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A study on current diagnostic approaches to Covid-19

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ABSTRACT

The unanticipated outbreak of the novel coronavirus later identified as Severe Acute Respiratory Syndrome Coronavirus 2(SARS-CoV-2) has rendered the world speechless. After only two and a half months since the first case was reported in the Wuhan city of China, the WHO declared the coronavirus disease a pandemic. So far the virus has covered all 196 countries infecting millions. Scientists all over the world are putting in all their resources to formulate a vaccine to put an end to this pandemic. Meanwhile, prevention remains the only option. It is necessary to contain the disease to slow down its spread rate and for that fast and accurate diagnosis is vital. This review summarizes the current approaches for the diagnosis of the coronavirus disease. They are categorized based on their target molecule (RNA, antigens, or antibodies). We have also shed light on some newly emerging rapid testing techniques using chest scans and biosensors for high specificity to get more accurate results. The use of any one of them depends upon the severity of the case. At times more than one technique is used to get a sure outcome. Culture-based virus detection in the lab act as the gold standard of accuracy for all of these techniques. The study of the underlying mechanism of these techniques also helps to understand the virulent behavior of the virus and its mode of action. Taking into consideration all of these approaches, each one has its limitation that is also discussed in this article.

Keywords: *SARS-CoV-2, Covid-19, Molecular Testing, RT-PCR, Lamp, Serological Methods, ELISA, CRISPR*

1. INTRODUCTION

The emergence of novel coronavirus disease, widely known as the COVID-19 disease, and its gradual development into a pandemic has led to severe global economic as well as public health concern. SARS-CoV-2 was found to have originated in bats and jumped to humans in one of Wuhan's seafood market. It has already affected more than 48 million people worldwide and resulted in the death of 12 million people as of now. As per the statistics available, mortality is high in older age group individuals (> 60 years of age) and people with other morbid conditions. Coronavirus disease (COVID-19) is an infectious disease caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus primarily affects the respiratory system causing flu-like illness with symptoms such as cough, fever, and in more severe cases, difficulty breathing. In addition to acute respiratory distress syndrome and respiratory failure, COVID-19 is now known to manifest as systematic inflammation, leading to sepsis, acute cardiac injury, and heart failure and multi-organ dysfunction in patients at high risk.

Coronaviruses are a group of enveloped viruses, having a positive single-stranded RNA genome and pathogenic. Outside the human body, the protective envelope is destroyed by household soaps apart from sanitizers. The transmission of the Severe Acute Respiratory Syndrome (SARS) Coronavirus 2 is elementary as it is through respiratory drops or aerosols generated when an infected person sneezes, talks or coughs. These droplets when inhaled or land in the mouth, nose or eyes of a nearby individual leads to infection by the coronavirus. It can also spread if a person touches a surface or an object with a virus on it and then touches the mouth, nose, or eyes. Symptoms may appear 2-14 days after exposure. Apart from having fever, cough and tiredness, it also causes the loss of taste and smells along with rashes, nausea, vomiting, headache and chest pains. Older individuals and people with existing chronic medical conditions are at higher risk of developing severe illness due to COVID-19. Complications after getting infected include respiratory distress along with cardiac infection and other secondary infections that may lead to death too. However, many patients with a milder form of the infection go unnoticed due to the absence of any major symptoms. Due to this, the provision of cost-effective, sensitive, and reliable testing methods has become the need of the hour. Detection methods based on the viral RNA and antigens have proved quite reliable so far but these require extensive lab skills. Scientists have also developed some rapid testing methods which are fairly easy to use and use antibodies present in from the blood sample of the patient. This would help in identifying and providing the infected people at an early stage before it advances and spreads. The goal of this article is to clarify the current state of testing for COVID-19. Therefore, many methods of diagnosis have been standardized so far for the detection of COVID-19 and are discussed below in this article.

2. MOLECULAR TESTING

The knowledge of the viral genome sequence has made it possible for us to use molecular techniques in the detection of SARS-CoV-2. Molecular diagnostic methods target to detect either specific regions of the viral genome or RNA-dependent RNA polymerase (RdRP) and/or structural proteins of SARS-CoV-2. Molecular diagnostics usually require samples from the patient that is likely to contain the virus, such as nasopharyngeal swabs or sputum samples. Some pathogens may also be detected in feces, urine, or blood. For respiratory diseases like COVID-19, nasopharyngeal swabs have been considered to be the most reliable, as they sample an area of the respiratory tract where the virus appears to first infect an individual. This site is relatively easily accessed, compared to the final site of viral infection: the lower respiratory tract. All molecular tests are developed to inform researchers of the presence of the pathogen, either by identifying its genetic material or identifying unique markers of the pathogen itself. Some of the most extensively used molecular diagnostic techniques are discussed below.

2.1 RT-PCR

Real-time reverse transcription-polymerase chain reaction (RT-PCR) is a type of nucleic acid amplification test that is used as a method of molecular diagnostic tool. Many different types of RT-PCR assays are being used across the world to amplify and detect different regions of the SARS-CoV-2 genome obtained from the respiratory samples of the affected individuals. The testing is done in two steps. The first one consists of a screening assay using the SARS-CoV2 specific E gene, which is necessary to reduce false negative results, and the second step is the confirmatory assay which targets the RdRp gene, N gene, and ORF1b. In vitro transcribed RNA of known copy numbers was used as a positive control to keep a check on the unknown variables. The assay requires 3-4 hours and is free of hydrolysis probe with high reproducibility.

The TaqMan probe is mainly used to ensure more sensitivity and specificity of the test. Studies have shown that when SYBER green dye was used, the results were more prone to false negatives. The false negatives may vary according to the viral load as they vary in disease progression and specimen type as well. The SARS-CoV-2 virus is an RNA virus which means that is more prone to inactivation and degradation if not handled and stored properly. Mutation in target sites of the virus, PCR inhibition, and cross-contamination between the samples also lead to inaccurate test results. It is found that the sensitivity of the RT-PCR test is between 50-79 percent depending on the abovementioned factors. Another significant factor is the cycle threshold which refers to the number of cycles in an RT-PCR cycle that is necessary to amplify the viral RNA to a detectable amount. Even though there are no scientifically proven reasons but due to the lack of standardization discrepancies may arise in the test results. On 10 January 2020, the full genome of SARS-CoV-2 was publicly released and any commercial assays have been developed to date concerning the protocols laid by the WHO, to target different regions of the gene. Most of these assays target short fragments of the gene which does not suggest if the virus is actively replicating within the host cells or not. Due to this many other methods of diagnosis are being relied upon for the detection of the SARS-CoV-2 virus in the body.

2.2 Loop-mediated isothermal amplification (LAMP)

LAMP is a molecular amplification technique by which the extracted genetic material is amplified. It has a high-efficiency rate along with the fact that it takes a shorter period. The target site of an extracted DNA is amplified by providing a specific temperature and primer. More than two specific primer pairs are required to carry out this method. The product obtained is cauliflower-shaped and has numerous inverted repeats. This method does not require expensive chemicals and instruments which is useful to lower down the cost of coronavirus detection. For the sake of rapidity of the test, laboratories opt for visual detection which does not require specialized equipment for COVID-19 detection. A colorimetric visual inspection method involving the use of suitable dyes is used. For the test of COVID-19, cresol red is used which is a pH sensitive indicator. In positive samples, the pH of the reaction mix decreases due to high DNA polymerase activity and it turns the red color to a yellow-orange color. This change in the color of the dyes hence makes the tests easy to decipher. It has been observed in many of the studies that this method effectively detects low levels of viral RNA. It is also found to be a hundred times more sensitive than the conventional RT-PCR method. This method is sensitive and yields specific results in a comparatively less amount of time. Since it does not require fluctuating temperatures, it can be easily carried out without using a thermocycler. The potential of getting false negatives from routine nucleic acid tests is highly reduced by this method.

2.3 Next-generation sequencing (NGS)

Next-generation sequencing (NGS) is the more advanced version of Sanger's sequencing method. Unlike the latter, NGS determines millions of base pairs of a genomic sequence in a very small period by carrying out sequencing of small fragments parallelly. These sequences are later analyzed and put together to map out the genomic structure of an individual. Each base is sequenced multiple times to provide higher depth into the data and eliminate the possible causes of the discrepancy. NGS is capable of detecting a broader spectrum of mutations constituting small substitutions, deletions, and insertions. Apart from this, it also helps link the new strains of viruses with human diseases. During the current COVID-19 global epidemic, NGS along with various bioinformatics tools are being used extensively to share the clinical and genomic data among the scientific community. SARS-CoV2 is a new strain of an RNA virus belonging to the Coronaviradae family, which mutates naturally in a consistent manner. The mutations may not be important enough to cause any major changes but it helps to understand how it affects an infected individual. These small mutations are recognized by NGS. The nasal samples collected from patients contain the viral RNA along with the DNA and RNA of the patient. The viral RNA is separated and an NGS library is constructed where the constantly evolving SARS- CoV-2 genomes are being updated. These are on public databases likes Global Initiative, NCBI GenBank, and China National GenBank databases. The current sequencing methods consist of metatranscriptomics sequencing, hybrid capture-based sequencing, amplicon sequencing, and nanopore sequencing. This technology is of great importance for the identification of unknown mutated pathogens having a known lineage. It has a broad detection range and is highly accurate and fast. However, the instruments and chemicals used are expensive and require skillful handling due to which it is limited to research laboratories only.

3. SEROLOGICAL METHODS

Serology is the scientific study or diagnostic examination of the blood serum. The serum is a fluid component of the blood (excluding WBCs, RBCs, platelets, and clotting factors). Serum includes all proteins not used in blood clotting; all electrolytes, antibodies, antigens, hormones; and any exogenous substances (e.g., drugs or microorganism s). The majority of serological studies are based on the immune responses via the antibodies present in the serum in regards to specific antigens. Taking a glance at the structure of the SARS-CoV 2 virus one can see many proteins but not all of them are immunogenic hence only a few of them trigger an elevated immune response. It is these antigens which are preferred for the serological diagnosis of COVID19 disease.

- **Spike protein (S):** It is a glycoprotein projecting outward of the virus membrane and allows the virus to attach and fuse with the membrane of the host cell. It has two subunits namely S1 and S2, out of which S1 is more important for diagnosis purposes as it catalyzes the whole attachment process. In the S1 subunit, there is a receptor-binding domain (RBD) that interacts with the angiotensin-converting enzyme 2 (ACE2) receptor protein of the host cell. It is one of the major ways through which the virus enters the host cell. The RBD is highly immunogenic and hence is extensively used in the diagnosis of COVID19 disease.
- **Nucleocapsid protein (N):** It is present inside the virus attached to the viral RNA holding the RNA genome together. It is a highly immunogenic phosphoprotein. Since it is present inside the cytoplasm of the virus it is normally very conserved. It modulates cell signaling pathways and replication of the viral genome inside the host cell. In humoral immune response to infection, pathogen specific antibodies, produced by B cells, neutralize and prevent further spread of the disease. The activation and differentiation of B cells into antibody-secreting plasma B cells are triggered by a cascade of events involving virus digestion by antigen-presenting cells (e.g., dendritic cells, macrophages) and presentation of virus-specific antigens to helper T cells. Antibodies protect the host by binding to specific antigens on the virus to neutralize its fusion and entry into the host cell and facilitate recognition and killing by phagocytic immune cells. In humans, three types of antibodies or immunoglobulins have been the target of COVID-19 serological tests: IgM, IgG, and IgA. Although the dynamics of the immune response in COVID-19 are not fully understood, typically IgM antibodies are produced by host immune cells during the early stages of a viral infection. IgG is often the most abundant antibody in the blood and plays a more prominent role in the later stages of infection and in establishing long term immune memory. While IgM and IgG antibodies have been the leading candidates in COVID-19 serological test development, recent studies show that IgA, predominately present in the mucosal tissue, may also play a critical role in the immune response and disease progression.

There are three major types of serological assays for COVID-19 diagnosis currently being used worldwide which are discussed below.

3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies, and hormones. It works by coupling antibody or antigen to assay enzyme. The assay combines the specificity of antibody and sensitivity of assay enzymes to primarily detect antigens through assay antibody or antibodies through assay antigens. The sensitivity and precision of the assay are enhanced by coating the plate with high-affinity antibodies. It is generally a 96-well or 348-well plate. The assay requires 2-5 hours to complete. For detection SARS-CoV 2 wells are lined with the immobilized immunogenic antigens present in the virus (such as S1, RBD, or Nucleocapsid). Serum from the patient's blood is poured onto the multi-well plate lined with the antigens. The plate is incubated for an hour. If the patient was infected by the SARS Cov-2 virus, antibodies specific to the virulent antigens will be present in its serum. Since the same antigens are present in the wells (immobilized form), the antibodies bind with them to form antigen-antibody complexes on the surface of the wells. After washing the plate to eliminate unspecific interactions, a detection antibody with a reported enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). Such a format in which the antigen is bound to the bottom of the microplate well, then an antibody specific to the antigen is added and a secondary antibody, conjugated to an enzyme or other detection molecule, is then bound to the first antibody is called indirect ELISA. These complexes are detected and quantified by adding a chromogenic substrate (e.g., 3,3′,5,5′-tetramethylbenzidine) that is utilized by the reported enzyme and leads to a change in the reaction color. Studies reveal that mostly IgG and IgM antibodies (IgA also in some cases) are detected in conjugation with the immobilized antigen in the case of SARS Cov-2. On repeated testing on the same patient for several days after the infection, it was also found that the IgM number dropped after a week. IgG however can persist for a long time following infection and may potentially have a protective role.

3.2 Chemiluminescent Enzyme-Linked Assay

Chemiluminescent immunoassay (CLIA) is a modified version of ELISA. The only difference is that instead of using reported enzymes like HRP and chromogenic substrates, antibodies labeled with light-producing enzymes are used and the luminescence is measured for the detection of pathogens. It is a quantitative test that can measure the number of IgG, IgM, and IgA antibodies. This test allows the mixing of patient samples with viral specific proteins. The formation of the antigen-antibody complex is then detected by using chemical probes that yield light emission through a chemical reaction to generate a positive signal. The amount of emitted light is then calculated for measuring the number of antibodies present in the sample. CLIA has an average time-to-result of 1–2 h. It is a bit faster as compared to ELISA but it has lower sensitivity at the beginning of the infection.

3.3 Lateral Flow Assay

It is a paper-based qualitative platform for the rapid and very low-cost detection of the SARS-CoV-2 virus. Like the other serological tests, it also works on the principle of antigen-antibody interaction. It is a Point-of-Care (POC) detection method and comes as a kit that is fairly easy to use and does not require core lab skills. The kit consists of a small cassette containing a strip, which is divided into 4 parts. The first part is the sample pad on which the blood pricked from the patient's finger is loaded (along with few drops of the buffer which comes with the kit). Then the cassette is left for incubation for about 10 minutes. The sample moves laterally along the strip through capillary action. The second part is the conjugation pad which is lined with gold-labeled SARS-CoV-2 antigens (S1 protein, RBD, nucleocapsid, etc.). The gold nanoparticles act as visual indicators as they scatter

visible light and produce a red color. This region also contains gold labeled rabbit IgG antibodies for quality control. If the patient is infected with Covid-19, the antigen-specific antibodies, generally IgG and IgM will conjugate with these gold-plated antigens and form a mixture as they move further along the strip. The third part is the detection zone having a porous nitrocellulose membrane. This is the part where we can visualize the results of the tests. It is lined with three markers namely anti-human IgM antibody, anti-human IgG antibody and anti-rabbit IgG antibody. The first two act as test lines, as they turn red on the detection of human IgM and IgG antibodies respectively. The last is the control line, it is to make sure that the sample flowed throughout the length of the strip. The last part in the cassette is the absorbent pad, it is just to absorb all the extra content. The mechanism of the test is very similar to that of a pregnancy test likewise it also gives fast results within 10-30 minutes.

Assessment of the results:

Positive results:

a) IgM positive: The infection is recent (a week or so) and the subject's immune system is actively producing antibodies to fight the virus. The subject should be quarantined and kept under medical observation
b) IgG/Ig positive: The infection is ongoing and likely began 14-20 days ago. The subject is still in the recovery period and needs proper medical care.
c) IgG positive: The subject was infected with the virus at some point in time roughly a month ago and has produced antibodies to fight the disease. Such a subject can act as a potential plasma donor for the people still recovering from the disease.

Negative result: Only the control line shows a visible response. It means the person is not infected with the virus or perhaps the person has a very recent infection and the antibodies are not yet produced.

Invalid result: No visible response was observed at the control line. It means the test did not run properly and should be taken again.

3.4 When serology testing is recommended?

Serology testing for COVID-19 is attractive because of the relatively short time to diagnosis and the ability to test for an active immune response against the virus. While hundreds of serology tests are currently on the market, only 21 have received Emergency Use Authorization (EUA) from the FDA (as listed on their site www.fda.gov). Serological tests give fast and reliable results in case of late-onset of the symptoms (since the antibodies would have been produced in abundant amounts). However, it still has its limitations. Results do not indicate the presence or absence of current or previous infection with certainty as IgM and IgG antibodies may take 1 to 3 weeks to develop after infection. So patients at an early stage in the disease course, or asymptomatic or paucisymptomatic patients, might have low antibody concentrations that could give false-negative results and we may have to rely on molecular testing methods like RT-PCR in case of more recent infections.

The presence of antibodies that bind to SARS-CoV-2 does not guarantee that they are neutralizing antibodies, or that they offer protective immunity. Apart from being a viable diagnostic test, serological methods are also used for epidemiology studies to trace the progression of the virus in the population.

4. RADIOLOGY BASED TESTS

The COVID-19 disease is marked with the severe acute respiratory syndrome. Early studies in Wuhan, China showed that the respiratory system i.e., lungs is the main target of the SARA-CoV-2 Virus. Hence radiology-based techniques such as chest X-rays or CT scans of the lungs can prove to be a fast and reliable tool for screening or even sometimes confirming COVID-19 patients. It is based on the clinical changes observed in the patient's respiratory system. CT scan results of COVID19 infected individual show a variety of abnormalities that are unique from other viral pneumonia infections of SARS, MERS, and Adenoviruses. Radiological studies on RT-PCR COVID positive cases showed a particular pattern. The appearance of bilateral peripheral ground-glass opacities (GGO), and solid white consolidations or crazy paving (in severe cases) were observed in the CT scans of the lungs in most of the COVID19 positive cases.

These observations were more prominent in severe patients. Radiology based techniques are often used to see the progression of the disease in hospitalized patients. However, these tests cannot be taken as confirmatory. Despite some unique features in CT scans of COVID 19 patients, they are still hard to distinguish as similar patterns are observed in some other diseases (viral pneumonia caused by adenovirus) and it may give false negative/positive results. Several US radiological societies have stated that CTs should be used sparingly in COVID and advised to use them when other methods like molecular testing or serological methods are not available. Even though these radiological based tests have low sensitivity and accuracy for COVID19, they are useful for screening the suspected patients.

5. VIRUS CULTURE-BASED TEST

The main basis of viral culture diagnosis depends upon the isolation of SARS-CoV-2 on cell lines promoting growth and replication of the virus. The virus culture is made in the labs from the tissue samples most commonly nasal swabs taken from the patient. The virus isolated from the specimens of suspected patients is grown on primary monkey cells and cell lines including Vero E6 (cell lines derived from Vero cells found to be very sensitive with the viral replication of SARS-CoV). This test relies on patient antibodies to prevent viral infection of cells in a lab setting. Neutralization assays (a culture-based method) are performed on these cultures to see whether the patient contains the neutralizing antibodies against the virus. When viruses and cells are grown with decreasing concentrations of patient antibodies, researchers can visualize and quantify how many antibodies in the patient serum can block virus replication. This blocking action can happen through the antibody binding to an important cell entry protein on the virus. Neutralization assay identifies the antibodies in the patient's serum that blocks the virus replication in the in

vitro culture the exposure of SARS-CoV-2 in the patient's serum specimen. This test must be performed in laboratories with designated biosafety certificates to culture SARSCoV-2-infected cells and has a time-to-result of 3–5 days. This test requires extensive lab skills and a lot of time to give results and is rarely used. Still, it is a gold standard for virological diagnosis.

6. EMERGING METHODS

The innovation of researchers in bringing out the best diagnostic tools is not restricted to the ones mentioned above rather many more tests are waiting to get green signals. Some of such promising and potentially faster and accurate methods are discussed below.

6.1 Biosensors based diagnosis

Nowadays several advanced biosensors-based diagnosis approaches have been utilized for the fabrication of innovative and novel handheld devices that can overcome the drawbacks of a lengthy gold standard detection protocol. These biosensors use the nanomaterials with tunneling and quantum properties leading to enhancement in signal amplification. Further, the nanomaterials are having a high surface-to-volume ratio which enhanced their high sensitivity much fold, moreover, the viruses (target analytes) are also in nanoscale, these all features make the nano sensors a potential diagnostics tool. Nano-biosensors using aptamers are one of such potent analytical tools for rapid diagnosis of diseases with high sensitivity and specificity in a cost-effective and user-friendly manner compared to conventional methods. Such a nano-sensor will have great potential for the detection of SARSCoV-2 even in person without any symptoms with high sensitivity, specificity, and selectivity only for COVID19. Aptamer-based nano-biosensor consist of oligonucleotides of nucleic acids or even small peptide molecules having high specific binding affinity for certain target molecules leading to an increase in sensitivity and accurate detection. These molecules can be any membrane protein, amino acids, toxins, immunoglobulins, cytokines, growth factors, coupling agents, ionic metals, intact cells, or other small molecules. Apta-sensors can be easily converted to any specific design through surface activation or modification by chemical treatment to induce linkers and coupling sites. Due to their high reproducibility and purity, stability, and reversibility under harsh environmental conditions with the vast availability of target-specific linkers, aptamers are being used as novel diagnostics tools. Aptamers can especially be designed and synthesized for the SARS-CoV-2, using its nucleocapsid protein to obtain fast test results within a few seconds only, and it won't require any sample preparation step. Pinpoint's aptamer-based POC for detection of SARS-CoV2 is in the developmental stage, for which the developers are claiming that will provide the SARS-CoV2 test result within 1 min and to be precise in only 30s!

6.2 CRISPER for SARS-CoV-2

CRISPER in the diagnosis of SARS-CoV-2 The use of CRISPER based technology is gaining wide importance for the construction of diagnostic kits where it works by recognition of a specific genetic sequence followed by cutting of the reporter molecule added into the reaction mixture. This process is highly specific and can reveal the presence of the genetic sequence of the virus in 5– 10 minutes. This CRISPER-based approach is called DETECTR for coronaviruses and is coproduced by a Biotechnology company in San Francisco known as Mammoth Biosciences and by a pioneer of CRISPER technology, Jennifer Doudna at the University of California. Similar inputs from Keith Jerome, a virologist at the University of Washington, and Feng Zhang at Broad Institute of MIT and Harvard in the development of a kit referred to as SHERLOCK has been accepted recently for emergency use authorization (EUA) by FDA.

7. CONCLUSION

As of March 2020, Covid-19 has been declared as a pandemic halting all major operations all across the world. The new virus outbreak has challenged the economic, medical, and public healthcare of the majority of the countries. Due to the highly infectious and easy primary nature of transmission, it has spread at a very fast rate all across the world. All current knowledge and data regarding the COVID-19 infection are being exchanged at the earliest since there are uncertainties as to how the disease will evolve and infect the masses eventually. Different scientific and pharmaceutical researches are in progress to determine the preventive and curative measures. Since the strain of coronavirus is found to infect the bats, pangolins and humans as well, it may also lead to the infection and subsequent mutations in many other species. Due to the possibility of the re-emergence of SARS, it has become essential to research the novel coronavirus strains. The development of reverse genetics to study coronavirus has the potential to answer the potential doubts regarding the possible mechanisms of transspecies adaptations, sensitivity to and escape fro biochemical and immune interfaces with replication, determinants of virulence and pathogenesis, mechanisms of genome recombination and mutation, functions of and requirements for replicas, structural and accessory proteins and development of stable viruses for inactive vaccine testing and production. All areas are being looked upon to gain more knowledge on the current pandemic that may help in preventing the same in the future years. With the help of various research projects working towards the production of successful vaccines, the COVID-19 pandemic is expected to get in control by the end of 2021 although the total eradication from the masses may take up to four or five years. At present, the most effective way to stay safe is prevention and practicing social distancing and proper sanitization at all instances of time.

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