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False-Negative Results of Initial RT-PCR Assays for COVID-19

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ABSTRACT

A new virus SARS-COV-2 was discovered in a group of cases of pneumonia in, China on December 2019. Reverse Transcription PCR (RT-PCR) technique that successfully amplifies the etiological agent from Oro-pharyngeal or nasopharyngeal swabs helps us for the identification of many infected people. Many people with COVID-19 symptoms have been testing negative due to several reasons.

An individual who is suspected for having infection and an initial negative result by RT-PCR test, with subsequent test positive is defined as a case of false negative corona virus or severe acute respiratory syndrome.

Results which are false negative have ramifications for accurate diagnosis as well as future transmission in community, as well as control activities, during emergence and in following transmission waves.

People who have been infected with SARS-CoV-2 but have tested negative for the virus are ignorant of their status of infection and could create a wrong feeling of safety based on the results of the test, putting them at danger of the virus spreading further. This would result in a situation where local epidemics are perpetuated, putting individuals having high risk of a severe viral infection.

There are number of causes which can result in false negative results of RT-PCR test which are being discussed below. Not only the causes but also newer techniques how to minimize the false negative rates are discussed along with proper management for the reduction of the same by proper techniques are mentioned below in this review article.

Key words: False negative test, Sensitivity test, RT-PCR, COVID-19

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INTRODUCTION

Cough, fever or shortness of breath is the most common respiratory symptoms related with this etiological agent. With more information regarding the virus, the range of clinical indications and symptoms having association with the virus has grown [1-4].

Nucleic acid amplification test for example RT-PCR (real time) is the gold standard for the diagnosis of COVID-19 on specimens of respiratory system, notably from oropharyngeal and nasopharyngeal swabs, aspirate or wash from nasopharynx Nonetheless, additional data is accumulating about its lack of sensitivity, raising the question of whether the present diagnosis of COVID-19 recommendations provide an appropriate effectiveness and safety level to fight against the spreading virus [5-7].

There has been a variety of RT-PCR tests, which included the viral nucleo capsid is being coded by gene N. The gene E, which codes for the envelope of virus; spike protein is being coded by the gene s, and the RNA polymerase gene (Helicase/RDRP) is being coded by the Hel gene [8,9].

A false-negative diagnosis occurs when an infected individual has a negative RT-PCR result at the time of first testing but a positive test result afterwards [10].

These tests are very specific, although sensitivity isn't perfect for a variety of reasons [11]. The likelihood of SARS-CoV-2 infection cannot be ruled out by one or more negative tests.

For SARS-CoV-2, the likelihood of achieving a false-negative RRT-PCR test depends on a variety of technical parameters and sampling, though as the virus titters decreases in clinical specimen with time the likelihood of receiving a true positive result also decreases.

Researchers believe that numerous pre analytical and analytical factors contribute to SARS-CoV-2 detection failures, including a lack of standardization for specimen collection, delays or poor storage conditions before arrival in the laboratory, and the use of improperly validated assays, contamination during the technique, insufficient viral specimens and load, the disease's incubation time, and the presence of mutations that elude detection or PCR inhibitors are all factors to consider [12-14].

LITERATURE REVIEW

Causes of false negative test results

Genetic diversity: Diversity within a species is referred as SARS-CoV-2 genetic diversity. Because viral RNA genomes are affected by evolution by causing viral sequences variation, it is critical to employ conserved portions of genomes of the virus when constructing primers or probes. Despite our best efforts, because of the mismatches present between target areas and primers due to fast development the results are false negative [15,16].

Errors in sampling: Mistakes that can happen when transporting, collecting and managing RNA samples. Collection of sample is sometimes insufficient, or the health personnel insert nose swabs not deep into the nose till posterior wall so that they get a good sample which has an appropriate virus load.

Types of sample: It relates to sorts of COVID-19 specimens that could be utilized to make a diagnosis. Sputum is the most accurate test for diagnosis, according to one study, followed by nose swabs. In early stages, another study proposed utilizing sputum, throat swabs and nasal swabs. In a prior investigation, virus replication was shown to be limited or non-existent in stool samples. This is a developing topic; as our understanding of COVID-19 grows, the most correct sample type will become increasingly evident. Viruses over time can move from the upper respiratory tract till lower part of the respiratory tract. As a result, specimens from the nasal swab may come out to be negative in certain circumstances [17,18].

Load of virus: This is the viral amount in an individual's swab of nose that has been infected. It's crucial to understand when an infected person's viral load is at its peak. COVID-19 multiplication or replication in the pharynx peaks around 5 days following onset of the symptoms, according to Wolfel, et al.

According to a new study, the viral load in severe and asymptomatic patients is nearly same, implying that both groups are at risk of virus transmission [18,19].

Optimal time: The viral load and virus exposure time are related to RT-PCR false negatives. In a pooled analysis and literature review, researchers found that in RT-PCR test detection the probability of getting the result as a false negative on the first day after getting an infection is almost hundred percent, and that it decreases to sixty seven percent on the fourth day, twenty percent on the eight day, and then increases to sixty six percent on the twenty first day following infection. To simplify, the chance of getting a false negative with RT-PCR is higher whenever the test is performed too fast. In that case, analysers believe that additional testing could be required for improvement in the test's conduction. According to a study from John Hopkins University, COVID-19 takes about a week to incubate after exposure. The timing of sampling, according to the study findings, is a crucial element which can lower the ratio of false negatives. When a patient exhibits symptoms, we can't rule out infection based on a negative test. In these situations, repeating the test boosts our confidence [20,21].

Whereas some studies suggest that testing for SARS-CoV-2 infection by RT-PCR from a single nasopharyngeal or oropharyngeal swab is not certain, also the likelihood for achieving a genuine result which is positive diminishes over time after symptom start. In simple words, the more the period between the beginning of symptoms and the testing of a case of suspicion, greater is the chance of getting a result as false negative. It may not be always possible to do a repeat testing of an individual who is RT-PCR negative as well as suspected for infection at a same time, such as when testing capacity is restricted, but findings imply that repeating tests greatly reduces the risk of missing sick people.

Other factors affecting

A false result can be caused by a lack of sample or a viral mutation; the swab may not gather the virus from the throat and nose despite individual carrying it. Endogenous chemicals, in addition to probable foreign chemicals, could block the conjugate pad membranes of the cassettes at significant concentrations. When antigens saturate the sample, certain 'sandwich' LFIAs might produce false-negative results, known as the Hook effect. Several factors impacting production of antibody, for example genetics, nutrition, sex, immunizations, adjuvants and other parameters impacting immune system, are reported by many authors and hence fast or laboratory IMA results are similarly influenced. Furthermore, antibody degradation caused by handling and sampling can result in false-negative results. False-negative SARS-CoV-2 antibody testing can potentially be caused by autoimmune diseases and their treatment.

The end result can be influenced by both endogenous and exogenous influences. In some situations triglycerides, haematocrit, cholesterol (since the cassette LFIA containing cellulose-based substance is hydrophilic and viscosity has impact on it), haemoglobin, and temperature of sample may alter the end result. Some typical false-negative kinds occur while using the 'gold-standard' real time RT-PCR and extraction free technologies:

- Poor RRT-PCR performance in the laboratory,
- Sample degradation or deficiency,
- Some issues which are technical with probes, kit primers and fluorescence type
- RT-PCR inhibitors and SARS-CoV-2 mutations.
- Poor collection of sample, transport, processing or degradation of viral RNA during the process of storage or shipping might result in poor test results and false-negative results.

Capability of testing, perceived incidence and decisions of policy all influenced RT-PCR testing regimes in different nations. Large groups of people were chosen or rather were examined by some countries, which include the

people who are self-quarantined and are mild to moderate symptomatic or asymptomatic. The test timing distribution is critical in nations like South Korea, where testing is rigorous [22,23]. A false negative result was more likely if many of individuals who were tested and would have been infected few days before the test but only had moderate or asymptomatic disease (hence had not report for management of disease).

According to the available data, people having mild to moderate disease can for minimum eighteen days shed virus [24], while individuals dealing with severe disease could shed for at least twenty days. Although the evidence is clear that proper virus culture is related with more viral loads as well as possibly no perceptible response serologically [25], implying that measuring of viral load that is still detectable quantitatively and serocon version can be useful for discharging patients safely from hospital or quarantine [26].

In some studies even after a median of six days after onset of symptom, infected individuals having an initial negative corona test for the virus had greater inflammatory markers than patients with a positive initial test. Treatment decisions, such as corticosteroids, that have been shown helpful in viral disease, can't be made on basis of the real time PCR results only. The diagnosis of etiological agent in patients should be based on the results of the RT-PCR test, as well as on the clinical findings and presentation from additional tests, like an HR-Computed Tomography scan, RTPCR test as well as extensive using of CT scans for diagnosis must be considered. Patients who have a false-negative first RTPCR test may have better results than those who get a positive first RTPCR test rigorously assessed [26].

Infected individuals having a false negative initial viral test but the end diagnosis of COVID-19 might have clinical, biological, and/or radiological characteristics that are different from those having a first positive RT-PCR testing.

Infected individuals having high counts of platelet or C-reactive protein levels were more likely to have an initial RT-PCR test as false negative. Infected person with symptoms which are non-specific including malaise, fatigue, headache, myalgia, and fever on the other hand, were less likely to have a false-negative initial RT-PCR. A false-negative result was not linked to the time between symptom onset and RT-PCR testing. Finally, the therapies received, the requirement for mechanical ventilation, and hospital mortality did not differ between the patients who had first false-negative test and those with first positive test.

Another crucial problem is the source of the respiratory samples to be analysed. For laboratory identification of the viral infection using RT-PCR, specimen collection at proper time and from the correct anatomical site appears to be critical [27].

DPCR

We were able to correctly detect infection in swab material in a large number of false negatives by using digital PCR, a high sensitivity approach for detecting low amplicon quantities. We show that using digital PCR technologies in the diagnosis of COVID-19 could help to resolve, at least in part, this pressing issue

When the molecular diagnostic approach yields negative results, other factors are considered for the diagnosis of virus, for example the usual appearance of the respiratory system radio logically as discovered by a high resolution CT scan [28]. To tackle this difficulty, researchers have proposed using DDPCR that is droplet digital polymerase chain reaction which is a nucleic acid amplification method, which may identify the etiological agent in upper respiratory tract with a sensitivity of one copy per reaction [29,30].

Highly comprehensive and thorough specimen collection, for example, by focusing over more than one respiratory locations [31], during the course of the illness repeating the tests at different times, or testing the aspirate from bronchus and alveolus along with upper respiratory tract material [32], to minimize this problem is good prevention plan.

Management

Patient's history, information about epidemiology, medical examination, as well as the diagnostic work-up results, which includes all biochemical, microbiological and radiological investigations, is used to make management decisions.

Multiple conserved sections of the viral genome should be targeted at the same time. Taking extra precautions when taking throat and nasal swabs can enhance test accuracy greatly. Choosing the optimal sample type at the right moment during an illness can yield the best results with the fewest false negatives.

Sample collection should be done by an experienced laboratory technologist or a trained healthcare professional to improve testing accuracy. Swabs should be placed in transport medium as soon as possible after being collected. In addition, the period between collecting the sample and performing the test should not be excessive. The sample should be stored for a maximum of 72 hours at 2-8°C. If transferring them within 72 hours is not practicable, they should be kept at-70°C to prevent viral RNA breakdown.

Taking into account the timing of exposure and the beginning of symptoms might help a healthcare provider choose the optimal sample by directing them to the correct anatomical place. As a result, nasopharyngeal and oropharyngeal swabs may be useful for detecting early infection in its early stages.

Repeated viral load measurements and calculations in the individuals who are infected permit for a much more accurate interpretation of reverse transcriptase PCR results, namely whether high cycle threshold *i.e.* CT or

negative result is constant with the trend which is previous in that clinical case or is anomalous, and thus tell us how infectious the patient is after the initial epidemic wave, countries began to reduce severe social separation restrictions and return to some semblance of normalcy, in this case it is very important to reliably contact the trace and test the new illnesses has become vital to preventing reappearance.

Such false-negative test findings must be reduced as much as feasible, because the respiratory doctors as well as other clinical personnel who are taking care for these infected individuals are informed to proper diagnosis as fast as possible, especially when hospitalization and additional management options are required. Apart from a mutation of virus that the assay cannot detect, the PCR assay on respiratory samples can be suppressed in numerous ways, and respiratory medicine doctors should be trained to avoid false-negative test findings in COVID-19 or other pathogens that require identification by PCR assay. Because large amounts of bile salts and bilirubin observed in the samples of human can block the PCR, previous history of medical illnesses such as hyperbilirubinemia relating disorders and jaundice can impact PCR results [33]. In addition, the sample collector's substance has an impact on the PCR assay. Background medical disorders that cause an overabundance of particular proteins (ferritin, lactoferrin, collagen, IgG, myoglobin, heme, and hemoglobin) in samples of human can be essential in predicting RT-PCR test results [34-35]. In accordance with the interim guidance's of each deployed test, citrates, phenolic, polyamines, or polysaccharides detected in samples of human because of previously existing circumstances or unique drug use and metabolism must be taken into account further.

These findings definitely motivate us to treat the patients who are having a more pre-clinical probability (because in the countries with more level of contagion everyone should be taken as consideration) and similar radiological and typical clinical features as seen in people affected by the corona virus, regardless of the results of the RT-PCR (real time), that too if the test is done on the specimen from upper respiratory tract. When one or more RT-PCR assays come out negative, collecting the sample from lower airway system should always be taken into consideration, especially in patients with high degree of illness, where the sputum and BALF yield the test results which are giving greatest positive rate. If we are suspecting COVID-19, a high-resolution computed tomography scan must be conducted at the time of hospitalization, either before swabs or along with the swabs, because doing this will definitely guide the clinical treatment properly from the initial disease grades and to give the maximum positivity rates even after a really small period from the onset of symptom.

DISCUSSION

SARSCoV-2 infection is detected using reverse-transcription polymerase chain reaction (RT-PCR) tests. Although RT-PCR tests are extremely specific and have a minimal risk of false positives, false negatives can occur depending on the type of swab used and the period since the onset of symptoms [11]. An individual who is suspected for having infection and an initial negative result by RT-PCR test, with subsequent test positive is defined as a case of false negative corona virus or severe acute respiratory syndrome.

A test which is negative doesn't remove the possibility of COVID-19 infection, as previously stated.

CONCLUSION

The above findings highlight the importance of repeat investigation in individuals suspected of having infection because of epidemiological or clinical reasons.

This research has improved our understanding of the significant impact that false negative RT-PCR testing can have on identifying SARS-CoV-2 infected people.

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