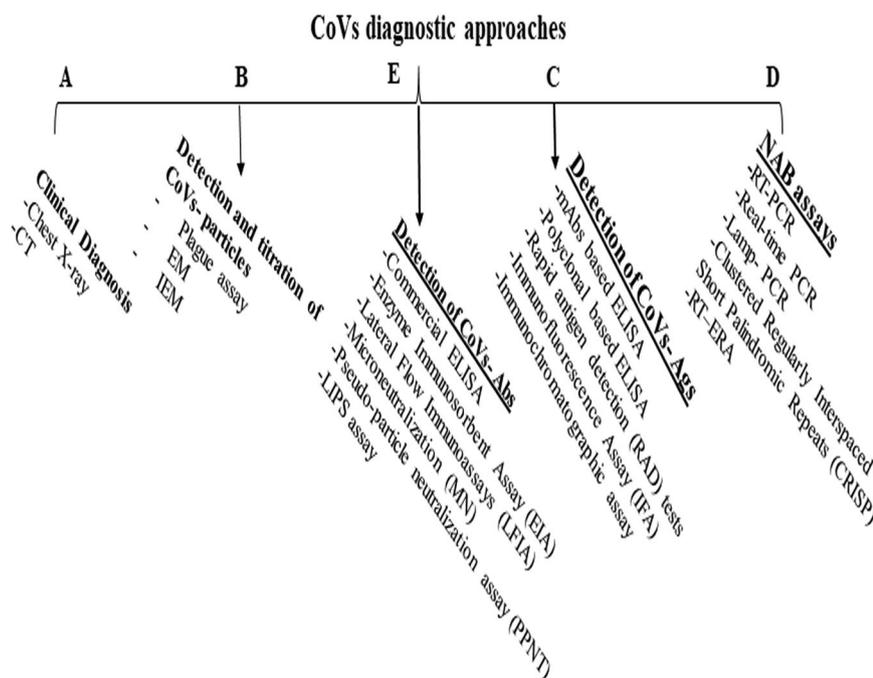


FIGURE 1 Schematic representations for the coronaviruses diagnostic techniques. (A) Techniques used for the detection and titration of the coronavirus particles and (B) techniques used for the detection of the coronavirus antigens, (C) techniques based on the NAT, (D) techniques used for the detection and titration of the coronavirus antibodies

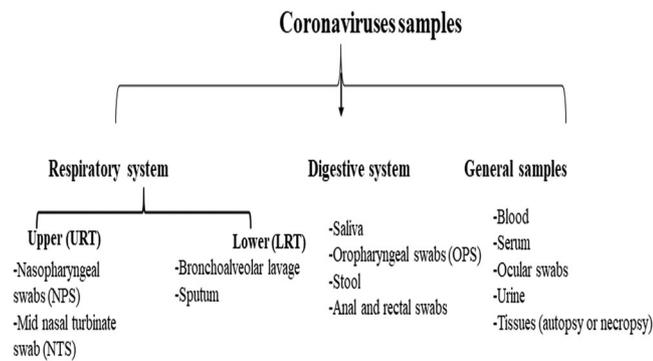


FIGURE 2 Schematic representations of different types of samples for the diagnosis of coronaviruses, especially SARS-CoV-2. Summary of the potential samples in case of SARS-CoV-2 infection, as suggested by the WHO and CDC. This diagram showing the type of samples to be collected from various body organs and locations, including the upper/lower respiratory tracts, the digestive tract as well as some generalized samples

emergence of SARS-CoV-2, there is a large number of diagnostic assays tackling different aspects in the SARS-CoV-2 diagnosis. Some of these assays or techniques are being used for other pathogens for many years, some old techniques have modified to overcome some its pitfalls, and some new techniques have already developed to fulfill new criteria of the diagnostic assays. Table 1 is showing a comparison between the coronavirus diagnostic assays based on the cost, the reaction time, high-throughput potential, specificity, and sensitivity. Each diagnostic assay requires the presence of certain equipment, facilities, and can be done on a limited number of samples or can be adjusted to screen a large number of samples to cope up with the high workflow of testing a large number of samples as in the case of the current pandemic. Table 2 is showing a comparison of the suitability of the coronavirus diagnostic assays to certain a facility, the possibility of their conduction under the rural and urban regions or in high biosecurity laboratories.

1.1 | Clinical-based diagnosis of COVID-19 in human

During the early phase of the SARS-CoV-2 pandemic, there was a high demand for the laboratory diagnosis of patients admitted with signs of pneumonia using real-time PCR. With this high number of cases admitted to the hospitals at a certain place, some hospitals were not able to meet the screening of a large number of cases per day. Based on this situation, some health authorities decided to rely on some clinical examination of the suspected cases, particularly using chest radiography. Patients who showed signs of pneumonia by chest X-ray and CT scanning were considered positive and started to receive the course of treatment available at that time immediately to save more lives.²⁹ This procedure has many pros and cons. The main advantage of this approach is the low cost of the technique since one machine can perform lab screening of a large number of patients per

day. It is usually very quick and can be performed in a remarkably short time. It can be used to monitor the progress of the health condition of some patients and the success of the course of certain treatments. On the other hand, this clinically based diagnosis is not pathogen-specific. The signs of pneumonia and lung consolidation could be due to many different pathogens and allergic conditions; thus, it requires further laboratory-supporting techniques specific for the target causative agent, particularly SARS-CoV2 in this situation.

1.2 | Applications of the electron microscope for the detection of coronaviruses

The unique crown or solar morphology of coronaviruses gives a very good privilege for the detection of the virus particles under the electron microscope (EM) through the negative staining technique.³⁰ The EM was used to detect coronaviruses in the stool of children suffering from acute enteritis caused by the HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1.³¹ The ultra-resolution electron microscope was also used to detect SARS-CoV.³² This technique was used as a routine diagnostic assay for several viruses, including coronaviruses, in the early 1980s. This technique was used as routine work for the detection of some enteric viruses of humans, animals, and birds, particularly the coronaviruses, rotaviruses, and enteroviruses. The sample of choice is usually the fecal or cloacal swabs from animals and birds, respectively, and stool samples from humans. Although this method is simple and very specific, this technique requires the availability of some expensive equipment, such as the high-speed centrifuge and the availability of electron microscope units. Moreover, the SEM is not suitable for screening a large number of samples and requires expert technical personnel.³³⁻³⁵ It may also take a long time for sample preparation to be able to detect the virus particles under the EM.³⁵

2 | PLAQUE ASSAY AND PLAQUE PURIFICATION FOR CORONAVIRUSES

The classical plaque purification assay is recently used to purify the SARS-CoV-2 for further testing. The plaque purified virus is suitable for some downstream applications, especially the development of the specific vaccine as well as diagnostic assays.³⁶⁻³⁸ Having pure virus particles is the ideal scenario for downstream virus research, including vaccine preparation, antiviral therapy, and diagnostic assays development. This approach was recently used to purify the SARS-CoV-2 to study virulence in a purified virus population in vitro in the Vero cell line.³⁸ The plaque assay was used for the infectivity titration of many coronaviruses, including SARS-CoV, MERS-CoV, and SARS-CoV-2.³⁸⁻⁴⁰ This approach using some mathematical calculations to demonstrate the infectivity of some coronaviruses in a given sample. Although this technique was developed a long time ago,^{41,42} it is still one of the best methods for the titration of the virus infectivity for viruses that grow well in the cell culture and able to

TABLE 1 Comparison of the coronavirus diagnostic assays based on the coast, the reaction time, high-throughput potential, specificity, and sensitivity

N	Test	Coast	HTP	DL	RT	Sensitivity	Specificity
A: Clinical diagnosis							
1	Chest radiography and CT	Low	(-)	NA	short	High	Low
B: Virus detection, isolation, and titration							
1	Isolation	Moderate	(-)	Concentrated samples	Long (several days)	High	High
2	Plaque assay	Moderate	(-)	Purified samples	Long (several days)	High	High
3	EM	Low	(-)	Purified concentrated samples	Short - few hours	Low	High
4	IEM	Low	(-)	Purified concentrated samples	Med- several- hrs	Mod	High
B Antigen detection tests							
1	IFA	Low	(-)	Few viruses in tissue	Short-few hrs	Med	High
2	AST	Low	(-)	Concentrated sample	Short-few hrs	Low	Low
3	Double Ab sandwich ELISA	low	(±)	Low concentration	Short-few hrs	Mod-High	Mod-High
4	LFIA	Low	(-)	Concentrated sample	Few minutes	Med	Low
C: Antibody detection tests							
1	Double Ag ELISA	low	(±)	Low concentration	Short-few hrs	Mod-High	Mod-High
2	PPNT	Med	(-)	Variable concentration	Several days	High	High
3	GICA	Low	(-)	Variable concentration	Very short (minutes)	Variable	Variable
4	LIPS	Low	(±)	Variable concentration	Relatively short (hrs)	High	High
D: NAB tests							
1	RT-PCR	High	(±)	Few copies of RNA	Short-few hours	High	High
2	LAMP	High	(±)	Few copies of RNA	Less than an hour	High	High
3	RPA	High	(±)	One copy of RNA	Short-few hour	High	High
4	RT-ERA	High	(±)	One copy of RNA	Short-few hours	High	High
5	NGS	High	(+)	Concentrated purified sample	Long up to several days	Very high	Very high
6	CRISPR	Very high	(+)	Few copies	Few hours	Very high	Very high

Abbreviations: ±, criteria fulfilled in some cases; DL, detection limit; HTP, high-throughput capacity; Med, medium; Mod, moderate; NA, Not applicable; RT, reaction time.

induce cytopathic effects, including some coronaviruses.^{43,44} The plaque assay has many applications in the diagnostic corona-virology as the titration of the virus infectivity and the evaluation of the efficacy of some antiviral compounds and vaccines for coronaviruses.⁴⁵⁻⁴⁸

3 | ISOLATION OF CORONAVIRUSES

Isolation of any pathogen is the milestone of the identification and characterization of this pathogen. After the emergence of SARS-CoV-2, there are well-known seven human coronaviruses identified in the past 60 years.^{49,50} The first reported human coronavirus was the HCoV-229E was identified through the isolation of the causative agent of a common cold in some patients in the United States.^{51,52} They used the human embryonic tracheal organ cultures to isolate

this virus, which was initially called B814.^{51,52} They examined this virus under the electron microscope and confirmed its morphology to belong to the coronaviruses. In a similar approach, the HCoV-OC-43 was discovered in 1967 by isolation of the virus in the tracheal cultures.⁵³ One of the advantages of both viruses is their ability to grow on cell cultures, which allows their characterization in more detail. In late 2002, the SARS-CoV was first identified.^{54,55} The virus was isolated using the fetal rhesus kidney (FRhK-4) cells.^{54,55} In 2004, another human coronavirus was identified in Netherland called HCoV-NL-63. They used the tertiary monkey kidney cells and the monkey kidney LLC-MK2 cell line to isolate the virus from a young child.⁵⁶ Another coronavirus similar to the HCoV-NL-63 was identified in a group of children in Hong Kong who suffered from typical respiratory distresses, including fever, coughing, and shortening of breath.⁵⁷ One study used the human-ciliated airway epithelial cell cultures (HAE) for isolating the HCoV-HKU-1 virus.³⁹

TABLE 2 Suitability of the coronaviruses diagnostic assays to a certain facility

N	Test	Urban	Rural	Field settings	Resource-rich	Resource-poor	Requirements for expensive equipment	High biosafety contaminant laboratory
1	Chest Radiography	(+)	(+)	(-)	(+)	(+)	(-)	(-)
2	Plaque assay	(+)	(-)	(-)	(+)	(-)	(-)	(+)
3	EM and IEM	(+)	(-)	(-)	(+)	(-)	(+)	(-)
4	Virus isolation	(+)	(-)	(-)	(+)	(-)	(-)	(±)
5	IFA	(+)	(-)	(-)	(+)	(-)	(+)	(±)
6	AST	(+)	(+)	(+)	(+)	(+)	(-)	(-)
7	ELISA	(+)	(+)	(+)	(+)	(+)	(-)	(-)
8	RT-PCR	(+)	(-)	(-)	(+)	(-)	(+)	(-)
9	NGS	(+)	(-)	(-)	(+)	(-)	(+)	(-)
10	LAMP	(+)	(+)	(+)	(+)	(+)	(+)	(-)
11	RPA	(+)	(-)	(-)	(+)	(-)	(+)	(-)
12	RT-ERA	(+)	(-)	(-)	(+)	(-)	(+)	(-)
13	CRISPR	(+)	(-)	(-)	(+)	(-)	(+)	(-)
14	SHERLOCK	(+)	(-)	(-)	(+)	(-)	(+)	(-)
15	LFIA	(+)	(-)	(-)	(+)	(-)	(+)	(-)
16	LIPS	(+)	(-)	(-)	(+)	(-)	(+)	(-)
17	GICA	(+)	(+)	(+)	(+)	(+)	(-)	(-)
18	PPNT	(+)	(-)	(-)	(+)	(-)	(-)	(±)

Abbreviations: (+), criteria fulfilled; (-), criteria not fulfilled; (±), criteria fulfilled in some cases.

In late 2012, a new coronavirus was identified in Saudi Arabia called the Middle East respiratory syndrome coronavirus.⁵⁸ The same study used both the Vero and LLC-MK2 cell lines for virus isolation.⁵⁸ More recently, the SARS-CoV-2 was identified in late 2019, which was initially isolated on human airway epithelium from the bronco-alveolar lavage of some patients.¹ An interesting study showed that the HAE cell culture is a universal system for the cultivation and isolation of the most common human coronaviruses.⁵⁹ Although isolation of coronaviruses is one of the gold standard diagnostic assays, it is time-consuming and requires the availability of certain cell types of cell cultures and, in most cases, a high biosafety contaminant laboratory, particularly (BSL-3) facilities.

3.1 | Detection and titration of coronavirus antigens

The antigen-based detection tests are usually the simplest diagnostic assays because they can do in a very short time. The test can be conducted in as fast as a few minutes. It does not require skilled personnel to be conducted; meanwhile, in most cases, it does not require expensive machines to be carried out. However, the specificity and sensitivity of the antigen-based assays are usually less in comparison to most NAB assays. Meanwhile, these assays require

known specific antibodies to be carried out. Thus, the antigen-based detection assays could be useful initial screening tests that require further confirmation by other NAB techniques.⁶⁰ One study was conducted to compare the performance of some antigen detection assays to that of the RT-qPCR assay in the detection of SARS-CoV-2 infections. The same study showed that the latter technique could detect 106 positive samples out of the 148 tested samples, while an antigen detection test can identify only 32 samples. This study confirms the low performance of the antigen detection compared to the gold standard RT-PCR assay.⁶¹ Other studies developed some antigen-based detection tests for the rapid diagnosis of SARS-CoV-2 infections in some patients. In this approach, they have used the nitrocellulose membrane (NCM) to develop some strips through the colloidal gold nanoparticles. They simply coated the NCM with some monoclonal antibodies against the SARS-CoV-2-N protein. They compared the sensitivity of this new antigen detection kit to the RT-PCR for SARS-CoV-2. Although this study used several modifications to improve the performance of the developed Ag detection test, this developed assay showed only 50% sensitivity when compared to the RT-PCR in the diagnosis of SARS-CoV-2 infections.⁶² Several research attempts have been recently tried to improve the sensitivity and specificity of the SARS-CoV-2 antigen. One study modified the double antibody sandwich ELISA assay for the detection of the de novo spike antigen of the SARS-CoV-2. In this approach, the

thio-nicotinamide adenine dinucleotide (thio-NAD) cycling in association with the double antibody sandwich ELISA. In this assay, the thio-NAD cycling allowed a high sensitivity starting 10 min of the application of the assay. This assay is considered one of the ultrasensitive antigen detection assays, characterized by high specificity and ultrasensitivity for the detection of SARS-CoV-S antigen.⁶³

3.2 | Immunofluorescence assay (IFA)

The IFA is one of the old techniques that is still in use and valid for many pathogens, including a large number of coronaviruses. Some recent studies used the IFA to detect the SARS-CoV-N antigen in the throat wash of some infected patients.^{64,65} Figure 3 is showing a summary of the common FAT used in the diagnosis of various types of coronaviruses.

3.3 | The immunochromatographic assay

The immunochromatographic-based assays have been recently developed to detect the SARS-CoV-2 in the saliva of infected patients.⁶⁶ These techniques should be coupled with another confirmatory NAT test to achieve an accurate diagnosis of coronavirus infection in clinical samples. Although these techniques are simple

and convenient, their specificity and sensitivity still require further evaluation.

3.4 | The antigen spot test (AST)

The AST used some bio-dot microfiltration units to detect the antigens of some coronaviruses, such as the bovine coronaviruses (BCoV). This technique may be handled as the ELISA-based assays for antigen detection.⁶⁷

3.5 | Enzyme-linked immunosorbent assay for coronavirus antigen detection

The ELISA technique is used for the detection of some coronavirus antigens, such as IBV, BCoV, and MERS-CoV.⁶⁷⁻⁶⁹ In this approach, the solid phase is usually coated with either polyclonal or monoclonal antibodies against the viral antigen under test. One of the main advantages of the ELISA technique is the possibility of detection of either the target antigens or the antibodies. This mainly depends on the coating materials of the solid phases used in the technique. Simply, there are several approaches to the ELISA techniques used for the detection of the viral antigen. Figure 4 shows a summary of several types of ELISA techniques, including the double antibody

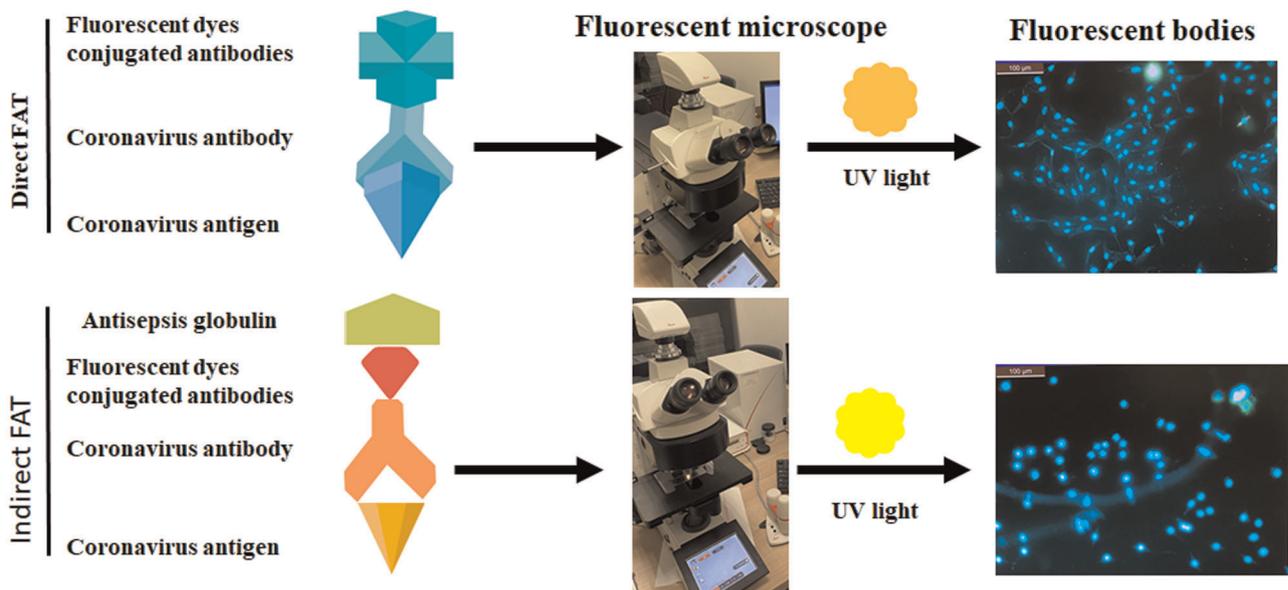


FIGURE 3 Types and applications of some common FAT techniques in the diagnosis of coronaviruses. (A) Direct FAT: first, the tissue material containing the tested antigen is fixed on slides. Second, the positive antibodies are added to these sections and allowed to react at the appropriate temperature for the appropriate time per each antigen. The fluorine conjugated dye should be added and incubated for the appropriate conditions. (B) Indirect FAT: the tested antigens are fixed on the slides. The known antibody is added and allowed to react with the fixed antigens under the appropriate conditions. The antispecies globulins are added and allowed to react with this complex for the appropriate time and conditions. The antibodies conjugated with fluorine dyes are allowed to react with this complex at appropriate conditions and time. Finally, the prepared slides from either A or B should be examined under the UV lamp of the fluorescent microscope. The positive reactions are indicated by the appearance of fluorescent granules or bodies in the examined tissues

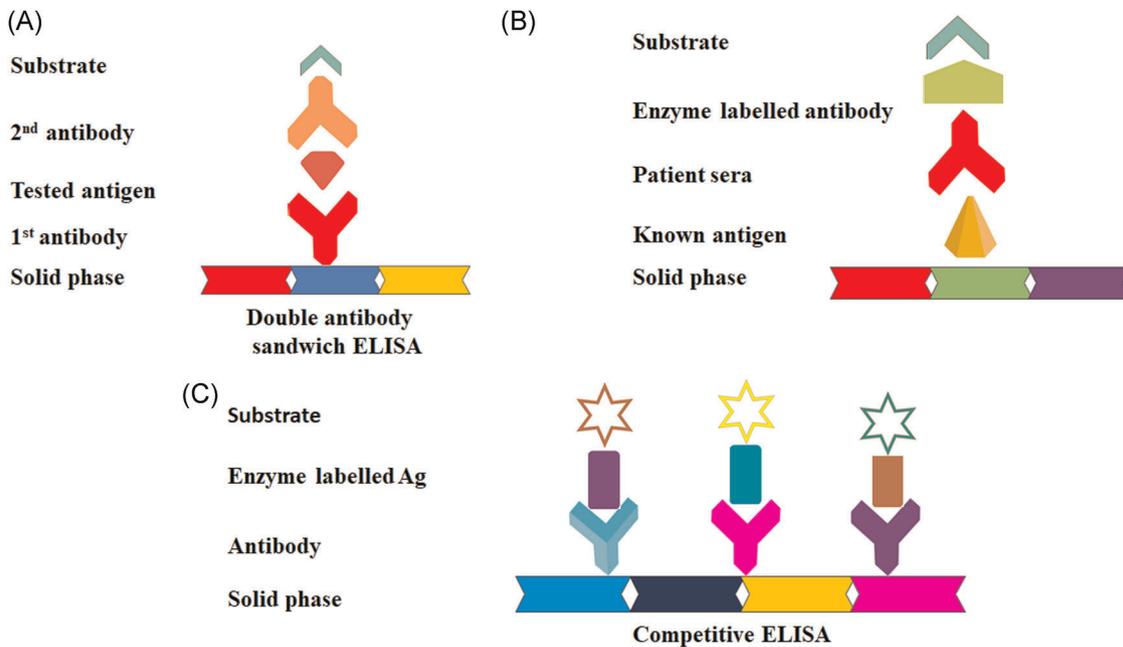


FIGURE 4 Types and applications of some common ELISA techniques in the diagnosis of some coronaviruses. Schematic diagrams of the common types of ELISA techniques used in the diagnosis of various coronaviruses. (A) Double antibody sandwich ELISA for antigen detection: A known antibody is allowed to adsorb to the solid-phase ELISA. The tested antigen is added and allowed to react at appropriate conditions and time. A second antibody specific to the antigen is allowed to react at appropriate conditions. Conjugated antibodies with enzymes showed to be added and react at appropriate conditions. The substrate is added for the standard time then the reaction stopped using the appropriate solution. The plates are read using a special spectrophotometer or ELISA reader. (B) Indirect ELISA: a known antigen is attached to the solid phase and allowed to adsorb for appropriate time and conditions. The unknown antibody is added to the plates and allowed to react at appropriate conditions. The conjugated antibodies are added and allowed to react with the mixture for appropriate time and conditions. The substrate is added and allowed to react for an appropriate time. The reaction is stopped using the stop solution. The optical densities of each reaction are read using the ELISA reader at the appropriate wavelength. (C) Competitive ELISA. The known antibodies are allowed to adsorb to the solid phase. The enzyme-labeled antigens plus the unknown antigens are allowed to react in some wells, while the enzyme-labeled antigens are added to other sets of wells. The substrate is added and allowed to react. The reactions are read at the appropriate wavelength using the ELISA reader

sandwich ELISA, the inhibition ELISA and the competitive ELISA (Figure 4A–C).

3.6 | NAT-based tests for coronaviruses

The NAT assays are widely used for diagnostic and research purposes for coronaviruses. The rationale behind these assays is the amplification of certain target regions of the coronavirus genome. The success of these assays depends on many factors, such as the selection of a unique amplification target, the collection of the representative sample at the right time, the processing of the specimens, the amplification parameters, and the interpretation of each reaction. Table 3 is showing a comparison between the classical and the recent techniques for the diagnosis of various coronaviruses, especially SARS-CoV-2.

3.7 | The RT-PCR

The RT-PCR, the real-time quantitative PCR (qRT-PCR), is the most common technique widely used not only for coronaviruses

but also for large numbers of viruses.^{7,70,71} Theoretically, the qRT-PCR technology should detect a minimal quantity of the viral-NA, even one copy of the genome.⁷² Despite the high sensitivity and specificity and broad application of the qRT-PCR in the process of coronaviruses diagnosis, this technique requires the availability of expensive real-time PCR machines, which may not be available at many laboratories or point of care settings.⁷³ Meanwhile, this technique also required high-trained personnel to experiment. This is in addition to the time required to conduct the experiment and to ship the samples from some small hospitals or clinics to be tested in a larger facility apart from the site of collection. This will increase the time required to get the test results and may negatively affect the transmission of certain pathogens in a community. Thus, there was an urgent need to develop a new generation of the NAT that can be conducted at the point of care and can be performed in cheap equipment and obtain the results in a remarkably short time. One of the main advantages of the NAB assays is the possibility of testing various type of samples collected from patients, including saliva, stool samples, nasal swabs, tissues, and even semen or vaginal secretions.⁷⁴

TABLE 3 Comparison between the newer next-generation assays over traditional methods

Technology	Advantages	Disadvantages
A: Traditional assays		
1. Clinically based diagnosis chest radiography and CT	<ul style="list-style-type: none"> • Easy to be conducted • Can examine a large number of people in a short time • Low cost of examination • Do not require expensive equipment 	<ul style="list-style-type: none"> • Low specificity • Not pathogen-specific • Require sanitation and disinfection after each use
2. EM and IEM	<ul style="list-style-type: none"> • High specificity • Fast technique • Requires purification and concentration of samples • IEM is much more specific, using a specific antibody against tested viruses 	<ul style="list-style-type: none"> • Low sensitivity • Require expensive equipment, such as the EM unit and ultracentrifuge
3. Plaque assay	<ul style="list-style-type: none"> • Accurate quantitative estimation of the virus infectivity 	<ul style="list-style-type: none"> • Requires BSL-3 lab facility in some cases • Time-consuming • Some viruses do not grow well in cell culture
4. Virus isolation	<ul style="list-style-type: none"> • Gold standard diagnostic assay • Help in preparation of virus stocks for further characterization • Help to study virus growth curve • Help in studying the viral pathogenesis and determination of virulence 	<ul style="list-style-type: none"> • Time-consuming • Require suitable host, particularly cell culture • Possibility of contamination to cell culture • Require BSL-3 lab facility in case of zoonotic viruses
5. Antigen detection assays	<ol style="list-style-type: none"> 1. Rapid tests 2. Easily conducted 3. Cheap 4. Do not require expensive equipment <ul style="list-style-type: none"> • Can be done at the point of care • Do not require well-trained personnel to be conducted 	<ul style="list-style-type: none"> • Low sensitivity • Low specificity • Possible of cross-reactivity between different antigens of
6. Antibody detection assays	<ul style="list-style-type: none"> • Cheap • Easily conducted • Do not require highly trained personnel to be conducted • Some of them are fast and can give results in a few minutes • Easily modified to be a high-throughputs as in some ELISA assays 	<ul style="list-style-type: none"> • Cross-reactivity between closely related antigens • Low sensitivity in some cases
B: NG assays		
1. NAB tests (RT-PCR and its modifications)	<ul style="list-style-type: none"> • High sensitivity • High specificity • High-throughput in some cases • Screening a large number of samples in a short time 	<ul style="list-style-type: none"> • Possibility of contamination • Requires expensive equipment and reagents • High cost • Requires validation and standardization with control positive and negative • Requires clean laboratory with some HEPA filters in most cases
2. The NGS	<ul style="list-style-type: none"> • High-throughput • Produce mega-reads • May identify some unknown and novel pathogens and new strains of known pathogens • Help in the diagnosis of none-cultured viruses 	<ul style="list-style-type: none"> • Expensive equipment • Expensive reagents and kits • Require a well-equipped laboratory for upstream sample preparation • Requires expert bioinformaticians for interpretations of the results • Require a relatively long time to get the results and bioinformatics analysis
3. LAMP	<ul style="list-style-type: none"> • Does not require expensive thermal cycle machines • Easy to perform 	<ul style="list-style-type: none"> • Designing the primers requires expert personnel • The possibility of contamination is much higher than other techniques

TABLE 3 (Continued)

Technology	Advantages	Disadvantages
	<ul style="list-style-type: none"> • Rapid amplification of multiple targets at one reaction • Can be adapted to amplify the RNAs in combination with reverse transcriptase reactions • More resistant to PCR inhibitors • Low costs of the reactions • Highly specific and sensitive technique • Can be used at the point of care or under the field conditions 	<ul style="list-style-type: none"> • Not recommended for multiplex detection approaches of several targets
4. RPA	<ul style="list-style-type: none"> • Designing the primers is simple • Fast technique, almost 20 min we can get the results • Amplification requires only a single temperature • No need for the initial heating stage • Can be conducted under field conditions • Minimize the potential human errors compared to other techniques 	<ul style="list-style-type: none"> • Relatively expensive technology • Reduce human manipulation, thus reducing the numbers of jobs in this field • Requires regular configuration
5. SHERLOCK/CRISPR	<ul style="list-style-type: none"> • High specificity and sensitivity • Fast technique (less than 5 min) • Low detection limit as one copy in 1 ul • Distinguish between two closely related pathogens, such as Dengue and ZIKA 	<ul style="list-style-type: none"> • Require high technical personnel to perform the test, especially in the RNA and protein purification • The primers are still not commercially available • Multistep reaction, which may affect the outcomes of the final reaction • Currently, not the technique of choice in the gene expression profiles

3.8 | Coronaviruses genome sequencing and the next-generation sequencing (NGS)

NGS is making a quantum leap in many aspects of research, including coronaviruses. The NGS allowed the identification of many coronaviruses for the first time during some active surveillances among some species of bats.⁷⁵ There are many applications of NGS in the field of coronavirus research. Decoding the full-length genome of some coronaviruses, particularly the MERS-CoV and SARS-CoV-2, is paving the way for the development of novel diagnostic assays, vaccines, and antiviral therapeutic.^{24,76} The NGS is playing an important role in studying the ecology and epidemiology of many coronaviruses in humans and animals.^{77,78} The NGS enables the discovery of novel coronaviruses as well as the study of the diversity among the known coronaviruses.^{1,6,58} Reporting the full-length genome sequencing of SARS-CoV was achieved in several months. This was developed through the classical cloning and sequencing of several overlapping PCR fragments encompassing the entire genome of the virus using the Sanger sequencing technology²; however, the first genome sequencing of SARS-CoV-2 was reported in few weeks after the emergence of the virus in the community.¹ This reflects the great progress in the field of genome sequencing from the Sanger method into the NGS. Currently, the NGS is being used on a large scale for monitoring any changes or single nucleotides polymorphism (SNPs) in the SARS-CoV-2 genome, which may affect the viral pathogenesis and virulence.^{8,79–82} On the other side, high-throughput sequencing of single B cells from convalescent SARS-CoV-2 patients

led to the discovery of some unique potent neutralizing antibodies against SARS-CoV-2 infections.⁷⁵ However, the current estimated costs of NGS are relatively high. It is worth doing in many cases since the output data is of great impact on our understandings of the ongoing changes in the genetic variations of the circulating variants of SARS-CoV-2 over time. Figure 5 is showing a simple diagram of the basic steps of the NGS.

3.9 | The loop-mediated isothermal amplification (LAMP) and its modifications

This technology is a new generation of colorimetric PCR-based assays. This technique and its modifications have been used extensively in the diagnosis of most human coronaviruses.^{83–88} This technology is mainly depending on a single range of temperatures during the entire reaction. It does not require a thermal cycle as used to be in the case of the regular PCR technique. This technology has many advantages over other NAT assays. It is a one-tube reaction technique characterized by high specificity and sensitivity, and could be used at the point of care or under the field conditions in the case of animals; the whole procedure can be done in a portable machine and executed in a short time and does not require sophisticated, expensive equipment.⁸⁹ Another privilege for the LAMP assays in the context of viral diagnosis is the ability to perform the test with the RNA extraction approach or without RNA extraction (directly on the sample). The latter approach is called direct RT-LAMP.⁸⁷ It is also

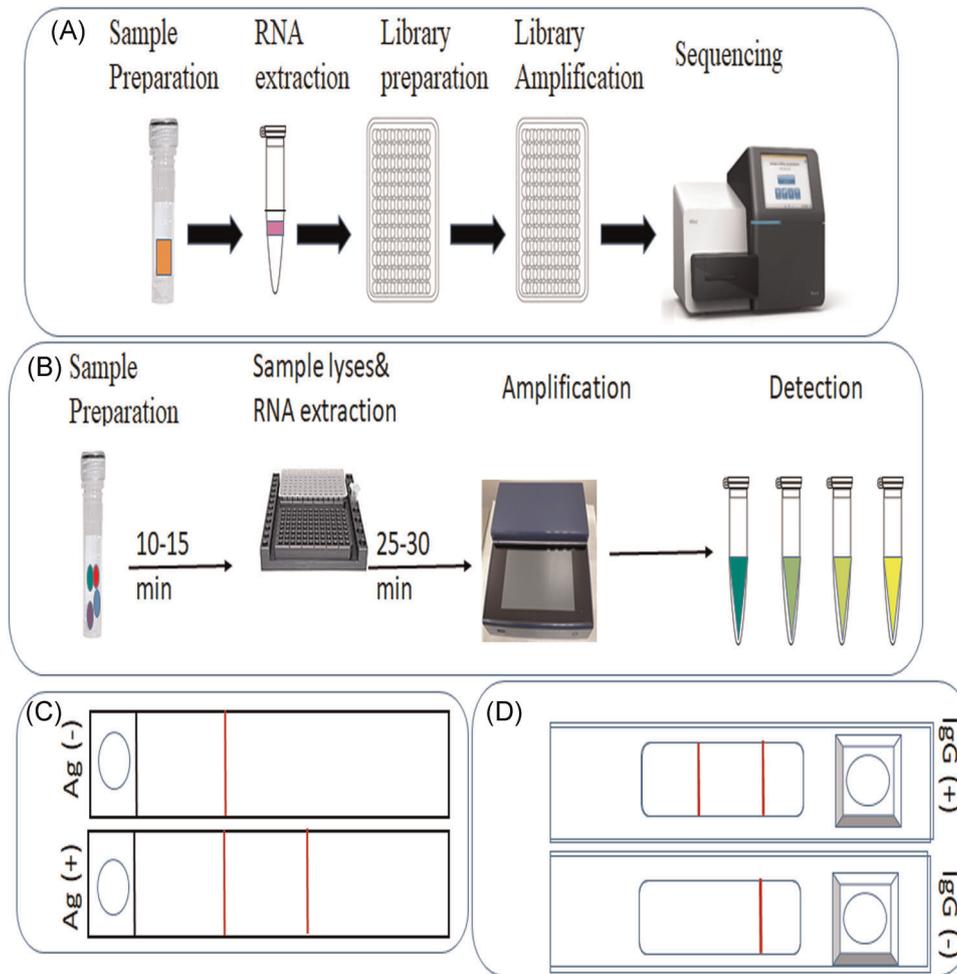


FIGURE 5 Schematic representations of some next-generation novel diagnostic assays for coronaviruses, particularly SARS-CoV-2. (A) Simple diagram showing the necessary steps for the next-generation sequencing starting from sample preparation until the development of the sequences (B) Diagram showing the basic steps of the loop-mediated isothermal amplification-polymerase chain reaction assay. (C) A simple description of the LIFA for SARS-CoV-2 antigen detection. (D) A simple representation of the GICA assay to detect the IgG for SARS-CoV-2 antibodies. The double lines in (C) and (D) indicated a positive result, while the single lines indicate a negative result

known that the performance of the PCR is usually affected by the presence of some specific inhibitors in the tested samples.⁹⁰ The high efficiency of the enzymes used in the LAMP techniques makes it relatively less affected by the presence of inhibitors.⁹¹ The detection limit of this assay is as low as 1–10 copies of the RNA molecules of the SARS-CoV-2.⁸⁷ The RT-LAMP reactions usually contain a mixture of several chemicals and enzymes, such as the high thermal tolerance DNA polymerase enzyme used in the regular PCR techniques, a set of up to six specific primers, and aliquots of the RNA of the tested samples. This reaction is usually exposed to a fixed temperature for 20 min at 65°C before the actual reaction.⁹² These are colorimetric reactions using color indicators, such as phenol red, which turns from red to yellow after 30 min of incubation. The intensity of this color (directly proportional to the concentration of the RNA in the tested samples) can be measured by the regular spectrophotometer or even a cell phone camera using special software (Figure 5B).⁹² This RT-LAMP showed higher specificity and sensitivity in the detection of the SARS-CoV-2 in clinical specimens compared to the real-time PCR

assays.⁹² This assay can detect the positive samples up to ($C_t = 30$) in the case of the real-time PCR technique. The same study developed a new version of the RT-LAMP assay called LAMP sequencing.⁹² This modified version of the test sequence the product of the regular RT-LAMP assay.⁹¹ Another study recently developed a LAMP-RT-PCR technique to detect and differentiate between the SARS-CoV-2 and the other coronaviruses, such as 229E, OC43, NL63, and MERS-CoV, as well as a large panel of respiratory viruses of human.⁸⁶

3.10 | The recombinase polymerase amplification (RPA)

The RPA is one of the most sensitive, accurate, and fast techniques mainly based on the isothermal amplification of the target DNA in almost 20 min under a temperature range of 37°C–42°C. This technique has a minimal detection limit of detection of the target DA up to 1–10 copies of the target DNA.⁹³ It has been used in the

detection of various classes of NAT molecules, such as the miRNAs, single-stranded DNA, double-stranded DNA, and single-stranded RNA molecules of various pathogens.⁹³ This approach has several modifications, such as the end-point lateral flow strips as well as the real-time fluorescent detection assay. The RPA has recently been used extensively in the diagnosis of COVID-19 patients.^{73,94,95} Another modification of the RPA is the nest-RPA, which has recently developed for the diagnosis of COVID-19 patients.⁹⁶ In this approach, two subsequent reactions, including amplification of a flanking region of a target sequence, then the nest reaction is usually conducted using internal primers inside the flanking region.⁹⁶ The high accuracy and sensitivity of this modified technique can be used to assess and give conclusive evidence about the possibility of discharging a COVID-19 patient from the intensive care unit.⁹⁶

3.11 | The reverse-transcriptase enzymatic recombinase amplification (RT-ERA)

This technique is a new version of RPA, which depends on the constant temperature for the amplification of some target regions within the viral RNAs.⁷³ This technique has a great privilege to be done in the absence of expensive thermal cyclers machines. The reaction can be done using several enzymes and two primers targeting one region within the SARS-CoV-2-N gene and another primer within the SARS-CoV-2-S gene. This novel technique has a very low detection limit, even as low as one copy of the viral genome, which enables the detection of very low concentrations of the virus in different samples. Thus, this technique can be done under the field conditions at the point-of-care settings as well as can be considered as a household-deployable technique.

3.12 | The CRISPR-based assays and their modifications

This approach mainly has the precision power of the CRISPR Cas12a/g RNA complex and the fluorescent probes to detect the amplicons of the regular RT-PCR or the LAMP assays. There are several versions of this approach, such as CRISPR-Cas12, the CRISPR-based DETECTOR Lateral Flow assay, the high-throughput CRISPR-p, and the CRISPR-based Fluorescent (CRISPR-FDS).⁹⁷⁻⁹⁹ Most of these assays and techniques are usually conducted in two consecutive steps—first, a preamplification RNA step. This is a very crucial step for the production of a large number of copies of the template flanking the target amplification region. The CRISPR-FDS usually requires at least two copies of the genome to give a high yield for the amplicons. The preamplification step through the RT-PCR or RPA, lowering the minimal detection limit of the CRISPR-FDS to be as few as two copies of the target RNAs.⁹⁹

Second, applying the CRISPR/Cas13a enzyme activity to target a specific sequence within the viral RNAs. The technique can be conducted in about 50 min and has a detection limit of as little as two

copies of the viral RNA in the given sample. This approach has been recently used for the rapid, sensitive, and accurate diagnosis of SARS-CoV-2.¹⁰⁰

3.13 | The specific high sensitivity enzymatic reporter UnLOCKing (SHERLOCK) assay

This assay can be conducted in three subsequent steps in less than an hour. The first step is the isothermal incubation of the extracted RNAs from the collected samples for 20 min. The second step is another incubation for the action of the RPA for 30 min. The final step is another incubation for the colorimetric visualization of the results for at least 2 min enhanced by the action of the Cas-12 or Cas-13 (Kellner et al., 2019). This technique provides a new cheap promising assay for the detection of the SARS-CoV-2 in some laboratory facilities with limited resources.¹⁰¹

3.14 | Detection and titration of coronavirus antibodies

Several ELISA techniques could also be used for the detection and titration of the coronavirus antibodies in the sera of infected or tested individuals. These techniques include the indirect ELISA, the antibody class captured ELISA, and the double-antigen sandwich ELISA.

3.15 | The commercial and recombinant ELISAs

Detection of the coronavirus-specific antibodies is the second side of the coin in the context of coronavirus diagnosis. There are many serological techniques used to detect and titrate the coronavirus antibodies in the affected host. Both the indirect ELISA and the antibody class capture ELISA were used to detect and titrate the antibodies against coronaviruses in serum samples. In the indirect ELISA, a known antigen is usually attached to the solid phase then a serial dilution of the tested sera should be added to these antigens. Conjugated antibodies are allowed to react with the tested sera, then a substrate should be then incubated, and the reaction should be then read. The intensity of the optical densities of each sample is directly proportional to the concentration of the antibody in each sample. The other type of ELISA is used to measure the concentration of various classes of the immunoglobulins, particularly (anti-IgA, anti-IgM, and anti-IgG). The newly developed ELISAs for the SARS-CoV-2 detected the prevalence of the viral-specific IgA, IgG, and IgM antibodies during viral infection.¹⁰² Based on the results of this study, both IgA and IgM were detected up to 5 days after the onset of the infection, while the IgG was detectable up to 14 days after the onset of the clinical symptoms in the affected patients.¹⁰² The combination of the results for both the IgM-ELISA and the PCR results increased the sensitivity of the patient's detection rates up to 98.6%.¹⁰² The recombinant ELISA was

also used to detect the MERS-CoV antibodies in the sera of dromedary camel, their herdsmen, veterinarians, and slaughterhouse employees.^{103,104} These recombinant ELISAs should be prepared from specific conformational epitopes within the highly conserved regions of the genomes across various groups of coronaviruses. Selection of these conserved regions will avoid any potential mutations, which may affect the performance of the developed assays in the detection of a wide range of coronaviruses strains, clades, and subclades. This recombinant DNA technology will allow the upgrading of these ELISAs in case of any significant alteration over the coronavirus genomes, affecting the production of the target proteins. That is why this is a promising trend in the field of coronavirus diagnosis and should be taken into consideration in the new generation of coronavirus diagnostic assays. The double antigen sandwich ELISA was used previously to detect SARS-CoV.¹⁰⁵ In this approach, a fragment of recombinant SARS-CoV protein was used successfully to test the seroprevalence of some clinical samples for the presence of SARS-CoV antibodies.¹⁰⁵

3.16 | The protein-based assays for serodiagnosis of coronaviruses

The protein-based assays have been developed to enable the detection of some coronaviruses in the sera of humans and animals.^{106,107} An earlier study developed a recombinant-based SARS-CoV-N recombinant ELISA that had high specificity and sensitivity for the detection of antibodies against SARS-CoV.¹⁰⁷ One study developed a novel MERS-CoV-S1-based assay, mainly targeting two different regions across the MERS-CoV-S gene. The first is designed to target the receptor-binding domains that enable the viral attachment to its cellular receptors, the dipeptidyl peptidase-4 (DPP4), and the second one is designed to target the N-terminal domains of the MERS-CoV-S1 gene.¹⁰⁶ The assay that used the first approach showed high specificity for the detection of MERS-CoV antibodies and showed a high degree of consistency to the PPNT discussed earlier for the detection of MERS-CoV neutralizing antibodies.^{106,108}

3.17 | The immunofluorescent assay (IFA)

The whole-virus-based IFA was extensively used as a standard gold technique in the diagnosis of SARS-CoV epidemics during 2003. Some studies reported the development of a novel recombinant IFA assay. This study mapped and designed two important epitopes within the SARS-CoV-S, and N proteins then used them to develop IFA-based assays that can be conducted in BSL-2 laboratories.¹⁰⁹ The main advantage of the newly developed assay was its safety, which does not require the handling or manipulation of the SARS-CoV.¹⁰⁹ Those assays showed similar sensitivity and specificity to that of the whole-virus-based IFA assay. Meanwhile, the IFA techniques were used to study the molecular pathogenesis and intracellular localization of some other coronaviruses, such as MERS-CoV.¹¹⁰ Some other

studies compared the performance of some ELISAs and IFAs techniques in the detection of the SARS-CoV-2-IgG antibodies in the moderate and severe cases of infections during 10–18 days postinfection.¹¹¹

3.18 | Lateral flow immunoassays (LFIA)

Initially, the LFIA was used to detect several biomarkers, especially cortisol in the saliva, with great success.¹¹² Using the LFIA, saliva was an ideal biological sample when fast results are needed compared to other biological fluids, such as urine, tears, and blood.¹¹² This approach was designed to detect either the viral antigen or its antibodies by using portable strips or dipstick, which carry the required reagents to conduct the assay.¹⁰⁰ Usually, one drop of human plasma is placed on a strip containing monoclonal antibodies (mAb) against SARS-CoV-2. If the patient were exposed to an active SARS-CoV-2 infection, the mAbs would recognize the counter antigen, and the reaction is developed.¹⁰⁰ Although this technique is very simple, it requires the preparation of some expensive mAb; meanwhile, this technique requires validation by using positive SARS-CoV-2-infected blood. Meanwhile, the LFIA can be used to detect the antigens in the saliva and the secretions of the URT of the infected patients.¹¹³ Saliva is proved to be one of the more convenient samples to be collected for screening a large number of people in short time. It is very simple and easy to collect, store, and transfer to the laboratory. Meanwhile, saliva collection is a noninvasive technique, which does not harm the patient during the collection compared to other samples, such as the bronchoalveolar lavage and the nasopharyngeal swabs.¹¹³

3.19 | Luciferase immunoprecipitation system (LIPS) assay

The LIP assay is a new technique based on the luciferase and the immunoprecipitation activities in the detection of many viruses, such as RSV as well as SARS-CoV-2 antibodies.^{114,115} It is a sensitive technique used for the quantitative estimation of antibodies against the SARS-CoV-2-N and S proteins.¹¹⁵ One of the privileges of this approach is its ability to detect the epitopes in their native conformational form as well as the linear epitopes.¹¹⁶ Another privilege that the LIPs assay provides is the detection of the immunological profile of various classes of SARS-CoV-2 infected people with various clinical outcomes.¹¹⁶

3.20 | The gold-immunochromatography assay (GICA)

The colloidal gold Immunochromatography assay has been used for the detection of many pathogens, including bacteria, parasites, and viruses, in addition to many other hormones in the milk or sera.^{117–120}

The GICA was showing a promising trend in the diagnosis of some high influenza virus strains, including influenza type B and some of the highly pathogenic influenza subtypes, such as H7N7.^{121,122} The GIA has many advantages as a rapid, sensitive technique that do not require sophisticated, expensive types of equipment or highly trained personnel to conduct it. Meanwhile, it may be one of the simple diagnostic essays that could be conducted under the field conditions and at the point of care settings. It may also be a suitable technique for the screening of a large number of individuals or samples during an epidemic.¹²² Several research groups developed similar GICA diagnostic assays for the rapid testing and initial screening of the COVID-19 patients infected with SARS-CoV-2.¹²³⁻¹²⁵ One of these studies concluded that the GICA detection for the anti-SARS-CoV-IgG and IgM is one of the great assets in the diagnosis of COVID-19 patients.¹²⁵ Another study suggested the GICA assays for those antibody classes could be a complementary diagnostic tool to the RT-PCR, especially in the case of delayed IgM antibody response in some COVID-19 patients (Figure 5).¹²³ The GICA was used successfully in combination with urea dissociation to overcome the high false-positive results of the original GICA in the case of the overexpression of the IgM in some instances.¹²⁴

3.21 | The microneutralization and the pseudo-viral particle neutralization (PPNT) assays

The neutralization assay still is one of the gold standard techniques for the diagnosis of many viruses, such as metapneumovirus, respiratory syncytial virus as well as many coronaviruses, including MERS-CoV and SARS-CoV-2.^{108,126-128} There are several versions of this assay currently in use for the serodiagnosis of some coronaviruses, including the plaque reduction neutralization tests (PRNT), the conventional microneutralization assays (MN), and the PPNT.^{108,126,128} These versions of the neutralization assays have more superior results than the commercial coronaviruses-S1 and N-based ELISAs.¹²⁹ One potential explanation is that the commercial ELISAs may fail to exclude the cross antigenic relationship among the closely related coronaviruses. Some studies showed the cross-reactivity of both the bovine coronavirus (BCoV) and the MERS-CoV in dromedary camels.¹²⁸ Usually, screening of sera for the presence of antibodies against coronaviruses is commonly done first by the commercial ELISA then confirmed by the microneutralization test as per the CDC guidelines for the diagnosis of MERS-CoV.¹³⁰ Typically, pseudoviruses are recently used in the diagnosis of various emerging and re-emerging diseases. This is due to their safety, flexibility, and the possibility of handling them in biosafety level-2 laboratories (BSL-2). A recent study developed a new version of the PPNT that can be used for the diagnosis of SARS-CoV-2 in a biosafety level-2 (BSL2) contaminant laboratory instead of BSL3.¹²⁶ The same study used the vesicular stomatitis virus (VSV) pseudovirus to detect the SARS-CoV-2 antibodies in the sera of tested individuals by the pseudo-virus-based neutralization assay.¹²⁶ This technique will pave the way for much research on SARS-CoV, particularly those related

to the vaccine, antiviral therapy against SARS-CoV-2 in many laboratories around the world that do not have BSL-3 laboratory facilities.

3.22 | Pitfalls of the current coronaviruses diagnostic assays

3.22.1 | Pitfalls related to the NAT detection assays

Most coronavirus NATs-based assays are prone to contamination. The entire laboratory testing starting from sampling until the data interpretations should be done at the complete sterile conditions. This to avoid any potential contamination of samples during the various stages of testing.¹³¹ There are several pitfalls related to the NAT detection methods, such as sample type, adequate collection period, the extraction process, problems during the collection of swabs, such as bleeding or coughing and sneezing. All these factors may affect the outcomes of NAT, such as the RT-PCR, the real-time PCR, and many other NAT detection techniques. Several governmental health authorities established some interim guidelines protocols for testing various clinical specimens for SARS-CoV-2.¹³² These guidelines are updated regularly to ensure the accuracy and safe testing of infected patients.¹³² It is well established that the high temperature has a deleterious effect on the viral NATs, which may affect the results of the downstream testing. This theory is valid for all viruses, including coronaviruses. Some recent research studied the effect of various temperatures, including the room temperature of sample processing and the temperature of sample transportation.¹¹³ High temperature during the processing of swabs collected from COVID-19 patients was responsible for at least 10% false-negative results. This may be attributed to the RNA degradation in the sample, which leads to the low cycle threshold of some samples and render the test negative.¹¹³ This study highlighted the mandate of immediate testing for the collected samples from COVID-19 patients or at least low-temperature preservation of samples in the case of transportation from remote regions to central facilities. This to ensure the intact integrity of the RNAs of the tested specimens.

4 | PITFALLS RELATED TO THE ANTIBODIES RELATED ASSAYS

Although serological assays are really important in the diagnosis and epidemiological studies of many coronaviruses, they do have several pitfalls that require fine-tuning to achieve maximum performance.¹³³ The problem is serological techniques are usually used to measure the immune response, not the causative agent/s. Thus, we cannot rely on a single serum sample to assess the disease condition of some patients or diseased animals.¹³⁴ The more practical way is to test two serum samples collected during the acute febrile stage of the diseases and another sample collected about four weeks later.

If the antibody level in the second (convalescent) sample is greater than the first (acute) sample, this considers an infection with the pathogen under study. This is the common concept for seroconversion, not only for coronaviruses but also for other pathogens.¹³⁵ Interestingly, the degree of seroconversion in the case of SARS-CoV-2 infection varies among the affected patients based on the severity of the virus infection.¹³⁶ Patients with a severe infection have more seroconversion than those with an asymptomatic form of infection.¹³⁶ Thus, inconsistency in the level of the seroconversion displayed by different patients may be attributed to many factors, including the genetic factors of each patient, the previous exposure history to the same coronavirus or closely related virus, and many unidentified factors.¹³⁶ Both the seroconversion and high viral loads and shedding have been recently reported in some patients during an outbreak in the Diamond cruise ship.¹³⁷ This is considered one of the major concerns regarding seroconversion in the context of COVID-19 patients. The patients may be asymptomatic and seroconvert while they are still shedding the virus in high amounts. Thus, we cannot rely on seroconversion alone as a marker for the complete recovery of COVID-19 patients. To overcome this seroconversion problem, an association between the seroconversion and RT-PCR testing or any other NAB assay negative could be considered before discharging the patients from the hospital or even ending a self-quarantine. One of the main weak points of the commercially available serological techniques, especially the ELISA, is the cross-reactivity of most coronaviruses. The presence of this cross will hamper the success of these techniques in the evaluation of the immune status of individuals against specific coronavirus candidates. This cross-reactivity was observed between various coronaviruses, such as MERS-CoV, which infect dromedary camels, and the BCoV.¹²⁸ To overcome the problem of cross-reactivity, the development of very specific serological techniques for each coronavirus candidate should be adopted. Both the micro-neutralization and the PPNT assays were used to overcome the problem of the cross-reactivity of various coronaviruses.^{103,108,128} Further studies are required to improve the performance of various serological techniques and improve their performance in the field of coronaviruses research and diagnosis.

4.1 | Pitfalls related to the antigen detection-related assays

The most common theme of the coronavirus antigen detection assays is the application of known and specific antibodies against the tested antigen. The cross-reactivity among human coronaviruses was reported on many occasions.^{138,139} In many techniques, the used antibodies are polyclonal; thus, they may pick up several antigens of closely related viruses^{138,139} that may hamper both the specificity and sensitivity of these antigen-based assays. To overcome this problem, the known antibody used in these assays should be produced against a single producing clone or monoclonal antibody against the unknown antigen. Sometimes improper sampling techniques may be reflected in the outcomes of the antigen detection

tests. A low concentration of the viral antigen in the tested sample may be prone to a false-negative.

4.2 | The next generation's coronavirus diagnostic assays (N-CoV-DA)

Based on the frequent emergence of new coronavirus candidates, the N-CoV-DA has to cope-up with this diverse group of viruses. There should be some novel next-generation diagnostic assays for coronaviruses. These assays should provide new features that do not exist or are weakly presented in the current techniques.

4.3 | Simultaneous detection of coronaviruses

Nowadays, there is an urgent need for the development of a diagnostic assay that enables healthcare workers to do simultaneous detection of a large number of respiratory viruses in a short time. It is crucial to identify the causative virus of the respiratory syndrome, either belongs to the low pathogenic coronaviruses, influenza viruses, or the highly pathogenic coronaviruses or influenza viruses.¹³⁹ This will help in the selection of the downstream treatment protocols. Several multiplex reactions were developed in the past to do simultaneous detection of several pathogens in one reaction by the multiplex PCR technique. However, the multiplex PCR is a laborious technique that requires careful design of the primers of targets in addition to laborious optimization of all the parameters and conditions that render the reactions successful. There are seven well-known human coronaviruses.⁴⁹ Some of them produce mild to moderate course of infection in humans, such as HCoV-229E, HCoV-OC43, HCoV-NL-63, and HCoV-HKU-1. Patients of these four candidates may recover spontaneously or require short-term healthcare.¹⁴⁰ However, infection with other coronaviruses, such as SARS-CoV, MERS-CoV, and SARS-CoV-2, require excellent medical attention and care in many cases.^{140,141} Rapid identification of the causative coronavirus and its strains will have a significant impact on the possible and reliable intervention as well as may save more lives in case of severe infection with some of these pathogens. An earlier study conducted in 2007 developed a new technology to do simultaneous detection of a large number of respiratory pathogens, including the most common strains of influenza viruses type A and B, the SARS-CoV, the RSV, and others based on the coupling of the real-time PCR products with the DNA microarray system.¹⁴² This approach enabled the clinicians to screen a large number of viral pathogens in one reaction and allowing the picking up the uncommon causes of viral infections.¹⁴² In the new generation of laboratory techniques, the seven human coronaviruses should be included in the respiratory viral panels parallel to other respiratory pathogens, such as various types of influenza viruses; metapneumovirus, RSV; and many other viruses, causing respiratory infections for testing patients admitted to hospitals with clinical respiratory manifestations. Thus, there is an urgent need for some tests that may help in the

simultaneous testing of one sample against a large number of viral candidates. Several techniques enable the simultaneous detection of multiple targets on one sample. A promising approach used the automated multianalyte point-of-care *mari*POC for the simultaneous detection of a large panel of respiratory viruses affecting humans.¹⁴³ This approach may be upgraded to include many respiratory pathogens, including the seven human coronaviruses, including targets for their clades and subclades. The microfluidic-chip-based was recently used to develop a diagnostic assay for the detection of four strains of Ebolavirus.¹⁴⁴ This approach can be easily adapted for the simultaneous detection of the seven human coronaviruses in one sample. Another recent approach is to development of microfluidic-based immunoassays, which may be able to detect multiple viral antigens simultaneously. A recent study developed a new assay based on this technology that enables the simultaneous detection of the IgG/IgM/Antigen of SARS-CoV-2 in one reaction.¹⁴⁵ This assay has many advantages besides the simultaneous detection of several targets in one reaction; it can be done on one of the most convenient samples, including saliva of tested patients, the results can be obtained in less than 15 min. This assay showed high specificity and sensitivity, which may be used for monitoring the progression of the SARS-CoV-2 course of infection and treatment in the affected patients.

4.4 | Multiplex detection of coronaviruses antibodies in the sera of infected patients

As there is a growing number of coronavirus due to the emergence of new candidates, there is a mandate for the screening of one serum sample for a large number of coronavirus targets in one reaction simultaneously. This will have a high impact on monitoring the dynamics and distribution of coronaviruses in specific communities. Meanwhile, it will help in the monitoring and assessment of the immune status of certain individuals for the seven human coronaviruses in one reaction. Before the emergence of SARS-CoV-2, one study developed a multiplexed magnetic microsphere immunoassay to detect the known human coronavirus antibodies.¹⁴⁶ This study showed great success in the simultaneous and multiplex detection of the antibodies against the six known human coronaviruses under study. One of the most promising approaches is the programmable bead arrays. This approach allows the simultaneous detection of up to 100 targets in one reaction.^{11,147} Another approach is the competitive Luminex immunoassays (cLIAs). This approach showed promising trends in the simultaneous detection and monitoring of the immune status of animals against the closely related viruses and their serotypes, such as the foot and mouth diseases virus (FMDV) and the VSV.¹⁴⁸ Both FMDV and VSV belong to different groups of viruses from coronaviruses. Fostering this technology will have a great impact on the identification of the exact coronavirus in a single reaction despite there are four human coronaviruses responsible for the common cold (HCoV-229E, HCoV-OC43, HCoV-NL-63, and HCoV-HKU1)¹⁴⁰ in most of the cases, while three coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2)⁴⁹

cause severe cases in many cases and requires excellent attention and healthcare management. Thus, the cLIAs will help the healthcare workers and decision-makers to take the right action and management protocols based on the type of the identified coronavirus. Some studies reported the development of two recombinant-based ELISAs for serological diagnosis of turkey coronaviruses (TCoV).^{7,149} These techniques are based on the expression of the TCoV-N¹⁴⁹ and the RBD of the TCoV-S¹⁵⁰ proteins. Although both ELISAs showed great success in the detection of specific TCoV antibodies, the TCoV-S based ELISA remained to be much more specific than the TCoV-N-ELISA.^{149,150} One possible explanation is that the N gene is highly conserved among most of the coronaviruses' cross-reactivity to other closely related coronaviruses, such as the infectious bronchitis virus of chicken, may be expected.^{149,150} In agreement with the previously discussed performance of various recombinant ELISAs, one recent study compared the performance of four recombinant-based serological assays (N and RBD-S proteins) to distinguish between SARS-CoV and SARS-CoV-2 in patient sera.¹⁵¹ The recombinant-based N-based techniques showed cross-reactivity between the two viruses in contrast to the S1 and RBD-based ELISAs.¹⁵¹ Based on these observations, using the right expressed proteins to develop the recombinant-based diagnostic assay could affect the specificity and sensitivity of these techniques; thus the cross-reactivity among closely related viruses could be avoided or at least minimized to the lowest levels.

4.5 | The high-throughput-based techniques

In the case of any epidemics or the current SARS-CoV-2 pandemic, there will be an urgent need for the development of high-throughput machines and assays that enable the handling and processing of a large number of samples in a short time. This technology will help the healthcare providers and decision-makers to take the rapid right action/s to slow down the spread of infection within a specific community. Several companies developed some new technology to do a screening of a large number of samples in a short time, such as the xMAP technology.¹⁵² Some respiratory and enteric viral panels may be developed to include the seven human coronaviruses.⁸⁴ This approach will also help in mentoring the disease exposure history and the immune status of a large population in a remarkably short time. The full-length genome sequences are among the technology that revolutionized the field of coronavirus research.^{76,153} During the emergence of SARS-CoV in 2002, it took several months to report the first full-length genome sequences.² The emergence of the NGS enables the scientist to decode the full-length genome sequences of the novel coronavirus (SARS-CoV) in a remarkably short time.¹ The continuous advances in the field of the NGS and the development of high-throughput sequencing technology will speed up the research on coronaviruses from different aspects. The fluorescence-based neutralization assay has been developed to detect the SARS-CoV antibodies in the sera of infected patients, which proved to be highly specific and sensitive compared to the plaque

reduction test.¹⁵⁴ This technology provides a robust, rapid, sensitive, and specific technique for screening a large number of samples for the SARS-CoV-2 antibodies.¹⁵⁴ On the other hand, the high-yield virtual screening was recently used to test a large number of compounds as antiviral therapy. This study identified several potential compounds that may help in the inhibition of several critical enzymes for the SARS-CoV-2 replication.¹⁵⁵

4.6 | Coronaviruses marker assays and the differentiating of the infected and the vaccinated individuals (DIVI) concept

The DIVA is an important concept used in veterinary medicine to differentiate between the infected and vaccinated animals or birds using a single serum sample. This concept is being used successfully for many viruses or pathogens, such as the highly pathogenic avian influenza, the bluetongue virus, the porcine reproductive and respiratory syndrome virus (PRRSV), the FMDV, and the pest des peste ruminants virus.^{129,156,157} This approach was developed for a certain type of vaccine called marker vaccines. The key factor in this concept is the ability to detect certain proteins in the case of infected individuals or animals. This protein is absent in the sera of the vaccinated groups. Meanwhile, this combination of the recombinant vaccine and its associated marker diagnostic assays was successful in the eradication of some viruses in the past, particularly the Aujeszky's disease in swine species.¹⁵⁸ In this approach, some deletion mutant vaccines were developed, lacking the envelope protein and thymidine kinase protein. Sera of animals containing antibodies against these proteins indicated that these antibodies are due to exposure to the natural infection, not vaccination. A similar approach was quite helpful for the eradication of the infectious bovine rhinotracheitis virus in some cattle populations.¹⁵⁹ Another example of a licensed DIVA concept-based vaccine and marker assay was developed for the classical swine fever virus (CSF) in pigs. In this model, a recombinant E2-subunit vaccine was expressed using the baculovirus expression system.¹⁶⁰ The E2 gene is one of the most important genes in the CSF, which triggers the production of neutralizing antibodies against the virus.¹⁶¹ This approach is used to distinguish between the infected and vaccinated animals with CSF. This approach contributed substantially to the control and eradication of the virus in certain regions.¹⁶⁰ Although there is no current licensed vaccine against any human coronaviruses until now, there are many vaccine candidates in the pipelines for many human coronavirus candidates, especially MERS-CoV and SARS-CoV-2. When these vaccines are administered on a large scale to humans, there will be detectable antibody responses in the sera of vaccinated people. This will hamper the interpretations of various serological assays. This may lead to confusion whether the detected antibodies in the sera of tested people were produced in response to active viral infection or due to the administered vaccines. Thus, there will be a mandate to differentiate between the infected and vaccinated people using a single serum sample. Some of the new generations of the coronavirus

serological assays should take into consideration the DIVI in single serum samples. The DIVA concept was successful in the case of some of the members of *Nidovirales*, affecting pigs, especially the PRRSV (cousin virus to the coronaviruses belong to the family *Arteriviridae*).^{157,162} In this approach, a marker DIVA-based vaccine was developed after the mapping of some potent epitopes within the PRRSV-M gene called M201.¹⁶² I believe this approach is applicable in other coronaviruses, including MERS-CoV and SARS-CoV-2. Thus, the next generation of vaccines against these viruses should consider these DIVA/marker assays, which proved a promising trend in the control and eradication of other viruses in the past.

4.7 | Factors hampering the success of some coronaviruses diagnostic assays

Some significant challenges are facing the success of coronavirus laboratory diagnostic techniques. These factors may include several potential factors, such as the poor-proof reading capabilities of their RNA polymerases¹⁶³⁻¹⁶⁵ and the possibility of recombination between various strains of coronaviruses, resulting in the emergence of new strains of the virus.^{166,167} These maybe some of the reasons for frequent changes among the viral genomes of coronaviruses, the improper procedures of sampling, the timing of the sample collection.

5 | CONTINUOUS EVOLUTION OF THE CORONAVIRUSES ON THE GENOMIC LEVELS

Most diagnostic assays, especially those based on the NAT-based approaches or those based on the recombinant proteins, depend on specific sequences across the coronavirus genomes. The continuous mutations and changes of the coronavirus genomes render some diagnostic assays outdated. Thus, frequent monitoring of the coronaviruses on their genomic levels is highly suggested, and to upgrade the diagnostic tests especially those NAB assays to match the circulating strains. The coronaviruses' NAT based on specific primers and probes should be prepared based on the most conserved regions of the genomes. Nevertheless, regular assessment of the specificity and sensitivity of these assays should be practiced. The same concept should be adopted from any upcoming recombinant vaccines. These potential coronavirus vaccines should be prepared from the closely related circulating strains of the target coronavirus. This approach is the widely used strategy for the preparation of seasonal influenza virus vaccines.¹⁶⁸

6 | IMPROPER SAMPLING PROTOCOLS

The proper sampling procedure and technique are the milestones for the diagnosis of coronaviruses. The WHO and the CDC provided detailed interim guidelines for the collection of samples from COVID-19 patients.¹⁶⁹ There are many types of suggested samples

(nasopharyngeal, oropharyngeal, nasal midturbinate swab, the nasal swab, and the nasopharyngeal wash/aspirate) for the diagnosis of respiratory viruses in general, especially the SARS-CoV-2. These samples should be collected under complete medical supervision. Each type of sample has a standard protocol, as suggested earlier.¹⁶⁹ Failure to apply the guidelines per each sample may result in false-negative for the downstream testing. One common error is the collection of the nasal swabs by just touching the nasal mucosa of the tested individual. Ideally, the nasal swabs should be introduced deep in the nostrils to touch the right mucosal surface, where a high concentration of the virus is present. This will allow the cotton piece of the swab to be soaked into the nasal fluids and come in close contact with the target mucosa in the nostril or the nasal turbinate where the tropism of the virus exists.¹⁷⁰⁻¹⁷² Another alternative is to select some proper, easily collected samples. The best example in this context in the case of the SARS-CoV-2 is using saliva for the easy and rapid detection of the viral RNAs. Saliva representing one of the most convenient and reliable samples for the rapid and accurate diagnosis of the SARS-CoV-2 infection compared to other samples, such as the nasopharyngeal swabs.¹⁷³⁻¹⁷⁵ High viral load was reported in the SARS-CoV-2 patients.¹⁷⁶ Saliva could be used as the sample of choice to monitor the progress of the viral infection as well as the success of some treatment protocols in the infected SARS-CoV-2 patients.¹⁷⁷ Besides the simplicity in the collection of saliva from infected patients, we can detect the live virus particles, the viral RNAs, as well as viral-specific antibodies.^{178,179} It may also be suitable for the identification of some new biomarkers specific for coronavirus infection; this is in addition to the possibility of detection of other immune response-related markers.¹⁷⁷ The procedure of saliva collection is much easier, convenient, and safer, and less invasive than the collection of other samples, particularly the OPS. The procedure of the latter samples may result in bleeding during the collection, induction of coughing, and sneezing of the tested patient, which may result in the generation of aerosol rich with virus particles. This may pose a high risk of infection to the medical staff or doctors collecting these types of samples. Recent studies showed the levels of IgM, IgA, and IgG in both the saliva and serum are consistent in SARS-CoV-2 patients.¹⁷⁸ These observations worth great attention to consider saliva as one of the best samples for the diagnosis of some coronaviruses, particularly the SARS-CoV-2.

7 | TIMING OF SAMPLE COLLECTION

The timing of the coronavirus sample collection is a very crucial step in the success of some diagnostic assays. The course of the viral shedding of most coronaviruses was studied in more detail.^{180,181} This provides essential information about the right time for the sample collection and when certain coronavirus reaches their peaks of shedding in the affected patient. There is a noticeable difference in the viral tropism and the curve of the viral shedding in the infected patients of the SARS-CoV, MERS-CoV, and SARS-CoV-2.¹⁸² Both SAR-CoV and MERS-CoV have cellular tropism in the LRT with

minimal viral presence in the upper airways of the infected patients.¹⁸² This is in contrast to the SARS-CoV, which showed tropism in the URT with high viral loads detected during the first 5 days after infection. The curve of the viral shedding in the case of SARS-CoV-2 starts to decline after 5-6 days from the initial admission of the patients to the hospital. This observation was confirmed by several groups from China and Hong Kong. This warns to the mandate for the early detection of SARS-CoV-2 patients and tracing its contact to minimize the virus spread among the certain community.^{183,184} Some other studies showed the curve of the SARS-CoV shedding detected at 6 days postinfection (dpi) and reached its peak around 12-14 dpi by the RT-PCR technique.¹⁸⁵ Testing these patients up to 5 dpi may show false-negative results. This may be attributed to the low viral loads in the tested samples.

8 | THE TYPE OF THE TESTED SAMPLES

Coronaviruses, in general, produce a large scale of clinical syndromes in different hosts. Some viruses cause nervous manifestations, others cause enteric syndromes, and large numbers produce respiratory signs in animals and birds. Meanwhile, some coronavirus candidates are multitropic viruses that can infect many tissues and cause pathology in many-body systems. The main theme of the seven human coronavirus candidates is the production of respiratory manifestations in the affected patients. However, the newly emerged MERS-CoV and SARS-CoV-2 can cause multiorgan pathology and failure.^{181,186,187} Ideally, sample collection should be from the most affected organs, particularly the respiratory tract, in most human coronaviruses. In the case of SARS-CoV-2, the virus induce respiratory, enteric, renal, and generalized conditions.¹ It was well known that nasal swabs are the ideal sample for most human coronaviruses. However, in the case of the SARS-CoV-2, a recent study showed that saliva could be one of the most important samples for the accurate diagnosis of the virus.¹⁷⁵ It may be used to monitor the progression of the diseases in the affected patients by frequent testing of the viral load of the saliva of the affected patients during the virus infection.¹⁷⁵ About 10% of the patients exhibit enteric manifestations in terms of vomits, diarrhea, and abdominal discomfort even ahead of the development of the respiratory signs. This observation directed the attention to the possibility of the excretion of the virus in the stool of the infected patients. Some studies showed the detection of the SARS-CoV-2 in the stool of some patients.¹⁸⁸ This also highlighted the potential fecal-oral route mode of transmission of SARS-CoV-2. It will also have a great impact on our understanding of the viral epidemiology, spread, and contribute to the mitigation of the risk of virus infection as well.

8.1 | Development of novel diagnostic techniques that allow the detection of coronaviruses on various surfaces and environmental samples

The environment plays an important role in the spreading of airborne pathogens, such as SARS-CoV-2. The infected patients shed the virus

through coughing and or sneezing small droplets, which may disperse on-air for sometimes then fall on the ground, surfaces, or objects. This is posing the risk of infection of other persons who may touch these virus-rich droplets. The virus persists on these objects and in the environment for a variable time based on the temperature and humidity of the area.¹⁸⁹ Meanwhile, the SARS-CoV-2-RNAs were detected in air samples from hospitals hosting COVID-19 patients.¹⁹⁰ Although the RT-PCR remains the test of choice for the detection of the coronaviruses on the environmental samples, there is an urgent need for the development of novel assays that may detect the presence of viruses on those objects and surfaces and even in the air. The Luminex xMAP technology was recently developed to do simultaneous detection of 10 fungal infections of major human concerns in hospitals' air through the direct hybridization technique.¹⁹¹ In light of the high-efficiency hybridization technique, it would be visible to upgrade this hybridization step to make the detection of viruses on surfaces and the environment much easier. Meanwhile, the fluorescent antibody technique was used extensively in coronavirus research for many reasons, especially the intracellular localization of various coronavirus proteins, pathological changes during the virus infection, as well as the signal transduction pathways in the context of coronavirus infections.¹¹⁰ In a similar manner, upgrading the fluorescent techniques to run in a one-step reaction allows the detection of the viral NAT on surfaces and environmental samples through the emission of visible lights. This approach will enable the apparent discovery of these pathogens on various objects and surfaces, thus making their decontamination quite possible.

8.2 | The criteria of the standard (N-CoV-DAs)

Based on the continuous challenges posed by some coronaviruses, there is a high demand for the development of novel diagnostic assays for this category of viruses. In addition to the general criteria of some of the currently used diagnostic tests, such as the high specificity, sensitivity, and accuracy, the N-CoV-DA should have unique features that overcome some pitfalls of the currently used techniques. The N-CoV-DA should be applicable at the point-of-care settings. They should be able to do simultaneous and multiplex detection of several coronavirus candidates at the same time. They should have a high-throughput screening potential of a large number of samples at a remarkably short time. They should have the upgrading potential to match any changes that might occur on the viral genetic materials and in the case of emerging new virus or strain of the currently known coronaviruses. Techniques that are bypassing some initial steps in the process of diagnosis, such as RNA extraction and cDNA synthesis, are highly desirable. Meanwhile, special attention should be paid to find more convenient samples that are applicable in the point-of-care setting and under field conditions, especially in low-income countries. These samples should be easily collected, less invasive, and produce less damage than some of the currently used samples. The best example is the use of saliva instead of nasal swabs in the diagnosis of SARS-CoV-2, as described above.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTION

Maged G. Hemida: conceived the ideas, analyzed the literature, drafted, and wrote the manuscript.

ORCID

Maged G. Hemida  <http://orcid.org/0000-0002-5986-7237>

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