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MINIREVIEW

Can Testing Predict SARS-CoV-2 Infectivity? The Potential for Certain Methods To Be Surrogates for Replication-Competent Virus

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ABSTRACT Since the beginning of the COVID-19 pandemic, molecular methods (e.g., real-time PCR) have been the primary means of diagnosing the disease. It is now well established that molecular tests can continue to detect SARS-CoV-2 genomic RNA for weeks or months following the resolution of clinical illness. This has prompted public health agencies to recommend a symptom- and/or time-based strategy for discontinuation of isolation precautions, which, for hospitalized patients, results in significant use of personal protective equipment. Due to the inability of current molecular diagnostic assays to differentiate between the presence of remnant viral RNA (i.e., noninfectious) and replication-competent (i.e., infectious) virus, there has been interest in determining whether laboratory tests can be used to predict an individual's likelihood of transmitting the virus to others. This review will highlight what is currently known about the potential for existing assays, such as real-time PCR and antigen tests, to predict active viral infection. In addition, data on the performance of new methods, such as molecular tests targeting viral RNA intermediates (e.g., subgenomic RNA), will be discussed.

KEYWORDS antigen, COVID-19, molecular, SARS-CoV-2, viral culture

Since March 2020, hundreds of laboratory tests have been developed to diagnose coronavirus disease 2019 (COVID-19). Among them, molecular assays targeting one or multiple regions of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) genome have been most common to identify infected individuals. Although the vast majority of these tests offer a sensitive means of diagnosing COVID-19 when performed 1 to 2 weeks following exposure to SARS-CoV-2, they are unable to differentiate between the presence of remnant viral RNA (i.e., noninfectious) and replication-competent (i.e., infectious) virus (1, 2). Several studies have demonstrated that molecular assays, most often real-time reverse transcription-PCR (RT-PCR), may be positive for weeks or months following the resolution of clinical disease (1, 3). In most cases, this is not believed to represent ongoing viral replication; however, SARS-CoV-2 has been recovered in viral culture from immunocompromised patients several months after their primary infection (4), signifying that certain individuals may be able to transmit the virus beyond the period of their acute illness.

Due to the possibility that molecular diagnostic tests can be persistently positive in COVID-19 patients, the Centers for Disease Control and Prevention (CDC) no longer recommend a test-based strategy to guide isolation decisions in most cases (5). Instead, a symptom- and time-based strategy is recommended. For inpatients with COVID-19, this can result in depletion of hospital resources, especially personal protective equipment. For outpatients, a minimum 10-day period of isolation can result in loss of work, separation from family members, and further disruption to routine social interactions.

Recently, significant effort has been directed at determining whether existing laboratory tests, or future methods, can be used to differentiate COVID-19 patients who are no longer infectious from those who pose a risk for ongoing viral transmission. This

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review article will highlight what is currently known regarding the application of laboratory methods (i.e., viral culture, rapid antigen tests, and semiquantitative molecular assays) to predict SARS-CoV-2 infectivity. In addition, the potential for new assays, such as those targeting viral RNA intermediates (e.g., subgenomic RNA), to serve as a surrogate for replication-competent virus will be discussed.

VIRAL CULTURE

SARS-CoV-2 can be cultivated in various cell lines that express the angiotensin converting enzyme 2 (ACE2) receptor, which is required for viral entry. The monkey kidney cell lines Vero-CCL81 and Vero E6 are most commonly used, and viral cytopathic effect (CPE) is often observed within 3 days of inoculation (6). Subsequent analysis of the culture material by real-time RT-PCR or immunostaining can be used to confirm the presence of SARS-CoV-2. Recovery of SARS-CoV-2 in viral culture has served as the reference standard for detecting replication-competent (i.e., infectious) virus throughout the COVID-19 pandemic. However, there are several significant limitations that have prevented this method from being widely deployed.

First, viral culture lacks sensitivity and has largely been replaced by molecular tests for the diagnosis of respiratory infections. In some cases, recovery of SARS-CoV-2 in viral culture has only been accomplished following blind subpassage, which is not universally performed (7). Therefore, a negative result by viral culture does not mean that replication-competent virus is absent (8). Second, routine viral culture is uncommon in many clinical laboratories due to the requirements for special infrastructure and personnel with the appropriate expertise. Finally, cultivation of SARS-CoV-2 in eukaryotic cell lines requires biosafety level 3 precautions since propagation of the virus poses a substantial safety risk to laboratory personnel (9). Due to these limitations, the routine use of viral culture to determine whether COVID-19 patients continue to be a source of infectious virus is not feasible. Therefore, several other methods have been studied to determine their potential to serve as a surrogate approach for predicting the presence or absence of replication-competent SARS-CoV-2.

Author's opinion on the use of viral culture to assess infectivity. To date, recovery of SARS-CoV-2 in viral culture is the only approach that can confirm the presence of replication-competent virus. A positive viral culture should be interpreted as ongoing viral replication and continued risk for transmission. However, due to low sensitivity, a negative culture should not be used to rule out ongoing infection. In symptomatic patients who test positive by a molecular test but negative by culture, it should be assumed they are still infectious. For asymptomatic individuals, a positive molecular test and negative culture require a careful assessment of when the patient was likely exposed to the virus to determine the risk of ongoing transmission. However, the limited availability of viral culture, as well as the biosafety concern of cultivating SARS-CoV-2 and the technical expertise required to do so, renders this method an impractical means of routinely assessing infectiousness.

RAPID ANTIGEN TESTS

A growing number of rapid antigen tests have received emergency use authorization (EUA) for the diagnosis of acute COVID-19. Most often, these tests are designed to detect the SARS-CoV-2 nucleocapsid protein in an upper respiratory specimen, such as a nasal swab. Although these methods are rapid (e.g., results in as little as 15 min) and easier to perform than centralized laboratory tests, concerns regarding their sensitivity have prevented wide-scale implementation. Among patients reporting at least one symptom of COVID-19, rapid antigen tests have shown a sensitivity ranging from 64.2 to 80.0% (10, 11). However, in the asymptomatic population, sensitivity of the BinaxNOW COVID-19 antigen test (Abbott Rapid Diagnostics, Lake Forest, IL) was 35.8% when used at two community-based testing sites in Arizona (10). Similarly, the Sofia SARS antigen fluorescent immunoassay (FIA) (Quidel Corporation, San Diego, CA) demonstrated 41.2%

sensitivity among individuals without symptoms during community screening at two university campuses in Wisconsin (11).

Despite lower sensitivity than molecular testing, it has been postulated that antigen tests serve as a better indicator of viral infectivity. In other words, rapid antigen tests should be positive when an infected individual is shedding the largest amounts of SARS-CoV-2, at which time they pose the greatest risk for transmitting the virus to others. To this point, several studies have demonstrated a correlation between increased antigen positivity and lower RT-PCR cycle threshold (C_T) values. During an evaluation of the Sofia SARS FIA, those samples that were positive by both antigen and real-time RT-PCR had a mean C_T value of 23.7 compared to samples that were positive only by real-time RT-PCR, which showed a mean C_T of 32.3 (11). Another study by Ford et al. (12) demonstrated the sensitivity of the Sofia FIA to be 96.3% in samples with an RT-PCR C_T value of $<$29. While these data show that the performance of rapid antigen tests improves at higher viral loads, other data suggest that a negative antigen result cannot be used to rule out the presence of infectious SARS-CoV-2. In an assessment of the BinaxNOW COVID-19 antigen test in a community screening population, Prince-Guerra et al. (10) identified 11 samples that were negative by a rapid antigen test but positive by viral culture. Similarly, Pray et al. observed that among 18 samples with a negative Sofia FIA result but a positive real-time RT-PCR, SARS-CoV-2 was cultured from two of these specimens (11). These findings suggest that even at higher viral loads that can be detected by viral culture, rapid antigen tests may still be negative.

An additional consideration is whether antigen tests, which most commonly target a single SARS-CoV-2 protein, are more susceptible to false-negative results due to viral mutations. Bourassa et al. (13) observed that a clinical sample with $>$200,000 copies/swab of SARS-CoV-2 repeatedly tested negative by the Sofia 2 SARS FIA. Further investigation by whole-genome sequencing revealed that the virus in this sample contained two coding mutations in the nucleocapsid gene, which impacted detection of this strain by the Sofia antigen assay. While the incidence of viral mutations impacting rapid antigen test performance is unknown, this case highlights an additional limitation that may confound the interpretation of negative antigen results.

Author's opinion on the use of rapid antigen tests to assess infectivity. In patients who have been previously diagnosed with COVID-19, the use of rapid antigen tests to predict the risk of viral transmission requires further study. In these cases, a positive antigen result implies that infectious virus is present. A number of studies have demonstrated that COVID-19 antigen tests have high specificity ($>$99%) (14). However, due to low analytical/clinical sensitivity and the possible impact of COVID-19 variants on antigen test performance, a negative result should not be used as the sole criterion to conclude that an individual is no longer contagious.

REAL-TIME RT-PCR QUANTIFICATION AND CYCLE THRESHOLD VALUES

Most SARS-CoV-2 molecular tests used during the COVID-19 pandemic have utilized real-time RT-PCR technology. These tests are often designed to be qualitative assays, generating either a positive or negative result for the presence/absence of SARS-CoV-2 RNA. However, many real-time RT-PCR tests provide a C_T value that is inversely proportional to the starting concentration of viral RNA target in the clinical sample. Since molecular tests, including real-time RT-PCR, can be positive in patients with COVID-19 beyond the period of their acute illness, there has been interest in incorporating a more quantitative assessment of the viral RNA into the test report. Some have suggested that the real-time RT-PCR C_T value be included in the report to assist in differentiating between active infection and the persistence of viral RNA (15). For example, a positive result with a C_T value of 20 might be highly suggestive of an active SARS-CoV-2 infection, whereas a C_T value of 35 could imply a low level of remnant RNA that is not associated with infectious virus.

Several studies have assessed the potential for C_T values to aid in the interpretation of SARS-CoV-2 RT-PCR results. Bullard et al. (16) performed a retrospective, cross-sectional

study in which 90 PCR-positive samples were inoculated on Vero cells to examine the presence of replication-competent SARS-CoV-2. Following routine RT-PCR testing, samples were stored at –80°C for up to 1 month prior to inoculation in cell culture. Among the 90 RT-PCR-positive samples, 26 (28.9%) showed growth of SARS-CoV-2 in viral culture. For each unit increase in the RT-PCR C_T value, the authors observed an approximately 32% reduction in the recovery of SARS-CoV-2. Furthermore, no virus was cultivated from samples with a RT-PCR C_T value of $>$24 or from patients who were $\geq$8 days from their onset of symptoms. From these data, the authors concluded that the likelihood of a COVID-19 patient with an RT-PCR C_T value of $>$24 being infectious is low.

Singanayagam et al. investigated the possible correlation between RT-PCR C_T values and the ability to recover SARS-CoV-2 in viral culture (17). This study included 324 upper respiratory tract (URT) samples that were positive by RT-PCR and subsequently inoculated onto Vero E6 cells. Among the 324 specimens, the median C_T value was 31.2, and the authors did not observe a statistical difference ($P = 0.79$) in C_T values between patients who had asymptomatic, mild-to-moderate, or severe disease. However, a strong correlation was observed between the C_T value and growth of SARS-CoV-2, with the odds ratio (OR) of a positive viral culture decreasing by 0.67 with each unit increase in the corresponding RT-PCR C_T value. When URT samples were collected from patients with at least 10 days of symptoms, SARS-CoV-2 was recovered in only 6% of cases. However, 5 (8.3%) of 60 samples with an RT-PCR C_T value of $>$35 yielded SARS-CoV-2 in viral culture (17). A similar study by La Scola et al. (7) showed a close relationship between the recovery of SARS-CoV-2 in culture and RT-PCR C_T values. Among 183 RT-PCR-positive samples, 100% with a C_T value between 13 and 17 yielded culturable virus. A stepwise decrease in the culture positivity rate was observed with increasing C_T values, with only 12% of samples with a C_T of 33 showing growth in viral culture. However, unlike the findings of Singanayagam et al. (17), who observed a low rate (8.3%) of culture positivity in specimens with a C_T value of $>$35, no virus was recovered in samples with a C_T of $\geq$34 (7).

Other groups have attempted to generate quantitative viral load data to aid in the prediction of SARS-CoV-2 infectivity. A study by van Kampen et al. (18) included 129 hospitalized patients with a positive SARS-CoV-2 real-time RT-PCR result. For each patient, a respiratory sample was inoculated into viral culture. Real-time RT-PCR C_T values were converted to $\log_{10}$ RNA copies/ml using a quantified envelope (E)-gene calibration curve. The results from this study demonstrated that a viral load of $>$7 $\log_{10}$ RNA copies/ml was strongly correlated with recovery of SARS-CoV-2 in culture. Furthermore, only 5% of samples with a viral load of $<$6.63 $\log_{10}$ RNA copies/ml yielded culturable virus. Wolfel et al. (19) observed a similar trend during their assessment of 9 hospitalized patients with COVID-19. Patient samples were tested by quantitative real-time RT-PCR and viral culture, and results demonstrated that viral recovery and viral loads peaked during the first week of symptoms. No samples collected beyond 8 days of symptoms were positive by viral culture, and $<$5% of specimens with a viral load of $\leq$5.4 $\log_{10}$ RNA copies/ml yielded replication-competent virus.

Although these data show a correlation between the concentration of viral RNA and culture positivity, there are several significant limitations to using RT-PCR C_T values and viral load thresholds to predict infectivity. First, there are currently no assays that have received EUA for the quantitative assessment of SARS-CoV-2 RNA in clinical samples. This represents a significant regulatory challenge for laboratories who may consider reporting C_T values generated by EUA methods. Second, it may become increasingly difficult to establish a C_T value threshold to predict infectivity, as the viral load present in clinical samples may vary as SARS-CoV-2 variants continue to emerge, and the impact of vaccination on viral levels in those who become infected remains to be defined (20). Third, it is well established that C_T values cannot be directly compared across testing platforms due to variability in target design, nucleic acid extraction technology/efficiency, and amplification chemistry (21). A study by van Kasteren et al. (22) compared the performance of seven commercially available COVID-19 real-time RT-PCR assays and demonstrated variation in the detection rate and C_T values generated by the different tests. In addition,

proficiency testing (PT) data from the College of American Pathologists showed that among 700 laboratories who tested the same PT material by various EUA RT-PCR methods, C_T values differed by up to 14 cycles (21). Additional factors impacting the precision of C_T values include the inherent variability in specimen collection (e.g., transport media used and storage conditions), the quality of the specimen, and the type of sample tested (e.g., throat swab versus nasopharyngeal swab). Finally, samples containing very low levels of viral RNA (i.e., high C_T values) may show increased variability in C_T values if tested in multiple replicates and may even fluctuate between positive and negative results.

Author's opinion on the use of RT-PCR C_T values to assess infectivity. Due to current regulatory requirements, the large number of SARS-CoV-2 RT-PCR platforms being used in clinical laboratories, and the variability in C_T values across these systems, the C_T value should not be included in the patient report. In addition, a universal C_T value or viral load threshold to predict infectivity cannot be established at this time. However, C_T values can provide helpful, supplemental information in certain cases. These situations require consultation with the laboratory director to ensure that the results are interpreted in the context of the patient's clinical status, the specimen type collected, and the assay used for testing.

VIRAL RNA INTERMEDIATES

Following infection of a host cell, SARS-CoV-2 undergoes a complex process of RNA replication, which occurs in the cytoplasm of the infected cell. Like other coronaviruses, SARS-CoV-2 replication involves the synthesis of genome-length, negative-sense RNA that serves to amplify positive-sense genomic RNA (23). In addition, a simultaneous process of discontinuous transcription results in the production of subgenome-length RNAs, each containing a common leader sequence (24). Due to the fact that these subgenomic RNAs (sgRNA) are transcribed following host cell infection and are not believed to be commonly packaged into new virions, they have been proposed to represent a potential marker for active infection and viral replication (25).

Several studies using a primate model of SARS-CoV-2 infection have demonstrated that detection of sgRNA may serve as a surrogate marker for replication-competent virus (25, 26). Dagotto et al. (25) compared the detection of sgRNA and total RNA in rhesus macaques that were experimentally challenged with SARS-CoV-2 and had either been (i) previously infected with SARS-CoV-2 (i.e., were convalescent and had existing immunity) or (ii) treated with anti-SARS-CoV-2 monoclonal antibodies (MAb) prior to infection. In both the convalescent rhesus macaques and those who received MAb therapy before viral challenge, sgRNA was not detected, while the total RNA PCR was positive. These findings suggest that sgRNA-based PCR assays assist in differentiating between actively replicating virus and the presence of viral RNA.

Speranza et al. (26) performed a study in African green monkeys to assess the potential of sgRNA to serve as a marker of active viral infection. This group challenged 8 animals with 2.6×10^6 replication-competent SARS-CoV-2 particles, while two control animals were inoculated with virus that had been inactivated using gamma irradiation. SARS-CoV-2 genomic RNA was detected in both groups of animals; however, only sgRNA was detected in animals inoculated with infectious virus, supporting its potential use to identify replicating virus.

A growing number of human studies have also evaluated the correlation of sgRNA with active viral infection. Perera et al. (27) tested 33 respiratory samples collected from patients with COVID-19 by both sgRNA PCR and viral culture. Among the 33 specimens, 12 (36.4%) were positive by both viral culture and the sgRNA PCR, while 12 (36.4%) were negative by both tests. Interestingly, of 22 specimens that were collected within 8 days of symptom onset, 18 (81.8%) were positive for sgRNA. In contrast, only 1 (9.1%) of 11 samples collected $\geq$9 days into the illness yielded detectable sgRNA.

Rodriguez-Grande and colleagues studied 60 patients who were persistently positive ($>$21 days from symptom onset) for SARS-CoV-2 RNA (28). In 48 (80%) of these cases, genomic RNA was detected but sgRNA was not, which the authors concluded to

suggest the presence of noninfectious virus (i.e., remnant genomic RNA). However, sgRNA was detected in the remaining 12 cases, including a patient who was 79 days postonset of symptoms. Although the authors' interpretation of these results was that a subset of patients may continue to be infectious for a prolonged period of time (i.e., as suggested by the detection of sgRNA), a significant limitation of this study was the absence of correlative viral culture data.

An additional approach to assess for active viral replication has been to test for the presence of strand-specific RNA transcripts. Hogan et al. (29) developed a real-time RT-PCR assay specific to the E gene minus strand, which is produced during active SARS-CoV-2 infection. To evaluate this method, specimens from 146 COVID-19 patients were tested by the novel method. The RT-PCR assay targeting minus-strand RNA was positive in 41 (28.1%) of these cases, including two immunocompetent patients who were >10 days from their disease onset. In addition, one immunosuppressed individual continued to test positive for minus-strand RNA for up to 30 days after developing symptoms, suggesting ongoing viral replication. A limitation of this study was that the results of the minus-strand RT-PCR assay were not correlated with viral culture; however, the approach may serve as a future laboratory tool and provide supplemental information to manage immunosuppressed patients or those with persistently positive SARS-CoV-2 molecular results.

Despite these data suggesting that viral RNA intermediates represent future markers for active SARS-CoV-2 infection, several other studies have raised important questions that should be addressed prior to broad implementation of this approach. Although it remains unclear exactly where SARS-CoV-2 transcription and replication occur in the host cell cytoplasm, it has been postulated that the viral replication complex locates inside double-membrane vesicles, which may protect viral transcripts for enzyme degradation (24, 30). To evaluate this possibility, Alexandersen et al. (24) tested clinical respiratory (i.e., oropharyngeal and nasopharyngeal swab) samples by next-generation sequencing (NGS) and routine SARS-CoV-2 RT-PCR assays. Prior to testing by NGS or RT-PCR, a subset of the samples was treated with Triton X-100 as a means of assessing whether lipid membranes play a role in protecting viral RNA from enzyme degradation. The authors observed that among the samples treated with Triton X-100, detection of SARS-CoV-2 RNA targets was significantly reduced. Samples that had stable lipid membrane fractions also were highly resistant to nuclease treatment, as determined by continued detection of SARS-CoV-2 RNA. In addition to observing that subgenomic RNA appears to be protected from cellular nucleases, this group also demonstrated that viral RNA intermediates could be detected for up to 17 days after disease onset, suggesting they do not serve as an accurate marker of active infection (24).

Dimcheff et al. (31) also examined whether subgenomic transcripts could serve as a surrogate for viral infectivity. Droplet digital PCR (ddPCR) was used to detect and quantify E- and N-gene genomic RNA as well as sgRNA from these two targets. The authors observed that sgRNA for the E- and N-genes could be detected for a median of 14 and 25 days, respectively. Furthermore, although the sgRNA targets became undetectable sooner than genomic RNA, the rate at which sgRNA and genomic RNA declined compared to symptom duration was equal. These data suggest that the earlier loss of detection of sgRNA (compared to genomic RNA) is due to the lower overall concentration of subgenomic transcripts rather than a true correlation with viral infectivity. Therefore, the authors concluded that sgRNA is no more predictive of the viral concentration or infectivity than RT-PCR C_T values measuring total RNA.

Author's opinion on the use of SARS-CoV-2 RNA intermediates to assess infectivity. Although promising, it is premature to use molecular assays targeting viral RNA intermediates (e.g., subgenomic RNA, strand-specific RNA) as a sole determinant for assessing SARS-CoV-2 infectivity. Published data suggest there is a difference in the analytical sensitivity of sgRNA targets (e.g., N-gene sgRNA > E-gene sgRNA), so the choice of sgRNA to measure may have a significant impact on detection and result interpretation (31, 32). Finally, due to data showing that sgRNA may persist beyond the period of acute disease, further studies are needed to correlate the detection of

sgRNA with viral culture results, or, more importantly, an individual's ability to transmit the infection to others.

SUMMARY AND FUTURE DIRECTIONS

Routine molecular tests for SARS-CoV-2 can be positive in COVID-19 patients for weeks or months following the resolution of their illness (1, 3); therefore, it is imperative that we develop tools that can differentiate between persistent shedding of viral RNA (i.e., noninfectious) and active viral replication (i.e., infectious). To date, no single laboratory method can serve as a reliable predictor of viral infectivity. When positive, viral culture can be used to confirm the presence of infectious virus; however, this method lacks sensitivity, and, therefore, a negative result cannot rule out ongoing infection (8). In the setting of a previously positive SARS-CoV-2 molecular test, a negative rapid antigen test suggests that a patient is no longer shedding large amounts of the virus, but there are now multiple reports of patients who have tested negative by rapid antigen yet continue to have virus recovered in cell culture (10, 11). Much attention has been focused on the possible correlation between RT-PCR C_T values and viral infectivity. Although there is a strong association between decreasing rates of viral culture positivity and increasing C_T values (16, 17), the lack of standardization in RT-PCR platforms and the large amount of variability that exists in the semiquantitative capabilities of this technology represent a significant limitation to using this approach as a routine way of differentiating infectious versus noninfectious individuals (21). Finally, the recent work that has been done to assess the potential application of subgenomic and strand-specific viral RNA assays offers promise as a future tool to manage complex cases, including those involving immunosuppressed patients and individuals who experience persistent COVID-19-related symptoms. A number of questions remain with regard to use and interpretation of these tests, including (i) how long does sgRNA persist after viral replication ceases, (ii) what is the correlation between sgRNA detection and transmission from one person to another, and (iii) what sgRNA targets might serve as the most accurate and reliable predictors of infectivity? Tools such as the one developed by Telwatte et al. (33), which uses ddPCR to detect and quantify multiple genomic and sgRNA transcripts within a single assay, might provide sufficient information on the viral replication dynamics to make informed decisions on the possibility for ongoing infection and transmission. Ultimately, future laboratory methods that are designed to predict infectivity will need to be evaluated in comparison to viral culture as the current reference standard but also using samples collected from patients who have been determined to be a likely source of viral transmission through contact-tracing investigations. These methods will need to be accurate and provide timely results to inform isolation decisions, and they should be designed to be performed at low cost so that the scale of testing can meet future demand. Until such tools are developed, refined, and fully validated against a collection of laboratory, clinical, and epidemiologic data, there will be a continued need to rely on existing symptom- and time-based strategies to inform the discontinuation of isolation decisions in patients with COVID-19.

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