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Laboratory Methods for COVID-19 Diagnosis

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Coronaviruses are a group of related RNA viruses that cause disease in mammals and birds. Covid-19 infection occurs due to an RNA virus which is single-stranded, called SARS-CoV-2; this virus is similar to SARS-CoV. This review throws light on the available laboratory techniques used for testing coronavirus. Certain challenges are encountered during the development of a diagnostic test for a novel pathogen, which depends on sensitivity of the method, that is, the potential in detecting very low pathogen level for early laboratory diagnosis, produce little or no interference with other strains of the virus, and produce results rapidly. Since the time of incubation and clinical manifestation of the infection are relatively the same with SARS, the widespread and effect of COVID-19 globally serve as the basis why the development of quick and reliable laboratory methods are necessary. Samples that could be collected for covid-19 testing includes blood (especially for screening purpose), nasal and throat swab. Currently, the gold standard method for laboratory diagnosis of Covid-19 infection is RT-PCR, which serves as a confirmatory method for Covid-19 testing. EIA and SVN laboratory techniques are other techniques used in detecting the viral infection. In addition, Rapid Diagnostic Testing (RDT) are currently developed for point-of-care testing, and often used as a screening method of Covid-19 infections. Early detection of the virus remains the primary focus for the treatment and control of SARS-CoV-2 infections. Therefore, this review was aimed at the available laboratory methods used in the diagnosis for coronavirus infection.

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1. INTRODUCTION

Coronaviruses are of *Coronaviridae* family which consists of enveloped positive sense RNA viruses that are single-stranded, with the largest genome among RNA viruses [1]. The isolation of these human Coronaviruses were first made in cell culture in the 1960s from individuals who were diagnosed with upper respiratory infections; these Coronaviruses were subsequently grouped into HCoV-229E and HCoV-OC43 [2]. Also, in the early 2000s, some Coronaviruses were isolated from individuals who were diagnosed with pneumonia and inflamed bronchioles; these coronaviruses were also grouped into HCoV-NL63 and HCoV-HKU1. Furthermore, in the 2002, a *Betacoronavirus* belonging to the lineage B (subgenus *Sarbecovirus*) originated from bats in the Guangdong Province, the Southern part of China, and this virus was transmitted into the civets of humans, thereby resulting to fatal respiratory disorder thus was called SARS-CoV, meaning "severe acute respiratory syndrome-related coronavirus (SARS-CoV)", [3,4]. In 2012, a lineage C *Betacoronavirus* showed certain clinical manifestation likened to SARS, thus was called "Middle East Respiratory syndrome (MERS-CoV)" [5].

In December 2019, the newest type of coronavirus emerged in humans in Wuhan City, Hubei Province, China [6,7] and has therefore been named SARS-CoV-2. Results of the genomic sequencing of SARS-CoV-2 showed that it is closely related to the betacoronaviruses detected in bats (with 88 percent sequence similarity), but different from SARS-CoV (with 79 percent sequence similarity) [8]; the sequencing of the genome was performed by combining Illumina, Sanger and Oxford nanopore sequencing [9]. Andersen et al. [10] reported that the source of SARS-CoV-2 was bats; this was revealed from phylogenetic analysis. However, some studies reported that the source may be associated with pangolins [11].

Coronaviruses have envelope, and contain single-stranded RNA; they are mostly spherical, with glycoprotein (S) containing pronounced spikes which are embedded in the envelope. Also, the virus contains more structural proteins such as;

- Envelope (E)
- Matrix (M)
- Nucleocapsid (N).

Events of genetic recombination and species transmission of Coronaviruses are promoting factors to the emergent rise of new strains of the virus [2].

On the 31st day of December, 2019, China gave the first COVID-19 reports to WHO, thereafter, Authorities reported 212 affected countries and territories with cases of about 4,302,774 on 12th of May, 2020. Therefore, a very essential tool used in tracking the spread of the disease during the pandemic was laboratory testing, which in turn permitted public health interventions such as isolation, quarantine, and proper clinical management of individuals who were infected. The main components of diagnostic tests for the virus include the following: (1) the appropriate sample (blood, nasopharyngeal and oropharyngeal swab) must be collected, (2) the availability of the viral genetic and viral proteomic sequences, and (3) the laboratory testing methods used must be rapid and accurate. Some challenges faced during the development of a diagnostic test for a novel pathogen include the potential of measuring low viral loads that could be detected early, to produce low or no cross-activity with other strains of the virus, and to produce results rapidly. Currently, several molecular devices used for point-of-care testing are being integrated to produce accurate and rapid diagnosis of the viral infection.

2. SYMPTOMS

The incubation period of human Coronaviruses (HCoVs) is between 2 to 5 days. The infections caused by these viruses include mild upper respiratory symptoms (like the "common cold"). However, endemic human Coronaviruses (HCoVs) are part of the most frequent causes of infections of the upper respiratory tract, whereas infections of the lower respiratory tract (such as bronchitis and pneumonia) are rare. After 4 to 5 days incubation period, infected patients begins to manifest symptoms of headache, fever and myalgias; symptoms of cough and dyspnoea usually manifest within a week after the illness onset; in addition, pneumonia and decline in respiratory function take place in 20 to 30 percent of cases. Interestingly, MERS and SARS share similar period of incubation, clinical presentations and course, except that a larger percentage of cases progress to decline in

respiratory function. A research first revealed an average time of incubation to be 5.2 days. Cough and fever are early presentations of the illness and the infections are characterized by dyspnoea, respiratory decline and chest X-ray revealing respiratory distress [6]; symptoms of lower respiratory infections often develop about one week from the onset of initial symptom.

3. LABORATORY METHODS

3.1 Analysis of Covid-19 by (RT-PCR)

The RT-PCR technique detects the nucleic material of the virus to either identify the presence of virus or to both identify and quantitatively determine the viral load.

3.1.1 Sample collection

It is important to mention, appropriate specimen collection is a vital component of laboratory diagnosis. Samples collected from the upper and lower respiratory tracts are used to detect human coronavirus (HCoV); Importantly, swabs collected from the nasopharynx are highly recommended as the specimen of choice in the laboratory for SARS-CoV-2 detection, while others such as oropharyngeal swabs, broncho-alveolar lavage, tracheal aspirates, and sputum are lower priority specimens when SARS-COV-2 is to be detected [12]. This is in agreement with the approach of Gaunt, Charlton, Falsey and their teams respectively (Gaunt et al., 2010; Charlton et al., 2019); [13]. It. reported that during the COVID-19 outbreak in China, oropharyngeal (OP) (n = 398) swabs were often collected than swabs from the nasopharynx (n = 8), but that OP swabs detected 32% of the viral RNA which was much lower compared to 63% detection of the viral RNA using NP swabs. The US Centers for Disease Control recommend the use of upper respiratory nasopharyngeal swab (which is a high priority specimen), and that, collection of OP specimen should be considered a lower significance, and thus if OP is collected, it should be added into the same specimen bottle (containing a viral transport medium) as the nasopharyngeal swab [12]. Also, nasopharyngeal aspirates are specimens appropriate to detect human Coronaviruses (HCoVs).

3.1.2 RNA isolation

Approved and recommended isolation kits for viral RNA extraction from specimens are used.

3.1.3 Real-time (RT)-PCR procedure

The isolated viral RNA is re-transcribed to copy DNA (cDNA) and then amplified using a RT-PCR technique. WHO reported different probe and primer sets for SARS-CoV-2 developed previously in China, Hong Kong, Germany, Japan, USA and Thailand [14]. There are several segments of the genetic sequence of the virus such as the

- Envelope (E) gene,
- RNA-dependent RNA polymerase (RdRp) gene and the
- Nucleocapsid (N) gene [15,16]

These segments are targeted by primers. However, Corman et al. [15] reported highest sensitivity when the E gene was targeted and the next was the RdRp gene which is commonly referred to as a confirmatory gene. Furthermore, multiple probe and primer sets found at various regions in the genome of the SARS-CoV-2 have been integrated (referred to as multiplexed PCR tests) by some laboratories, such that these primer sets may have the potential to simultaneously target many genes (RdRp/hel, S, N) or to target several regions in a single gene (for instance, the N gene). However, in cases where there is viral RNA degradation or loss during sample collection and/or extraction of nucleic acid, or in the case of mutation in the viral genome, the use of multiplexed assays will help to promote sensitivity of the test. These methods use positive controls of RNA (that was synthesized *in vitro*) obtained from transcripts and also used to develop standard curves. RNAse P (RP) internal quality control promotes the validity of the quality and detection of nucleic acid in specimen, and a negative amplification control was obtained from nuclease-free water. Sample from a negative patient serves as negative control on extraction to detect cross contamination in samples and for test reagents validation.

3.1.4 Advantages

RT-PCR being the gold standard technique used for the diagnosis of COVID-19, it possesses the potential of testing thousands of samples daily, and reveals a high testing sensitivity of about 95 percent [15]. From a study conducted recently, the limit of detection of the virus with this technique is less than 10 copies per reaction which implies that, it can detect early infection, as well as low viral load.

3.1.5 Disadvantages

When the RT-PCR technique is used, a cross-reaction between the primers and nucleic acids from other existing microbial infection, may lead to the production of false-positive results, in which the detected pathogen may not have caused the disease. Also, when the RT-PCR primers and probes of SARS-CoV-2 are matched with reliable libraries (for example, BLAST), related coronaviruses such as SARS-CoV or other pathogens (for example *Staphylococcus aureus* and *Candida albicans*) is usually ruled out. Also, the occurrence of false positive results may be attributed to the contamination of reagents in the laboratory, especially with the huge testing rate faced during a pandemic; however, a negative patient's sample is useful in diagnosing or ruling out this anomaly. In furtherance with the aforementioned, false positive result may also be attributed to mutations in specific or target regions in the SARS-CoV-2 genome designated for the primer and probe. Nevertheless, negative test results may not rule out possibility of COVID-19 infection, therefore, re-analysis should be carried out using different sets of primer against the same target gene, in combination with patient's medical history and other clinical details to accurately ascertain patient's infection status.

3.2 The Use of ELISA in Detecting Antibodies against SARS-COV-2

This is one of the diagnostic methods used to detect antibodies in the blood samples, and oropharyngeal and/or nasopharyngeal swabs obtained from patients. Besides its use in detecting antibodies against SARS-CoV-2, it also has the potential of measuring the amount of the antibodies (such as Immunoglobulins M, G and A), which defines previous exposure to the virus.

Of all the immunoglobulins present in the plasma, the immunoglobulin M (IgM) happens to be the first to be produced as a response against an antigen, and is primarily detected as the disease begins to get manifested which takes about three to seven days. On the other hand, the immunoglobulin G (IgG) happens to be the most abundant, and is detected much later as a response against an antigen ranging between seven to twenty-five days; this IgG is however maintained during post-primary exposure, and may also play a protective role in acquired immunity. Another immunoglobulin known to function in immune function in the mucous membranes is the immunoglobulin A (IgA).

The SARS-CoV-2 attaches and enters into the human cells by means of its glycoprotein found on the surface of the virus; it also functions as the main target in producing neutralizing antibodies of the host; this feature has rendered the S protein to be the main target for the development of antibody and vaccine, whereas the N protein found in human Coronaviruses act as an interferon antagonist and VSR (viral-encoded repressor) of ribonucleic acid interference (RNAi) that enhances the replication of the virus, and is also the main target for antibody design. Suitable diagnostic targets used to detect immunoglobulins M, G and A antibodies are developed from both recombinant antigens; S protein (rS) and N protein (rN). Also, undergoing some developments is a dual detection of IgM/IgG and IgG/IgA immunoglobulin, which is used together with NA (nucleic acid) testing.

3.2.1 Specimen collection

Whole blood is collected but centrifuged to get the serum which is needed for the serological assay. It should be of note that serological assays are not regularly used for the diagnosis of human Coronaviruses (HCoVs) owing to lack of commercial reagents that have undergone vetting through clinical trials and regulatory review process. When there is inappropriate collection of nasopharyngeal and/or oropharyngeal swab specimens, and unsatisfactory molecular assays performed, serological assays are recommended to enhance COVID-19 infection detection.

3.2.2 Procedure

On the surface of a multi-well plate, purified recombinant S or N protein (used as capture antigens) will be immobilized. This will be followed by incubation of control samples as well as inactivated COVID-19 serum with the antigen to enhance binding of antibody of COVID-19 and antigen. In addition, a labeled secondary antibody-conjugate (such as horseradish peroxidase) is added, which binds to the SARS-CoV-2 antibodies and are used to detect signal through the addition of substrate, hence its quantification.

3.3 The Use of Lateral Flow Immunoassays (LFIA) for the Rapid Detection of SARS-COV-2

This method enhances rapid qualitative detection of SARS-CoV. It is designed as a simple portable diagnostic test strip used for detecting either the antigens or antibodies of SARS-CoV-2. However, because viral titers are usually considerably low in nasal swabs and serum or plasma, it may be more difficult to detect antigens compared to that of antibodies. Interestingly S protein domains (S1 and S2) bind to ACE-2, which happens to be a trans-membrane protein in the alveolar epithelium of the lung [16, 17], serological assays used to detect viral antigen, target these S1 and S2 domains.

3.3.1 Lateral Flow Immunoassay (LFIA)

Lateral flow is a strip/dipstick technique for rapid or qualitative diagnosis. The strip is composed of a coated purified mAb or recombinant antigen placed at certain areas on a nitrocellulose membrane. This method is characterized by a principle that is based on binding between the monoclonal antibody and the viral antigen, or between the recombinant antigen and the antibodies present in samples of infected patients. Also, the strip is made of labeled detector antibodies that will bind the same viral antigen; this labeled detector antibodies is used as a control measure. When the coating antigen and the antibodies in the sample of the patient bind together with the detector antibodies, coloured bands in the test and control regions are produced, and a positive antibody test result is indicated. Additionally, when the coating antibody and antigen in the sample of the patient bind together with the control regions, a positive antigen test result is indicated, both represented by two coloured bands.

3.3.2 Advantages

One of the advantages of this technique is that it only requires little quantity of blood, ranging from two to three drops for SARS-CoV-2 detection and its antibodies. Also, in this technique, visual detection by the naked eye is used, and results are generated within 15 minutes, compared to RT-PCR (which takes about 2-5 days). When antibodies are detected, it implies previous exposure to the virus, whereas when antigens are detected, it implies an active case of the infection. The specificity and sensitivity of LFIA when compared with other antibody and antigen methods are relatively suitable.

3.3.3 Disadvantages

This technique that assays the viral antigens in samples are difficult to develop compared to techniques that detect antibodies against the virus, as purified monoclonal antibodies have to be produced against target antigens.

3.4 Serum Virus Neutralization Assay (SVN)

SVN is utilized to qualitatively and quantitatively determine functional antibodies (in the patient) [18] that enhance the neutralization or prevention of SARS-CoV-2 infection, and thus ameliorate the infection. This test is said to be the most reliable during the assessment of protective antibody due to its high sensitivity and specificity, and it can be informative using convalescent plasma in COVID-19 treatment especially among critically ill patients. However, it is not used routinely for diagnostic purpose, but used in frontline during an outbreak. Even with paucity in data, suggestions from some early studies indicate that convalescent plasma when transfused can induce suppression of the replication of SARS-CoV-2, thus enhancing individuals' protection against getting infected.

There are various cell lines used when transducing SARS-CoV-2, some of which may include human hepatoma cell line (Huh7), monkey kidney cell line (Vero), and human kidney cell line (293T); in this process, the convalescent serum from patients was subjected to serial dilution, followed by the addition of known viral strains after incubation for 5 days, or fluorescence or formation of plaque after incubation for 24 hours. The binding of the neutralizing primer is followed by the application of polymerase, which enhances separation of the annealed strand to the target sequence, and hence its amplification and detection; these amplified gene products can be detected using a photometer. Additionally, various molecular diagnostic platforms utilize amplification of isothermic nucleic acid which is regarded as the fastest rapid molecular based Point of Care Testing (POCT) for the assay of the virus.

3.5 RT-LAMP

The RT-LAMP technique is used for the detection of SARS-CoV-2 in clinical specimens

from individuals who tested positive for COVID-19. In this method, both the open reading frame (ORF1ab) and S genes are targeted by primers with multiple loop, and were utilized to displace DNA strand and consequently amplify the target that was successfully detected; these findings were relatively similar to RT-PCR amplification. Also, this technique is said to be definitive because, specificity and sensitivity were said to be 100%, with a mean detection time of less than 30 minutes.

A study reported the use of this technique in the detection of SARS-CoV-2 obtained from cell culture supernatant and 19 samples of nasopharyngeal swab which composed 8 positives and 11 negative outcomes. Also, binding of primer to the homologous sequence in double-stranded DNA is made possible using isothermal techniques for reverse transcription together with recombinant potential that promote. Subsequently however, enhanced polymerase-mediated extension primer obtained 100 percent diagnostic specificity and sensitivity. This technique is more advantageous compared to RT-PCR on the basis of scale, portability and speed, and hence allow for clinical decisions (based on facts) to be achieved on a patient visit.

3.6 Clustered Regularly Interspaced Short Palidromic Repeats (CRISPR)

This technique is a coupled reaction between pre-amplification of nucleic acid and CRISPR-Cas enzymology, and used for the detection of RNA or DNA using pre-amplification of nucleic acid integrated with CRISPR-Cas enzymology system for sequences recognition specificity. This technique is used for the detection of RNA or DNA.

The CRISPR/Cas13a coupled system was a currently identified in the CRISPR-RNA (crRNA)-guided analysis approach which is RNA specific and as such, it is applied for the detection of SARS-CoV-2. A major characteristics of this system is the Cas13a (previously called C2c2) enzyme that has the ability to recognize and bind targeted RNAs in a manner that is sequence-specific; this is followed by a non-targeted RNA cleavage mediated by non-specific *trans*-endonuclease activity ("collateral" cleavage) for signal amplification and nucleic acid detection. The Cas13a method can be coupled with target nucleic acid amplification for stronger sensitivity using the technique, isothermal exponential amplification, usually RPA. This coupled technique allows for rapid detection of many of the targets.

4. CONCLUSION

Accurate laboratory diagnosis of COVID-19 is the basis for control and treatment plan. Molecular tests are confirmatory methods for COVID-19. Although serological tests for COVID-19 are widely available and play an important role in screening and in understanding the frequency and distribution of SARS-CoV-2 across the nations of the world due to low cost and rapid diagnosis, the role of nucleic acid detection is vital and confirmatory.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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