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“Not All Tests Were Created Equally”

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Abstract:
The most reliable method for routine microbiological diagnosis of coronavirus illness 2019 (COVID-19) is the measurement of viral RNA in respiratory samples using reverse-transcription polymerase chain reaction (RT-PCR). Additional diagnostic tests involve the detection of specific IgM and IgG antibodies for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in patient samples. Evidence suggests that virus shedding commences a few days before to the onset of clinical symptoms. Consequently, viral RNA can be identified in the respiratory tract during the symptomatic phase of the disease, starting around 2-3 days before the emergence of clinical indications. Due to the increased viral load present in lower respiratory tract samples such Broncho alveolar lavage and tracheal aspirate, there is a likelihood of observing a higher rate of PCR positivity compared to nasopharyngeal samples. Confirmatory PCR tests necessitate specialized equipment and skilled workers, in addition to being time-consuming and expensive. Antibody assays are uncomplicated and expedited tests that necessitate minimal equipment and can be implemented in any laboratory. These tests can be conducted using only 2-3 drops of blood obtained from the fingertip of patients. The tests are performed utilizing affordable chromatographic fast assays. These tests are applicable at the later stages of the disease, as particular antibodies become detectable between the 7th and 10th day after the onset of clinical symptoms in individuals with COVID-19. The rapid antibody card tests exhibit average levels of specificity and sensitivity, whereas antibody tests employing micro ELISA demonstrate better levels of both sensitivity and specificity.

الملخص:
الطريقة الأكثر موثوقية للتشخيص الميكروبيولوجي الروتيني لمرض فيروس كورونا ٢٠١٩ (كوفيد-١٩) هي قياس الحمض النووي الريبي الفيروسي في عينات الجهاز التنفسي باستخدام تفاعل البوليميراز المتسلسل النسخ العكسي .(RT-PCR) تتضمن الاختبارات التشخيصية الإضافية الكشف عن أجسام مضادة IgM و IgGمحددة لفيروس كورونا المتلازمة التنفسية الحادة الوخيمة ٢ (SARS-CoV-2) في عينات المرضى. تشير الدلائل إلى أن انتشار الفيروس يبدأ قبل أيام قليلة من ظهور الأعراض السريرية. وبالتالي، يمكن التعرف على الحمض النووي الريبي الفيروسي في الجهاز التنفسي خلال مرحلة أعراض المرض، بدءًا من حوالي ٢-٣ أيام قبل ظهور المؤشرات السريرية. بسبب زيادة الحمل الفيروسي الموجود في عينات الجهاز التنفسي السفلي مثل غسل القصبات الهوائية ونضح القصبة الهوائية، هناك احتمال لملاحظة معدل أعلى من إيجابية تفاعل البوليميراز المتسلسل مقارنة بعينات البلعوم الأنفي. تتطلب اختبارات PCR التأكيدية معدات متخصصة وعمالًا ماهرين، بالإضافة إلى أنها تستغرق وقتًا طويلاً ومكلفة. فحوصات الأجسام المضادة هي اختبارات غير معقدة وسريعة وتتطلب الحد الأدنى من المعدات ويمكن تنفيذها في أي مختبر. يمكن إجراء هذه الاختبارات باستخدام ٢-٣ قطرات من الدم يتم الحصول عليها من طرف إصبع المريض. يتم إجراء الاختبارات باستخدام فحوصات كروماتوغرافية سريعة ميسورة التكلفة. تنطبق هذه الاختبارات في المراحل اللاحقة من المرض، حيث تصبح أجسام مضادة معينة قابلة للاكتشاف بين اليوم السابع والعاشر بعد ظهور الأعراض السريرية لدى الأفراد المصابين بكوفيد-١٩. تُظهر اختبارات بطاقة الأجسام المضادة السريعة مستويات متوسطة من النوعية والحساسية، في حين تُظهر اختبارات الأجسام المضادة التي تستخدم تقنية ELISA الدقيقة مستويات أفضل من الحساسية والنوعية.

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Introduction:

While governments around the world have tried to take preventative measures to contain the virus by wielding those thermometer "guns" at passengers traveling to or from infected regions, this has proven to be ineffective when it comes to slowing the spread of the virus or even identifying infected travelers. Statistics speak louder than words; the CDC reports identifying only one infected individual among nearly 50 thousand travelers (Quilty et al, 2020). This can happen for several reasons; passengers often lie when asked about having a fever or other symptoms out of fear of being denied entrance to their destination country or, worse, being quarantined. Another reason is that core body temperature cannot be measured by handheld digital thermometers, as they can only measure skin temperature, which is usually off by a few degrees. Accordingly, such measures can never be "preventative." At best, they can only delay the epidemic from spreading (Quilty et al, 2020).

Widespread testing helped countries like S. Korea and Germany to curb the spread of the virus, using serological assays, whereas the US has seen surges in the number of cases due to the shortages of clinical supplies needed to perform the tests. Clinical testing, including chest tomography (CT) scans and chest x-rays are to be coupled with RT-PCR testing to overcome such false-negatives (Pecoraro, 2022). Equity with respect to administering serological tests could not be achieved throughout the US due to high cost and the necessity of using specific supplies (e.g., reagents, protective gear) was, thus, less available in rural/low-income neighborhoods (Tromberg et al, 2020). The first documented case in the US was confirmed through laboratory diagnosis on Jan 22, 2020. In less than one month, more than 26 million other cases were confirmed (Stokes et al, 2019). Consequently, and because of these variations in testing equity, the CDC practices and guidelines regarding organizational policies changed during the COVID-19 (Evans, 2020). Such discrepancies left participants unable to understand when or where to go to get tested.

Serological testing (blood tests that can detect the presence of viral proteins or how our immune systems are responding to disease) is proven to be more effective in that aspect (Ghaffari, 2020). The current protocol for diagnosing COVID-19 is the standard test of the reverse transcription polymerase chain reaction (RT-PCR), which aims to detect the virus' RNA in samples received via bronchial aspiration or nasopharyngeal swabs (WHO, 2020). It works by extracting RNA from the genetic samples, which is then converted back to complementary DNA. At this stage, fluorescent probes detect the viral nucleic acids using quantitative amplification. Despite having high specificity, sensitivity is compromised, resulting in receiving false-negative results. This real-time test provides both sensitivity and specificity, where the former refers to "the ability of the test to correctly identify those patients with the disease" (Theagarajan, 2020) and the latter refers to "the ability of the test to correctly identify those patients without the disease" when detecting SARS-CoV-2 (Lalkhen & McCluskey, 2008).

In comparison to other samples received from urine, blood, feces, or even rectal swabs, results obtained from the respiratory system proved to be the most sensitive and specific (Böger et al, 2021). Such tests rely on the PCR method or the polymerase chain reaction, in which fluorescent-laced probes detect viral RNA on the genetic material obtained via a nasopharyngeal swab. The targeted genes include the "nucleocapsid (N), spike (S) and envelope (E) proteins or the RNA-dependent RNA polymerase gene (RdRp)" (Parikh, 2008). This viral RNA is then subjected to a series of reactions of heating and cooling, to get copied. The test itself takes a few hours to be completed. The long wait is attributed to other logistical issues like transport time from clinics to testing facilities, freeing up of the high-tech machines that perform the PCR test, not to mention shortages in solutions and nasal swabs. Although testing time has been decreased to a more efficient rate (by increasing the national testing capacity, with Arkansas medical facilities reporting receiving test results within three days), the duration, from when the patient is tested to the time when they get the results, is still significantly high posing a risk of continued delays (Health Resources & Services Administration, 2020). These findings necessitate that patients be warned of the need to follow quarantine guidelines as they wait for the results (Adalja, 2020).

Serological tests detect current and even past infections, as they detect the antibodies that were released by the immune system in response to the virus; namely the IgM, the antibody produced at the onset of the disease and IgG, which gets produced later. These antibodies linger in the bloodstream even after killing the pathogen's cells and their detection helps track the pandemic and those at risk the most for being infected (Afsin & Demirkol, 2020). A small drop of blood is deposited onto a strip, like those used to detect pregnancy, and then, if positive, two red bands appear. If negative, only one band appears. Once the blood is collected, it takes a few minutes for the result to appear, making it optimal for usage in hospitals, clinics, and other medical facilities. However, while positive results (coupled with symptoms like fever, myalgia, dyspnea, etc.) are considered definitive, negative results need to be confirmed (Satyanarayana, 2023).

Due to the high infectivity and serious pathogenicity of the virus, strict precautions involving heat-inactivation were mandated to ensure biosecurity (WHO, 2019). However, this affected antibody detection values for both IgG and IgM (Wölfel et al, 2019). When attempting to enhance biological safety, heat inactivation is employed to ensure that serum sample and infectious supplies are safe before subjecting them to serological testing. The problem with that is that this process diminishes the virus titer for 30 mins when at 56°C and when raised to 60°C, there remains no residue of the virus (Schunemann et al, 2020). However, Heat-inactivation has been associated with increased IgG and decreased IgM antibody values, leading to both false-positive and false negative results. Therefore, prior to performing Ab testing, testing platforms need to be tested and regulated in accordance with relevant guidelines to ensure accurate results (Tenny & Hoffman, 2020). Moreover, heat-inactivation may result in false-positive and false-negative results as heating affects the protein structure and spatial composition of AB-antigen region (Xue et al, 2020).

While molecular-based tests are characterized by high specificity, sensitivity is compromised, resulting in false-negative results. Accurate and timely diagnosis of the virus is crucial in ruling out (or confirming) the disease, thus helping contain the outbreak of the virus, trace it, as well as control it (Mina et al, 2020). Other factors that affect analytical sensitivity include the stage of infection, type of sample, the skill of the phlebotomist, and even genome mutations of the virus (Albert et al, 2022). Sensitivity rates of antigen tests using samples from the first 7 days of exposure to the virus were reported in a recent study to reach as high as 98% (Corman et al, 2020). This shows that self-administered antigen testing will be invaluable in effective future diagnostic approaches (Cortés, 2021). High analytical sensitivity is crucial to avoid false-negative results. One of the reasons behind false-negative results is due to how some testing providers may not have the skills to obtain adequate nasopharyngeal specimen. Such results can compromise controlling the outbreak of the virus, with seemingly asymptomatic patients, erroneously categorized as uninfected, are allowed to the roam the city, thus, unintentionally transmitting the disease. (Lan et al, 2020). Swab technique training is recommended to overcome this drawback (Li, 2021).

False-positive results can serious repercussions; for example, patients who were tested before their operations had their surgeries postponed until the test results were out. Other patients awaiting to be discharged and who were screened prior to being discharged were detained unnecessarily at the hospital. Even transplant patients testing "positive" had to be removed from the waiting list for 2 weeks, and as a result, missed finding a match and had to start over. Not only that but they had to be re-screened until they tested negative, which is a waste of time and resources (Healy et al, 2021)

Implications of false positive results can differ according to the level of prevalence. In low-incidence settings, positive and negative predictive values are affected with negative values decrease. Such serious results are more significant in low-incidence settings. Conversely, false positive results in this case is not without adverse effects, which may include "unnecessary treatment and investigation, missing or delayed surgery, unnecessary isolation and contact tracing with subsequent negative impact on workforce and resources, a risk of subsequent increased exposure if the individual changes their behavior as a result of believing that they have been infected so they are not careful who they associate with and hence might get exposed to the virus" (Healy et al., 2021).

General information:

The SARS-CoV-2 virus is a pandemic RNA virus in the Coronavirus family. Coronaviruses (CoV) are wrapped RNA viruses that have pretty big genomes. They are made up of a single strand that is positively polarised and 27–32 kb long. The spiral nucleocapsid is made up of infectious genomic RNA and N phosphoprotein. The lipoprotein coat around the virus is made up of the envelope protein (E) and the transmembrane matrix protein (M). The CoV virus has two different kinds of spike-shaped protrusions. The spike (S) and hemagglutinin-esterase (HE) glycoproteins make up the long (20 nm) and short protrusions, respectively. Coronaviruses, which means "crowned virus" and comes from the Latin word "corona," which means "crown," are named after these protrusions because they look like crowns. There are at least six open reading frames (ORF) in the SARS-CoV-2 DNA. Two-thirds of its genome is made up of ORFs that code for non-structural proteins. The other one-third is made up of ORFs that code for at least four structural proteins, which are S, M, E, and N (Figure 1).

HCoV-229E and HCoV-C43 were the first human infectious coronaviruses (CoVs) to be named. They were found in the mid-1960s. There are four main groups of coVs: alpha, beta, gamma, and delta. Each of these groups has several subgroups. Mammals like bats, cats, dogs, pigs, and some types of birds, as well as people, can get sick from COVs. SARS-CoV was found in 2003 and caused a global pandemic. It has since been shown to be 88% genetically similar to SARS-CoV-2, which is causing the current pandemic in 2019. That's right, SARS-CoV-2 is a normal coronavirus. It's in the same family as SARS-CoV and MERS-CoV, which stands for Middle East respiratory syndrome coronavirus.

Human COVIDs can spread a lot of different diseases, from a simple cold to major problems with the lungs and other organs, like in SARS, MERS, and COVID-19. The death rate for COVID-19 was between 0.1% and 21%, with a mean of 3.5%. This is lower than the rates for SARS-CoV (9.6%) and MERS-CoV (34.4%).

COVID-19 was first found in people with pneumonia in Wuhan City, China, in December 2019. The cause of these cases was later found to be a new virus. Because it spreads so easily, SARS-CoV-2 had caused 25 million cases of COVID-19 by September 2020.

Microbiological diagnosis of COVID-19:

COVID-19 has an incubation period of 2–14 days, with an average of 5.2 days. The virus is thought to start leaving the body a few days before signs show up. As long as the symptoms last, viral RNA can be found in the nasal tracts, starting two to three days before the first clinical symptoms show up. Because samples from the lower respiratory tract, like Broncho alveolar lavage and tracheal aspirate, have more viruses, they may show a higher PCR infection rate than samples from the nose and throat.

In samples from the lower respiratory passages, like lavage and aspirate, 93% and 69% of the samples were positive for PCR. The virus has been shown to multiply in the digestive system and to be alive when it leaves the body through the stool. Still, it's not clear how the fecal-oral method of transmission works. Some problems with PCR tests used to prove a diagnosis are that they need the right tools and trained professionals to do them, they take a long time (5–6 hours for the fastest labs), and they cost a lot.

To make a diagnosis and find SARS-CoV-2-specific proteins in clinical samples, it is also important to find the IgG and IgM type-specific antibodies that form against the virus in the patient's blood serum (Figure 2). Although tests that look for antigens in clinical samples are possible, they are not advised because they are not very specific or sensitive. On the other hand, antibody testing can help when PCR can't prove a case even though there are clinical symptoms. There is no doubt that antibody testing has a guiding effect on surveillance studies because it helps keep infected people from getting in touch with others, finds sick people quickly, treats them early, and stops the disease from getting worse. These tests are also very helpful for quickly and correctly diagnosing COVID-19, even though there are different ideas about how to treat it and people haven't been vaccinated yet. Early detection in cases with few or no symptoms can stop the virus from spreading to groups that are more likely to get sick. Antibody testing is a quick and easy test that can be done in almost all labs without the need for expensive tools. For quick tests, 2-3 drops of finger-prick blood can even be collected at the bedside. The only problem with these tests is that they can't be used on people who already have COVID-19 because the antibodies don't show up until seven to ten days after the sickness starts.

IgG and IgM type-specific antibodies that show up in a patient's serum can be found very accurately and sensitively using equipment found in a normal microbiology lab. They can also be found by hand from human blood serum using the micro ELISA method for finding antibodies. The qualitative rapid card-based tests that were widely used during the pandemic to find antibodies on chromatography have an average specificity and sensitivity that are about the same as PCR and micro ELISA tests. The antibody card-based tests are easy to use and don't require any special skills or tools. They also have other benefits, like quick results and low costs.

Even though SARS-CoV-2 temporarily increases the amount of virus in the blood, there is no proof that the virus can be spread through blood transfusions.

This means that samples from the respiratory tract and patient blood are the most important ones that can be used to diagnose COVID-19. Besides this, the following things are also very important to remember: The samples from the lower respiratory tracts probably have more viruses than the samples from the upper respiratory tracts. The viral detection rate can be raised in the samples from the upper respiratory tracts by taking swabs from the nasopharynx and oro-pharynx at the same time and transferring them to the same viral transport medium, if possible. It is best to take samples from the nasopharynx very deeply, which should cause lacrimation and gagging. To keep the PCR reaction from going wrong, polyester or Dacron swabs should be used. Antibody tests might not be useful or important for COVID-19 patients until after ten days of the clinical process.

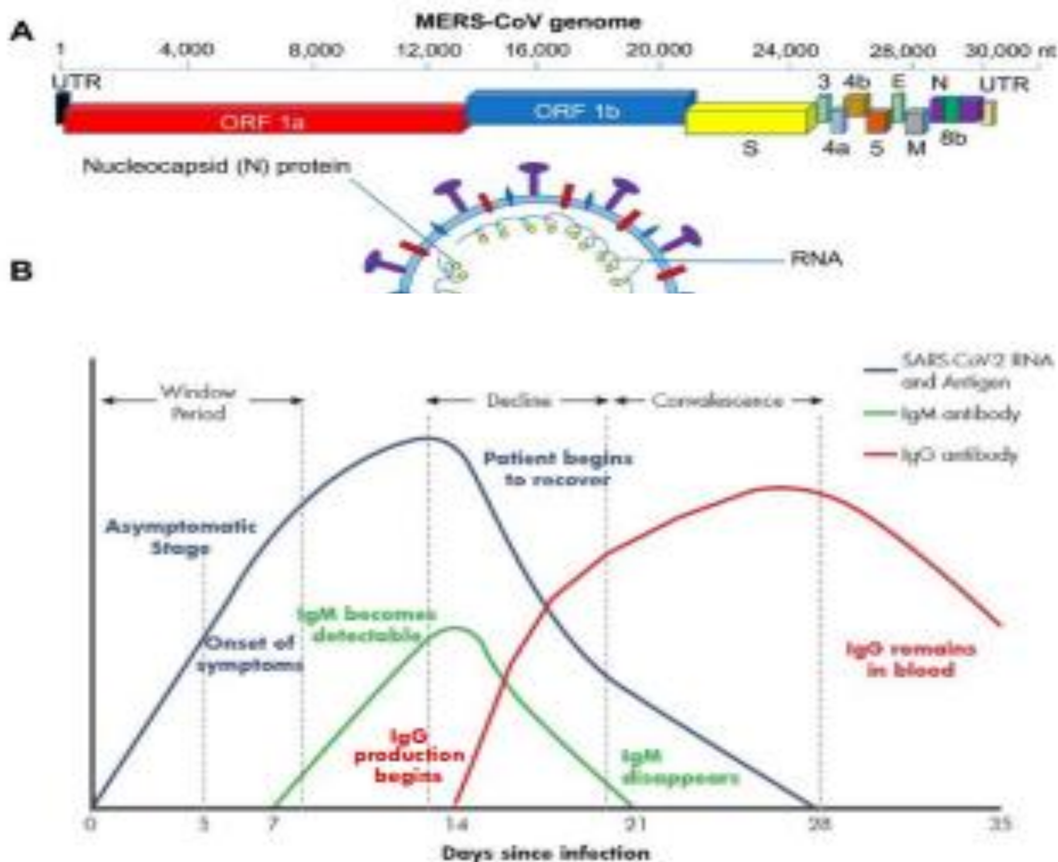

Figure 2. The clinical correlation of the COVID-19-specific PCR and serological tests (13)

PCR Test:

To make a correct molecular diagnosis of COVID-19, it is best to look for SARS-CoV-2 RNA in respiratory tract samples using PCR (RT-PCR). The right clinical samples should be used to get RNA, and PCR procedures should only be done in molecular microbiology labs that have knowledge and the right tools for the job. Biosafety level 2 (BSL 2) steps should be taken, starting with the lab accepting the samples. It should also be made clear that only authorized laboratory staff trained in this area should work in BSL 2 cabinets while wearing protective gear.

PCR to keep going as long as the symptoms last, starting two to three days before the symptoms show up.

There are more viruses in samples from the lower respiratory tract (Broncho alveolar lavage, tracheal aspirate, and sputum), so the PCR success rate is higher for those samples than for samples from the nose and throat. In mild cases, the virus can be gone in ten days. In severe cases, this time can last up to six weeks. Even though samples taken from the back of the throat are clear, the virus may still be present in sputum or stool for a longer time. With PCR tests, viral RNA can be found in 30 to 60% of COVID-19 cases.

The PCR test's ability to find viruses depends on many things, including how long it takes for SARS-CoV-2 to leave the respiratory tract, the sampling method, the storage or transfer conditions during the pre-analytical steps, and the lab's previous PCR experience (Table 1). So, PCR tests should be done again within 24 to 48 hours in people who might have COVID-19. RT-PCR tests used today have only been tested on the respiratory tract, but different studies have shown that SARS-CoV-2 RNA can be found in cerebral fluid and tears. SARS-CoV-2 RNA, on the other hand, is not found in breast milk, amniotic fluid, or vaginal samples. It has been said that in COVID-19 cases, the virus load is high all the time, whereas it is usually only high in the early stages. This is especially true for older people and people with serious illnesses. A high and long-lasting viral load is known to be a key indicator of how well someone will do with COVID-19.

Tests using RT-PCR look at parts of the RdRp, E, N, and S genes. It is known that the E and RdRp genes give the best results.

You can also use nucleic acid sequence research if you need to. The local PCR kit, which was made in Turkey and given away for free by the Turkish Ministry of Health to authorized COVID-19 diagnostic labs, also works on the RdRp and N gene regions. It is 99.4% sensitive and 99% specific.

Antibody Tests:

Table 1. Factors contributing to the false-negative PCR (18)

- Poor quality sample with very little patient material
- A sampling at a very early or late period of COVID-19 infection
- A sample not properly processed and/or sent to the laboratory under unsuitable conditions
- Taking samples with cotton-tipped or wooden-shaft swabs
- Technical reasons inherent in the test such as PCR inhibition or virus mutation
- Wavy scattering of the SARS-CoV-2 virus into the respiratory tract in symptomatic and asymptomatic cases

The PCR test takes a long time to get results, is expensive, and needs skilled medical staff to run and interpret. Because of these problems, people are looking for easier and faster tests to diagnose SARS-CoV-2. The number of tests used to find SARSCoV-2 antibodies quickly grew around the world because people agreed with the FDA's approach to regulating these tests at the start of the pandemic. However, these tests were only used on a small number of people.

There was no agreement on this problem because the different tests used to check for antibodies gave different results. This is known as the "use field of serology." The Infectious Diseases Society of America (IDSA) suggests using serological studies to choose plasma donors, evaluate vaccinations, and do epidemiological studies on people who have clinical signs, even though SARSCoV-2 RNA tests came back negative.

At the start of the pandemic, both IDSA and the World Health Organization (WHO) said that positive antibodies should be seen as a sign of immunization. However, no study has yet been done to see if immunization can be done in people. The WHO is still looking at the proof about how the antibodies react to the SARS-CoV-2 illness.

It was thought that the antibodies made during these treatments would protect against SARS-CoV-2 because immune plasma from people who had recovered from COVID-19 had helped them get better. Even so, there are still some questions because the sensitivity and titration of the epitope have not been figured out. Out of the 12 approved and available antibody kits on the market, only five could test for IgG alone and three could test for both IgG and IgM. Different studies have come to different conclusions about titrations. There is a strong resemblance in antigens between SARS-CoV-2 and other seasonal coronaviruses. This shows how important it is to choose the right antigens for high specificity in serological tests. These tests, which use antibodies made against the N or S proteins of the virus and are approved by reference centres like the FDA and EUA,

were not very sensitive either. This is because it was not possible to stop responses with other coronaviruses.
The antibody tests used for SARS-CoV-2 do not meet the standards for tests meant to stop a pandemic that is spreading quickly. These tests should have a high positive predictive value and high specificity. In a different study that looked at how well serological tests worked, it was found that the four most common antibody tests that had good specificity and sensitivity turned positive no earlier than the tenth day of the disease. This meant that these tests could only be used in a smaller window of time to diagnose COVID-19.
Things that can change the way antibodies are made should be thought about. As an example, the rate of seroconversion is much lower for cancer patients than for healthcare workers.
As a result, the SARS-CoV-2 IgM and IgG diagnostic kits that are currently on the market are not good enough and should not be used for broad screening of the pandemic yet because they are not sensitive enough.

Conclusion:

During peak periods of virus transmissions, aforementioned strategies should be re-instated as they will offer invaluable steps at warding off infection. On average, 58% of COVID-19 patients have had an initial false-negative RT-PCR result. These results suggest that a more sensitive method is needed to improve accuracy, thus decreasing false-negative results, and containing the outbreak of the pandemic. To overcome heat-inactivation issues, a new method for copying the genetic material at a constant temperature has been developed. While it still relies on the PCR method, the fact that the temperature is kept constant has allowed for the test to be done more swiftly. To overcome these barriers to testing, healthcare officials and public leaders can join forces and collaborate on creating an online website that lists updated information specifying locations, hours of operation, and cost (McElfish et al, 2021). Furthermore, local organizations need to be encouraged to partake in making available such tests throughout the financially challenged neighborhoods. Moreover, detection kits need to have reduced development time.
Media reporting of how painful nasopharyngeal swabs needs to be addressed so as not to deter potential patients from taking the tests (Cummins, 2020). Another issue that needs to be addressed is the long wait for the results, as voiced by test takers (Raymond, 2020). It was reported that those who were tested had to wait “two weeks for results, which made testing seem futile” (Lazer, 2020).
Interpretation of laboratory results should be carried out with caution, where values of positive genes are reported, rather than the usual positive/negative binary fashion, along with the Ct value, which will yield useful information when interpreting these results. (Watson, 2020). Clinicians need to consider the possibility of getting both false-positive and false negative. During peak times, false-positive will be an issue, and vice versa, during low-incidence times, false negatives will be more likely. Accurate interpretation of PCR results necessitates proper assessment of risk exposure before mandating molecular testing and that negative results of high-risk patients are cautiously evaluated where they are subjected to a second testing. To minimize false-negative results (currently at around 12% with the range ranging from 2% to 58%) a highly sensitive test combo needs to be applied that detect bot antibodies and the viral genome (Watson, 2020). Serological antibody detection can be used in addition to nucleic acid detection in obtaining both swift and accurate results. This is because they integrate rapid magnetic automated separation and also utilizes chemiluminescence which is highly sensitive, as well as immunoassays which are characterized by high specificity. False-positive could be minimized by adopting more rigorous methods of sampling, collection, and labelling to avoid mislabeling and contamination. Human error in this respect can be minimized by employing highly skilled clinicians and phlebotomists to guard against messy laboratory protocols and imposing strict standards, where Ct levels are below 35 on a single gene are treated with caution (Healy, 2020). When grouping positively-tested patients, caution still should be taken, as some of them could have mistakenly tested positive. Large-scale, routine screening can cause more harm than good at a time of low-incidence and needs to be temporarily deferred when incidence is at its lowest.

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