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LITERATURE REVIEW ON **MOLECULAR DIAGNOSTIC TEST FOR SARS-COV-2 /CORONAVIRUS/**

LITERATURE REVIEW SUBMITTED TO WOLKITE UNIVERSITY, COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES, DEPARTMENT OF BIOTECHNOLOGY, FOR PARTIAL FULFILLMENT OF DEGREE OF BACHELOR OF SCIENCE (BSC.) IN BIOTECHNOLOGY

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LIST OF ABBREVIATIONS

WHO: World Health Organization

SARS: Severe Acute Respiratory Syndrome

COV-2: Corona Virus

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

PCR: Polymerase Chain Reaction

ELISA: Enzyme Linked Immunosorbent Assay

RT-PCR: Reverse Transcription Polymerase Chain Reaction

HIV: Human Immunodeficiency Virus

HCV: Hepatitis C Virus

ZIKV: Zika Virus

EBV: Epstein Barr Virus

CDC: Central Disease Control

PUI: Person Under Investigation

NP: Nasopharyngeal

OP: Oropharyngeal

VTM: Viral Transport Medium

RBD: Receptor Binding Domain

CLIA: Chemiluminiscence Immuno Assay

ABSTRACT

SARS-CoV-2 is a single-stranded RNA virus of ~30 kb genome size which belongs to genus Coronavirus, family Coronaviridae. SARS-CoV-2 has recently emerged and has been declared as a pandemic by the World Health Organization. Genomic characterization of SARS-CoV-2 has shown that it is of zoonotic origin. The prominent structure of SARS-CoV-2, include the spike, membrane, and envelope surface viral proteins are embedded in host membrane-derived lipid bilayer encapsulating the helical nucleocapsid comprising viral RNA. The Corona virus disease 2019 caused by SARS-CoV-2 is a perfect example how viral infection could pose a great threat to global public health and economic sectors. Therefore, the first step in combating viral pathogenesis to get a timely and accurate diagnosis. Early and accurate detection of the viral presence in patient sample is crucial for appropriate treatment, control, and prevention of epidemics. In this short review, we summarize some of the molecular and immunological diagnostic approaches available for the detection of SARS-COV-2. Molecular diagnostic techniques provide rapid viral detection in patient sample. They are also relatively inexpensive and highly sensitive and specific diagnostic methods. Immunological-based techniques can detect antiviral antibodies or viral antigens in clinical samples. There are several commercially available molecular and immunological diagnostic kits that facilitate the use of these methods in many clinical laboratories worldwide.

Keywords: *COVID-19, SARS-CoV-2, Diagnosis, RNA, Molecular Techniques*

1. INTRODUCTION

Viruses are small obligate intracellular parasites, which contain either a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) genome surrounded by a protective, virus-coded protein coat. Viruses may be viewed as mobile genetic elements, most probably of cellular origin and characterized by a long co-evolution of virus and host. For propagation viruses depend on specialized host cells supplying the complex metabolic and biosynthetic machinery of eukaryotic or prokaryotic cells. A complete virus particle is called a virion. The main function of the virion is to deliver its DNA or RNA genome into the host cell so that the genome can be expressed (transcribed and translated) by the host cell. The viral genome, often with associated basic proteins, is packaged inside a symmetric protein capsid.The nucleic acid-associated protein, called nucleoprotein, together with the genome, forms the nucleocapsid. In enveloped viruses, the nucleocapsid is surrounded by a lipid bilayer derived from the modified host cell membrane and studded with an outer layer of virus envelope glycoproteins (Becker Y and Darai G (1995).

Common classification of a viruses

A useful classification is by the type of nucleic acid the virus contains and its mode of expression. They can use either RNA or DNA as their genetic backbone. A virus is an infectious agent that can be inside or outside cells. It is minimally constructed of two components:

1) a genome consisting of either DNA or RNA, usually not both and
2) a protein –containing structure (the capsid) designed to protect the genome
Many viruses have additional structural features, for example an envelope composed of protein containing lipid bilayer, whose presence or absence further distinguishes one virus group from another. Viruses can be rod-shaped, sphere-shaped, or multifaceted (Arvin M. 1996).

RNA Viruses

Ribonucleic acid (RNA) is a nucleic acid polymer consisting of covalently bound ribonucleotides. RNA nucleotides contain ribose rings and uracil unlike deoxyribonucleic acid (DNA), which contains Deoxyribose and thymine. In eukaryotic cells, it is transcribed from DNA by enzymes called RNA polymerases and further processed by other enzymes. RNA serves as an intermediate template for translation of genes into proteins, transferring amino acids to the ribosome to form proteins, and translating the transcript into proteins. The RNA of most RNA viruses is synthesized by viral RNA dependent RNA polymerases.
All RNA viruses are single-stranded except for dsRNA reoviruses.Viruses are limited to a particular host or cell type. RNA viruses are important human pathogens. Many acutely infecting enteric (Calici, Entero, Corona, Hepatitis A and E), respiratory (Orthomyxo - influenza, Paramyxo, Corona) viruses, and persistent ones (Retro -HIV, Flavi- hepatitis C) are RNA viruses. A common property of RNA viruses is their sequence variability. This can be explained by the lack of "proof- reading" in RNA dependent RNA/DNA polymerases. In contrast, proof- reading is present in DNA dependent DNA polymerases (Fenner, 1994 and Howley, 2001).

DNA Viruses

All DNA viruses are double-stranded except for parvoviruses, which have ssDNA. dsDNA-containing viruses have genomes ranging from 100 to 280 kb, including *herpesviruses, asfarviruses, baculoviruses, iridoviruses,* and *ascoviruses.*
Most DNA viruses are budded off the nucleus. Viral infections range from very mildto life- t hreatening. Many viruses are strictly human origin, others are zoonoses transmitted by vectors. Most DNA and few RNA viruses can become permanent resident of host cells. (Monier A, and Pagarete A, 2009).

Family Member of Corona viruses

According to the International Committee on Taxonomy of Viruses, CoVs are classified under the order of Nidovirales, a family of Coronaviridae and subfamily of Coronavirinae. Based on previous serologic and recent genomic evidence, the family of Coronaviridae

encompasses two subfamilies: subfamily Orthocoronavirinae and subfamily Torovirinae. The subfamily of Orthocoronavirinae consists of four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. Based on the genetic sequence identity and the phylogenetic reports, SARS-CoV-2 is sufficiently different from SARS-CoV; thus, WHO has classified it as a new Betacoronavirus that infects humans (Chen L, and Zhang Q.2020).

2. DIAGNOSIS OF VIRUSES

Diagnostic virology is rapidly moving into the mainstream of clinical medicine because of the convergence of several independent developments. First, dramatic progress in antiviral therapeutics has increased the need for specific viral diagnoses. Second, technological developments, particularly in nucleic acid chemistry, have provided important new tools for viral diagnosis. Viral diagnostic methods generally belong to one of four categories: Virus isolation, Virus antigen detection, Virus nucleic acid detection and Virus antibody detection (serology). Virus isolation is a rather cumbersome and slow technique. Virus antigen detection is more rapid, but still is manually intensive and relatively insensitive. Virus serology is an indirect approach with many limitations. There is a great clinical need to develop new virus diagnostic techniques. Rapid, sensitive, and rational virus detection and quantification methods are needed. This is true for both human and veterinary medicine. Broadly targeted methods are the major theme of this thesis. They can reduce the time and cost of diagnosis of infectious disease. Once a diagnosis has been reached, appropriate medical action can be taken (Gardner PS, and McQuillin J. 1980.)

Molecular techniques principles

Molecular testing consists of reverse transcriptase-polymerase chain reaction (RT-PCR) tests specific for the RNA. It can detect infection within the first few days after the onset of fever in viral infection patients, but the duration of detectable viraemia and virus shedding is unknown, so RT-PCR tests performed too early or late, or from the wrong sample, could give negative results (Tizard ,1994).

Nucleic Acid-Based Amplification Techniques

Molecular techniques that involve the amplification of viral genomic material are extremely sensitive and specific, provide rapid diagnosis, and allow the detection of several viruses at

same time. Nucleic acid amplification techniques are very useful for the detection of viruses that are uncultivable or difficult and harmful to culture, slow growing viruses in culture, and viruses that display antigenic variations. The nucleic acid amplification tests are very popular in the diagnosis of viral infections caused by several viruses, including hepatitis C virus (HCV), human immunodeficience virus (HIV), dengue virus, Epstein–Barr virus (EBV), influenza viruses, Zika virus (ZIKV), Ebola virus, and coro- navirus. Several nucleic acid amplification methods are currently available for the laboratory diagnosis of viral infections worldwide (Nolte FS.1999).

Polymerase Chain Reaction (PCR)

PCR is a typical example of nucleic acid amplification assay. It has revolutionized the field of molecular diagnosis since developed by Mullis and Faloona. PCR is based on extraction and purification of DNA moleculeand exponential amplification of the target sequence, using a thermostable DNA polymerase and two specific oligonucleotide primers. After the PCR reaction, the amplified product can be detected by several techniques, including gel electrophoresis, colori- metric methods, and sequencing. Since its inception, PCR has been used for the detection of human viral infections with overall clinical sensitivity ranging from 77.8% to 100% and clinical specificity ranging from 89% to 100%. These reports suggest that PCR can be employed for the detection of medical viruses in a variety of specimen types. Conventional PCR is still in use by some clinical laboratories worldwide, but it is rapidly replaced by more advanced variants of the technique. PCR is a highly versatile technique. A number of variants of the conventional PCR have been developed, but the most important variants are reverse transcription-PCR and real- time PCR. The first method was devised to amplify ribonucleic acid (RNA) targets, the second technique was introduced to quantify deoxyribonucleic acid (DNA) in real time throughout the PCR reactions (Ebner K and Lion T. 2005).

Reverse Transcription-PCR (RT-PCR)

RT-PCR was designed to amplify RNA targets. In this technique, reverse transcriptase (RT) is used to convert viral RNA targets into complementary DNA (cDNA),and then there sulting cDNA is amplified by conventional PCR. Since its development, RT-PCR has been used for the diagnosis of human infection by RNA viruses. Conventional RT-PCR

demonstrated overall sensitivity ranging from 73% to 100% and specificity ranging from 99% to 100% in the detection of viral infection (Suda M, and Lion T.2005).

Serological principles

Serological testing for anti-virus antibodies includes indirect fluorescent antibody testing and enzyme-linked immunosorbent assays (ELISA) which detect antibodies against the virus produced in response to infection. The performance of viral serologies is useful in the diagnosis of recent, past or chronic viral infections. Serum allows the detection of infection after clearance of virus. In most viral infections immunoglobulin M (IgM) appears within several days after onset of symptoms, peaks at 7-10 days, and declines to undetectable levels within 1-2 months. IgM methods are useful adjuncts to the direct detection of virus and virus nucleic acid (Butterworths, 1980).

3. MOLECULAR DIAGNOSIS TESTS OF CORONA VIRUS

In any infectious disease outbreak, accurate and accessible diagnostic testing must be one of the pillars of control-measure policies to understand and minimize the spread of disease. The epidemiological studies of the outbreak in China estimated the proportion of undetected COVID-19 cases to be as high as 86%. As asymptomatic or mild cases could play a significant role in the transmission and spread of the SARS-CoV-2 virus symptoms alone are not reliable diagnostic markers. There are two major types of diagnostic technologies available to address this: molecular and serological tests. Currently, much of the focus is on the SARS-CoV-2 molecular test, which can detect, with high accuracy, the virus-specific RNA molecules circulating in the host body. The gold-standard molecular test is based on reverse transcriptase polymerase chain reaction (RT-PCR) technology. However, the PCR test is not useful in distinguishing between highly infective viruses versus ones that have been neutralized by the host, and it cannot assess immunity status against SARS-CoV-2. Serologically based antibody tests can complement molecularly based tests by providing a more accurate estimate of SARS-CoV-2 incidence and by potentially detecting individuals with immunity against the disease, as these tests detect markers of the immune response.
A positive test for SARS-CoV-2 generally confirms the diagnosis of COVID-19, although false-positive tests are possible. If initial testing is negative but the suspicion for COVID-19 remains, the WHO recommends resampling and testing from multiple respiratory tract sites. The accuracy and predictive values of SARS-CoV-2 testing have not been systematically

evaluated. Negative RT-PCR tests on oropharyngeal swabs despite CT findings suggestive of viral pneumonia have been reported in some patients who ultimately tested positive for SARS-CoV-2. Serologic tests, once generally available, should be able to identify patients who have either current or previous infection but a negative PCR test. Co infection with SARS-CoV-2 and other respiratory viruses, including influenza, has been reported, and this may impact management decisions (Huang, W.E.; 2020).

Virus sample collection

At present, the CDC recommends two criteria for testing. The high priority category includes hospitalized patients with symptoms, healthcare facility workers, and residents in long-term care facilities or other congregate living settings with symptoms. The priority category includes persons with symptoms of suspected COVID-19 infection, and persons without symptoms who are prioritized by health care providers, for any reason, including public health monitoring or screening according to state and local plans. The CDC does not recommend testing for asymptomatic persons (COVID-19 (SARS-CoV-2, accessed on 9 May 2020).   .

Once a person is identified as a person under investigation (PUI) for COVID-19, it is recommended that the clinician should immediately adapt infection control and prevention (PPE) measures. In addition to excluding all other sources of respiratory infection, the decision on who to test should be based on the CDC guidance and local epidemiological data. For sampling, the WHO recommends collecting specimens from both the upper respiratory tracts, nasopharyngeal (NP) and oropharyngeal (OP) swabs, in the spontaneously breathing patients, and the lower respiratory tract such in the mechanically ventilated patients. Researchers have proven that NP swab specimen is superior to the OP swab specimen for the examination of SARS-CoV-2 as higher viral loads were detected in the nasal area than in the throat. Therefore, NP swab is the preferred sampling method for COVID-19 diagnosis. Upon collection, the swabs should be placed immediately into a sterile transport tube containing 2-3mL of either viral transport medium (VTM), Amies transport medium, or sterile saline and stored at four degrees Celsius. Potential risks of NP swabs include 1) the production of aerosol during the sampling, which can impose infection risks to health care workers) the inconsistent quality of NP swabs between collections, which may lead to false-negative results, 3) the patient may experience discomfort during the sampling procedure. Aiming to address these potential risks, further studies demonstrated the efficacy of less-invasive routes

for sampling such as throat wash and sputum collection. Saliva collection was shown to yield greater detection sensitivity and consistency throughout the course of the infection when compared to patient-matched NP samples. Furthermore, saliva could enable self-administered sample collection for accurate large-scale SARS-CoV-2 testing. Some authors suggested a potential value in testing both fecal and respiratory specimens to improve the test sensitivity. However, this issue remains under debate, as the detection of viral RNA in stool may not reflect actual viral replication or infection (Watson, J.; BMJ 2020).

RNA isolation

Testing for the presence of SARS-CoV-2 viral RNA typically begins with the collection of a patient swab sample which is stored and transported to a testing facility in Viral Transport Medium (VTM). These samples are lysed, and viral RNA is typically purified using either RNA extraction columns or magnetic beads. One advantage of RNA purification is that the viral RNA present in the more dilute swab sample can be concentrated and eluted in a buffer compatible with RT-PCR. However, in order to decrease reliance on commercial lysis buffers and viral RNA extraction kits and simplify COVID-19 testing, there has been great interest in finding alternative strategies or eliminating RNA purification altogether by adding patient swab samples directly to the RT-PCR reaction. Additionally, eliminating RNA purification can dramatically speed up the overall workflow time per test and may be an ideal solution for streamlining testing times.RNA can be extracted from samples mentioned above using any standard extraction protocols or kits. In general, the sample lysis step in RNA extraction inactivates any live virus. Thus, lysed samples are generally considered non-infectious. The inactivation of COVID-19 virus through sample lysis has been verified for some commercial kits. Sputum samples require liquification prior to molecular extraction, while tissue samples require lysis and homogenization (Bisoffi Z, and Pomari E, 1969).

PCR test for SARS-COV-2

At present, a positive result in nucleic acid testing using reverse transcription-polymerase chain reaction (RT-PCR) is the gold standard for diagnosing COVID-19. Although the assay is highly specific, the sensitivity is relatively low secondary to several factors including the viral load, virus replication, RNA isolation method, and the source and the timing of swab collection in relation to the disease stage. Therefore, further optimization to mitigate the high false negative rate has been a crucial goal of high priority.After RNA isolations from the

clinical sample and amplification through a reverse polymerase chain reaction, RT- PCR), the SARS-CoV-2 specific primers serve to search for the viral RNA sequences that are conserved. In patients with confirmed COVID-19 diagnosis, the laboratory evaluation should be repeated to confirm viral clearance prior to releasing from isolation. The conserved or abundantly expressed genes such as the structural genes N, E, and the non-structural RNA-dependent RNA polymerase as well as the ORF 1a/b genes are the preferred targets for the SARS-CoV-2 RT-PCR assays. Traditionally, two molecular targets should be included in the assay to avoid potential cross-reaction with other CoVs as well as potential genetic drift of SARS-CoV-2. Among these assays, the RdRp(RNA dependent, RNA polymerase) gene assay exhibited the highest sensitivity (3.8 RNA copies/reaction at 95% detection probability) and involves two probes. One of the probes is non-specific, which would detect other CoVs, whereas the second probe is specific for the SARS-CoV-2. These RdRp assays have been used in >30 laboratories in Europe. In the United States, the CDC recommends assays targeting two nucleocapsid proteins [N1 and N2], while the WHO recommends an initial screening with the E gene assay followed by a confirmatory assay using the RdRp gene. Importantly, the RdRp assays were designed and validated using synthetic nucleic acid technology (in vitro transcription) in the absence of SARS-CoV-2 isolates or patient specimens (SARS-CoV-2 Real Time PCR LAB-KIT™. Available online: accessed on 28 April 2020).

Data reporting

According to the International Health Regulations (IHR), all COVID-19 confirmed cases should be notified in 24 hours through official IHR channels. Additionally, all positive and negative results for COVID-19 must be reported in the Flu Net database that is sent weekly to PAHO/WHO. Updated Flu Net spreadsheets with the addition of a new column for COVID-19 reporting have been sent to the countries to replace the previous version.

4. SEROLOGICAL ANTIBODY TEST FOR SARS-COV-2

Serological, or antibody, tests detect immunoglobulins produced by the host's plasma B cells following exposure to foreign antigens. The SARS-CoV-2 genome encodes approximately 25 proteins that are required for infection and replication, including four major structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) . The S protein plays a critical role in fusion and entry into the host cell, and it comprises an N-terminal S1

receptor-binding domain (RBD), N-terminal domain (NTD), and a C-terminal S2 subunits. The primary function of the SARS-CoV-2 N protein (NP) is binding and packing of the viral RNA genome into a helical nucleocapsid structure during viral replication. Studies on the serum of recovered COVID-19 patients suggest that host-neutralizing antibodies primarily work against S and N proteins. Consequently, the likelihood of predicting immunity status could increase in serological tests that target various regions of S or N proteins. Therefore, the characterization of specific SARS-CoV-2 antigen domains targeted by the humoral immune response becomes an integral part of the serological test development. There are four major types of serological diagnostic tests: the rapid diagnostic test (RDT), enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), and neutralization assay. The neutralization assay is a lab-based test that uses live virus and cell culture methods to determine if patient antibodies can prevent viral infection in vitro. This test must be performed in laboratories with designated biosafety certificates to culture SARS-CoV-2-infected cells and has a time-to-result of 3–5 days. An RDT is a simple and rapid test based on lateral flow immunoassay technology, commonly found in pregnancy test kits, for example. RDT can potentially be administered as a point-of-care test or self-test. Typically, RDT test strips use a drop of blood to detect the presence of patient antibodies (IgG, IgM, or IgA) produced against a specific SARS-CoV-2 antigen. AnRDT is simple to use with a time-to-result anywhere between 10 and 30 min. Therefore, it has the potential to be deployed in large-scale serological surveys (Vashist, and S.K.2020).

ELISA assay, currently the most used format of the serological test, is a lab-based test with an average time-to-result of 2–5 h. ELISA typically uses a surface coated with specific viral antigen(s) to bind to and detect the corresponding patient antibodies (IgG, IgM, IgA) in blood, plasma, or serum samples. The bound antigen–antibody complex is then detected by using a second antibody and a substrate that produces a color- or fluorescent-based signal. ELISA assays can be found in different formats including direct, competitive, and, the most commonly used, sandwich or double-antigen-bridging assay (DABA). CLIA technology follows a similar concept to ELISA by taking advantage of high binding affnity between the viral antigen(s) and host antibodies but uses chemical probes that yield light emission through a chemical reaction to generate a positive signal. CLIA has an average time-to-result of 1–2 h. CLIA and ELISA are both high-throughput laboratory-based assays with high level of analytical agreement. (Ebner K, and Suda M, Lion T.2005 et).

5. SUMMARY

The use of viral culture is not feasible option for rapid diagnosis as it takes 3-5 days for SARS-CoV-2 to cause obvious cytopathic changes in vitro. In addition, virus isolation requires biosafety level- 3 (BCL-3) facilities of limited availability in many medical centers. Serology tests have not yet been validated. Moreover, the issue of cross-reactivity with SARS-CoV remains to be solved. This review provided an insight current situation in the diagnosis of COVID-19 and represents a picture of the current state of the art in terms testing the virus or viral particles focusing on the molecular approaches. There is a rapidly growing body of literature on this topic and hopefully it will help in finding an effective, fast and cost-effective diagnostic tools.The choice of specimen type for SARS-COV-2 diagnosis is subjected to the patient condition and stage the disease course. Specimens from upper respiratory tract are the best choice during the early days of the illness, whereas sputum is the most sensitive at later stages. Owing to occasional false result with rRT-PCR, CT radiography should be obtained to reach an accurate diagnosis and for proper management. As in other RNA viruses, mutations and other genetic changes are likely to occur, which may result in pitfalls of nucleic acid amplification assay. Genomic homology of SARS-COV-2 with other coronaviruses is also a challenge to serological and antigen detection tests. However, improvements in such point of-care tests are expected to aid in better management of the pandemic as they are rapid and simple to perform.

6. RECOMMENDATIONS

SARS-CoV-2 has recently emerged and has been declared as a pandemic by the World Health Organization. Based on the genomic sequences submitted to NCBI database, the scientific community has analyzed the samples and suggested preventive and therapeutic strategies. Therefore, investigation of genomic diversity in the collected specimens from around the globe needs to be conducted in order to design common, effective therapies and vaccines. In addition, genomic characterization helps us accurately identify the origin and evolution of the virus. Deciphering the mechanism of SARS-CoV-2 replication in various cell-based models may help us understand the pathogenesis and identify specific targets to develop effective antiviral drugs.

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