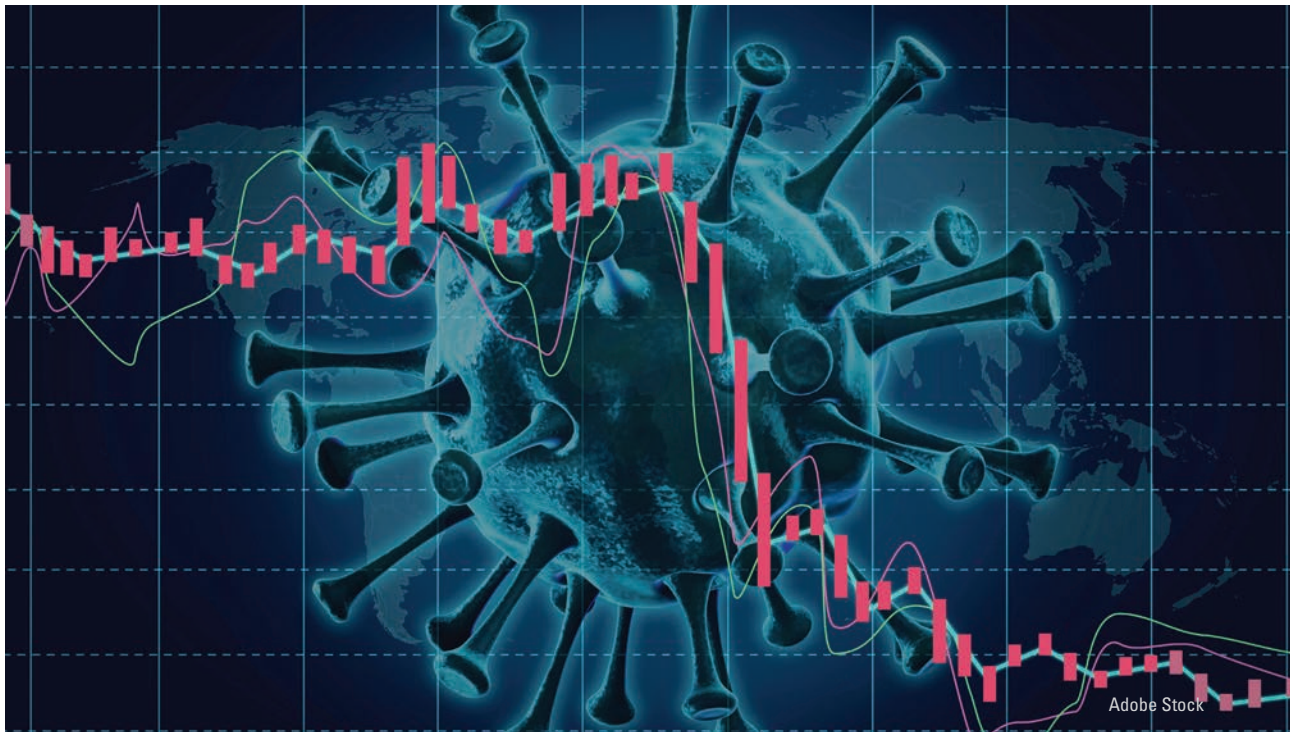

The role of cycle threshold values in infectious disease diagnostics

By Kelly Hughes, PhD, Sonia Rao, PharmD, BCIDP, BCPS, and Davide Manissero, MD, MRCPCH, MSc, DTM&H

Effective interventions in the management of infectious diseases rely on rapid and specific detection of causative pathogens, driving patient care decisions and outcomes. Successful pathogen detection is facilitated by reliable, accurate diagnostic tools; and among the variety of testing tools available, molecular assays such as polymerase chain reaction (PCR) have become dominant. Sensitivity and specificity are paramount in diagnostics and molecular testing provides both. In theory, PCR can detect the presence of a single copy of nucleic acid from a single organism. The use of sequence-specific primers and probes in these assays yields unparalleled specificity and allows for testing to be multiplexed to detect multiple pathogens in one test run. This specificity allows not only for detection, but also for determining the presence of specific genes for typing different strains and antimicrobial resistance profiling.[1] Alternatively, primers and probes can be designed to be less specific, or target highly conserved regions, to provide broad coverage and detect divergent pathogen genomes.[2]

PCR testing is successful in identifying organisms that cannot be grown *in vitro* or in situations where current culturing techniques lack sensitivity and/or require prolonged incubation periods.[2] Other testing methods, such as culture or serology, often do not match the level of sensitivity or timeliness of molecular testing. In some cases, alternative methods may increase the risk of false negatives or incorrect results in critical testing situations where fastidious pathogens may be difficult to grow or immune responses difficult to detect.

Utility of cycle threshold values

Effective PCR tests have now been developed for a wide variety of pathogens. However, these PCR tests, specifically real-time PCR (RT-PCR) tests, can reveal more than just the simple presence of a pathogen. Results from molecular tests are typically reported in a binary, positive or negative manner; however, this approach may not recognize the full benefits these assays have to offer. In addition to reporting pathogen detection, RT-PCR reports cycle threshold (Ct) values. The cycle threshold is defined as the thermal cycle number at which the fluorescent signal exceeds that of the background and, therefore, passes the threshold for positivity. Ct value is inversely related to the quantity of target nucleic acid in the sample, with lower Ct values reflecting higher viral loads, which

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LEARNING OBJECTIVES

Upon completion of this article, the reader will be able to:

1. Recall the strengths and limitations of RT-PCR tests.
2. Describe the definition of cycle threshold (Ct) value and how it is used in infectious disease diagnostics.
3. Discuss research into the utility of Ct values in assessing COVID-19 disease progression.
4. Discuss research into the utility of Ct values in assessing disease progression for other respiratory diseases as well as gastrointestinal illnesses.

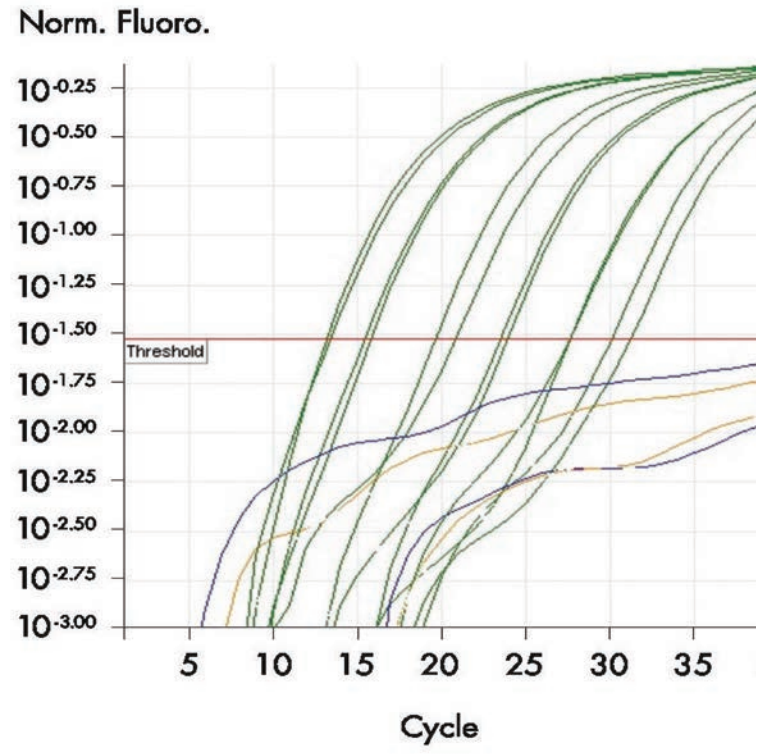
can allow for comparison of relative levels of pathogen. Using this principle, a more specific quantification may be obtained with the use of a standard curve of known DNA quantity. This quantification can help establish pathogen burden and distinguish between what are normal, commensal levels and what are pathogenic levels of a microbe.

In addition to quantification, Ct values can provide additional information that may be useful when complex or unexpected results are received. Analysis of amplification curves and Ct values can be helpful in assessing sample quality and/or potential contamination. For patients with co-infections, the Ct values from a multiplex panel can help clarify which pathogen is most likely the causative agent in an illness. For patients on antimicrobial therapy, comparison of Ct values over time can be used as an indicator of the response to therapy.[1]

While PCR testing and Ct values can offer a deeper insight during the diagnostic process, they are not without limitations; perhaps the most important of which is the inherent inability to distinguish between live and dead organisms. The exquisite sensitivity of PCR allows for the detection of minute quantities of nucleic acid; however, this does not always correlate with the presence of live organisms. The assay will amplify nucleic acid from dead microbes as readily as from living.[1] As dead organisms can be shed for weeks after recovery, a recovering patient might have the same positive test result as an actively infected patient. It is important to be able to distinguish between these two patients, as the positive test from the recovering patient may have no clinical relevance, while the actively infected patient may require treatment. In this situation, utilizing Ct values for relative quantification of the pathogen may help the clinician distinguish between these two patients.[3]

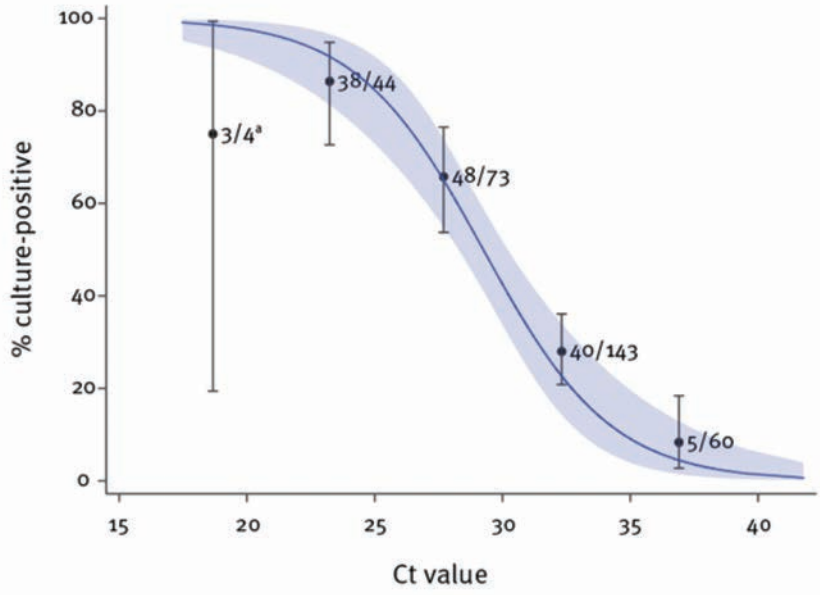
It is important to understand that Ct values should not be interpreted as a unit of pathogen load without a standard curve. This is due to the number of variables that can affect reaction efficiency and amplification. As a result, Ct values are dependent on multiple factors, including sample type and quality. For example, the presence of inhibitory factors in a sample will reduce reaction efficiency, affecting both Ct values and final test results. Inconsistencies between different assays and machines must also be taken into consideration. Without a standard curve, Ct values can still provide an indirect, relative quantification of pathogen load; however, it is important to keep in mind that Ct values can only be compared if the reaction efficiency is

Figure 1. RT-PCR Amplification plot



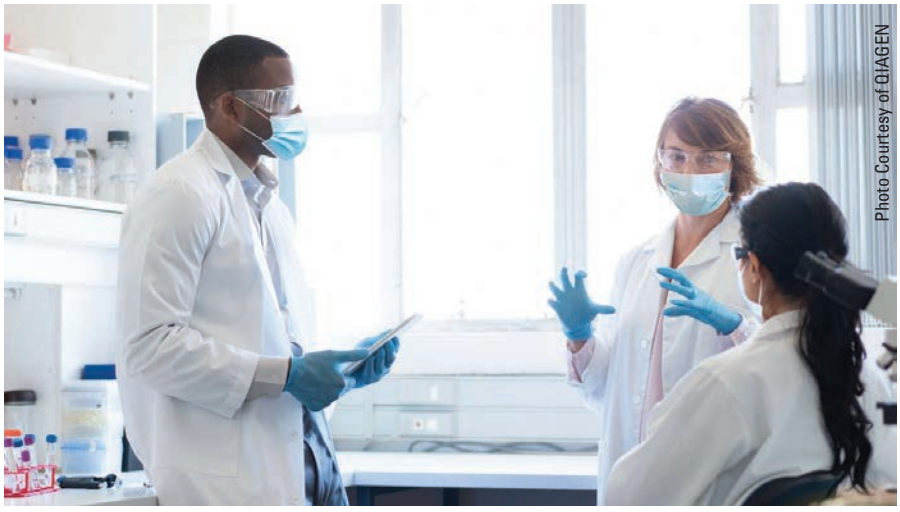
Amplification plot from RT-PCR assay, showing positive samples (green) with threshold used to determine Ct values, and negative samples (purple, yellow) Source: QIAGEN

Figure 2. Ct value is strongly related to the ability to recover infectious SARS-CoV-2 virus



The ability to recover infectious virus is strongly related to Ct value, as the estimated odds ratio of finding infectious SARS-CoV-2 virus decrease by 0.67 for every unit increase in Ct value. All five samples with Ct >35 from which virus was successfully propagated were from symptomatic patients and none had severe illness. Source: Centers for Disease Control and Prevention. Wide confidence levels is due to small sample size. https://www.cdc.gov/library/covid19/090420_covidupdate.html source:covid19/090420_covidupdate.html

Ct values can inform patient management



The additional information provided by Ct values can help clinicians determine the best course of action in patient management.

similar and the target genes have the same number of copies.[4] This additional layer of complexity in understanding Ct values may limit a broader use of this data, narrowing use to only those personnel who have been trained to understand and appropriately interpret data from RT-PCR tests.

Ct values in COVID-19

The ongoing COVID-19 pandemic, with its clinical unknowns and a lack of standardized quantitative assays for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has brought on an unprecedented level of interest in the clinical utility of Ct values. Patients infected with SARS-CoV-2 can display an array of clinical presentations, ranging from an absence of symptoms, to the requirement of intensive care and respiratory support, to an eventually fatal outcome.[5]

In fact, the proposed disease pathogenesis of COVID-19 indicates two overlapping disease states: an initial viremic phase where signs and symptoms are attributable to viral replication, and a subsequent inflammatory phase where the severity and critical illness is attributable to a "cytokine storm."[6] While these two phases have different management implications from a treatment choice perspective – antiviral/antibody therapy versus anti-inflammatory therapy – a clear prognostic approach is still missing. The ability to predict prognosis and infectiousness of patients during the viraemic phase could fundamentally impact triage and management of patients and, potentially, of their contacts.

Early in the COVID-19 pandemic it was suggested that evidence from the 2002 SARS-CoV epidemic indicated that higher viral loads were associated with increased need for intensive care and overall worse outcomes. While there are differences between SARS-CoV and SARS-CoV-2, it has emerged that, similar to SARS-CoV, the viral load of SARS-CoV-2 could also play a key role in determining COVID-19 disease severity and prognosis, as well as infectiousness.[3,7-11]

While the recent appearance of antigen tests has added to the panel of diagnostic solutions for COVID-19, RT-PCR has been the gold standard diagnostic approach since the beginning of the pandemic. Several of the COVID-19 RT-PCR solutions available on the market provide Ct values as part of their results data, and experts argue that these could be used as a semi-quantitative proxy for viral load in the absence of quantitative assays.

A recent systematic review assessed evidence to establish whether SARS-CoV-2 Ct values correlate with clinical outcomes and, therefore, whether they could provide valuable patient management information to clinicans.[12] The findings indicate that lower Ct values may be associated with adverse outcomes and, in several instances, a correlation with clinical course and prognosis was identified. While the review concluded that, in general, higher Ct values correlate with lower viral loads, limitations have been highlighted around the fact that Ct values and lower viral load may not be directly proportional due to the presence of inhibitory factors within samples as well as the linear dynamic range of the assay.[4]

While the results of SARS-CoV-2 PCR diagnostic assays remain – in the majority of cases – reported as positive or negative results in the clinical setting, a discussion has emerged about the possibility of reporting and interpreting Ct values to aid clinicians in patient management. The increasing evidence supporting the utility of Ct values has led public health authorities, such as Public Health England and the Centers for Disease Control and Prevention (CDC), to develop guidance and recommendations regarding their use. Experts agree that if Ct values are to be reported, linearity, limits of detection and reference quantification curves must be standardized and validated. Interpretations of a single positive Ct value may be of limited use in the clinic.[13,14] However, indications are emerging that serial Ct values may offer greater utility for the purpose of clinical management.[13]

What is certain is that – in the absence of quantitative assays for SARS-CoV-2 – Ct values have become of increasing interest with regard to providing interpretative guidance, including as a potential benchmark for the development of antigen and rapid tests.

Ct values beyond COVID-19

With, finally, a light at the end of the tunnel with the emergency use authorization (EUA) of several COVID-19 vaccines, it would be remiss not to highlight the potential application of Ct values beyond SARS-CoV-2. Prior to the COVID-19 pandemic, the most common causes of respiratory illness in seasonal circulation were influenza virus, respiratory syncytial virus (RSV), parainfluenza virus (PiV), human metapneumovirus (HMPV), adenovirus (AdV), rhinovirus and coronavirus (hCoV). RT-PCR is one of the most common diagnostic modalities for detection and identification of respiratory pathogens. There are several studies that have attempted to elucidate the clinical utility of Ct values for respiratory pathogens with contradictory reports that Ct values are associated with disease severity, intensive care unit (ICU) admission and length of hospital stay (LOS).[15-17]

The results of many studies evaluating associations between Ct values and clinical outcomes for patients with influenza were conflicting. However, a large influenza study identified that patients with low Ct values were significantly more likely to self-report moderate to high disease severity and fever.[18] Several studies of patients with RSV reported association between low Ct values with clinically meaningful outcomes such as hospitalization, radiographical evidence of pneumonia or LOS, while others have failed to identify such associations. Despite many small rhinovirus studies having no association between Ct values and patient

outcomes, there are a few large studies that found that low Ct values were associated with symptom severity and hospital LOS.[19] For non-SARS-CoV-2 respiratory pathogens, Ct values for some respiratory infections may be useful in guiding treatment and healthcare decisions; however, the evidence supporting utility of Ct values is conflicting and warrants further investigation.

Multiplex PCR testing for viral, bacterial, and parasitic causes of gastrointestinal illnesses is one of the newer uses of syndromic diagnostic testing. As infectious diarrhea is estimated to cause more than 48 million illnesses and 3,000 deaths per year in the United States alone, any markers that could potentially help stratify patients to aid in clinical management is greatly needed. Compared to respiratory illness, there are fewer studies investigating a correlation of Ct values and clinical outcomes for gastrointestinal pathogens. A case-control study evaluated the application of molecular detection on relative amounts of gastrointestinal pathogens.[20] There were significantly higher pathogen loads in cases versus controls for *Campylobacter spp*., *Salmonella spp*. Enterotoxigenic *Escherichia coli* (ETEC), typical Enteropathogenic *E. Coli* (EPEC) and *C. parvus/hominis*. Of note, Ct values for *C. difficile* and Enteroaggregative E.Coli (EAEC) were significantly lower for cases than for controls in patients 21–50 years old, and Ct values for atypical EPEC were significantly lower in cases than control for patients younger than 5 years old.

This study highlighted that low Ct values indicate high pathogen load and have the potential to suggest microbiological causes of gastroenteritis. A separate study specifically assessed the utility of Ct values on *C. difficile* disease and found significantly lower Ct values reported for patients with severe disease versus those with mild/moderate disease (median Ct values 25.9 vs 28.1, respectively; p=0.00001), with significant difference in the median age between samples with Ct values <25 and Ct values >25 , 79 and 74 years, respectively (p=0.004).[21]

As treatment of *C. difficile* infections is guided by severity of symptoms and disease, the ability to predict severity of disease through Ct values as a surrogate for bacterial load has the potential to impact patient management and antimicrobial selection.

Conclusion

In conclusion, there are many considerations when utilizing Ct values as a surrogate for pathogen load in the laboratory setting and for use in the patient management decisions. However, the COVID-19 pandemic has highlighted the potential ability of Ct values to assess disease severity and those at higher risk of mortality. Although for other infectious pathogens the evidence is not as conclusive and many studies are conflicting, we should continue to evaluate the utility of Ct values to determine impact on patient management, as well as educate both laboratorians and clinicians on how to best interpret this numerical degree of PCR test positivity.

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Kelly Hughes, PhD, is a Medical Science Liaison in infectious diseases for Medical Affairs at **QIAGEN.**

Sonia Rao, PharmD, BCIDP, BCPS , is a Senior Medical Science Liaison at **QIAGEN**.

Davide Manissero, MD, MRCPCH, MSc, DTM&H, is a Vice President and Head of Medical Affairs and Chief Medical Officer – Infection and Immune Diagnostics – at **QIAGEN.**

TEST QUESTIONS

Circles must be filled in, or test will not be graded. Shade circles like this: ● Not like this: ✗

1. **Among the variety of testing tools available, molecular assays such as____ have become dominant.**
   - A. next generation sequencing
   - B. polymerase chain reaction (PCR)
   - C. flow cytometry
   - D. genomic testing

2. **The use of sequence specific primers and probes in PCR assays yields unparalleled specificity and allows for testing to be multiplexed to detect multiple pathogens in one test run.**
   - A. True
   - B. False

3. **PCR testing can verify the presence of specific genes, enabling laboratorians to:**
   - A. type different strains of disease; determine appropriate pharmacologic agents
   - B. complete antimicrobial resistance profiling; determine if the virus is active
   - C. type different strains of disease; complete antimicrobial resistance profiling
   - D. determine if the virus is active; complete antimicrobial resistance profiling

4. **PCR testing might be a good choice in situations in which current culturing techniques:**
   - A. lack sensitivity and/or require prolonged incubation periods
   - B. are not able to recognize viable cells and/or require highly skilled personnel
   - C. are very sensitive and/or resource intensive
   - D. are resource intensive and/or require highly skilled personnel

5. **In addition to reporting pathogen detection, RT-PCR reports____.**
   - A. cycle threshold values
   - B. complete blood count
   - C. activity levels of proteins
   - D. whole chromosomes

6. **The cycle threshold is defined as the thermal cycle number at which the fluorescent signal exceeds that of the ____ and, therefore, passes the threshold for ____.**
   - A. foreground; negativity
   - B. background; negativity
   - C. foreground; positivity
   - D. background; positivity

7. **Ct value is ____related to pathogen quantity, with lower Ct values reflecting ____ viral loads, which can allow for comparison of relative levels of pathogen.**
   - A. inversely; lower
   - B. inversely; higher
   - C. directly; lower
   - D. directly; higher

8. **A quantification obtained with the use of a standard curve of known ____ can help establish pathogen burden and distinguish between what are ____, commensal levels and what are pathogenic levels of a microbe.**
   - A. DNA quantity; normal
   - B. RNA quantity; abnormal
   - C. DNA quantity; abnormal
   - D. RNA quantity; normal

9. **For patients with co-infections, the Ct values from a multiplex panel can help clarify which pathogen is most likely the causative agent in an illness.**
   - A. True
   - B. False

10. **One limitation of PCR testing is its inherent inability to:**
    - A. quantify nucleic acid
    - B. distinguish between live and dead organisms
    - C. produce high false positive results
    - D. produce high false negative results

11. **The presence of inhibitory factors in a sample will reduce reaction ____, affecting both Ct values and final test results.**
    - A. time
    - B. specificity
    - C. efficiency
    - D. inefficiency

12. **It is important to keep in mind that Ct values can only be compared if the reaction efficiency is similar and the target ____have the same number of copies.**
    - A. nucleic acids
    - B. genes
    - C. chromosomes
    - D. proteins

13. **The ongoing COVID-19 pandemic has brought on an unprecedented level of interest in the clinical utility of Ct values because of the ____.**
    - A. clinical unknowns of the virus
    - B. lack of standardized quantitative assays for SARS-CoV-2
    - C. lack of treatments
    - D. A and B

14. **There are no COIVD-19 RT-PRC solutions available on the market that provide Ct values as part of their results data.**
    - A. True
    - B. False

15. **If Ct values are to be reported, ____, ____ and reference quantification curves must be standardized and validated.**
    - A. regression, linearity
    - B. linearity; limits of detection
    - C. limits of detection, regression
    - D. linearity; analytical sensitivity

16. **Indications are emerging that serial Ct values may offer greater utility for the purpose of clinical management of ____.**
    - A. SARS-CoV-2
    - B. influenza
    - C. respiratory syncytial virus
    - D. adenovirus

17. **A large influenza study identified that patients with ____ Ct values were significantly more likely to self-report ____ disease severity and fever.**
    - A. low; low to moderate
    - B. high; low to moderate
    - C. low; moderate to high
    - D. high; moderate to high

18. **Multiplex PCR testing for viral, bacterial, and parasitic causes of ____ illnesses is one of the newer uses of syndromic diagnostic testing.**
    - A. neurological
    - B. gastrointestinal
    - C. respiratory
    - D. circulatory

19. **Infectious diarrhea is estimated to cause more than ____ million illnesses and 3,000 deaths per year in the United States.**
    - A. 18
    - B. 25
    - C. 48
    - D. 59

20. **One study highlighted that ____Ct values indicate ____ pathogen load and have the potential to suggest microbiological causes of gastroenteritis.**
    - A. low; high
    - B. high; low
    - C. low; low
    - D. high; high

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