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Editorial

The Use of Arbitrary and Generalized Ct Values in COVID-19 Non-Quantitative Reverse Transcriptase Polymerase Chain Reaction (nonQ-RT-PCR) Testing Must End

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An elementary problem with the polymerase chain reaction (PCR) – recognized at its inception – is that non-specific binding of PCR primers to arbitrary targets will cause off-target amplification. This is true whether the target sequence is a gene sequence within a single individual, and it is true when the target is viral, such as the SARS-CoV-2 viral genome in a clinical sample. PCR is used routinely to produce enough copies, or amplicons, of the target nucleotide molecule so the sequence can be determined using either Sanger sequencing or Next-Generation sequencing.

In RT-PCR, the viral nucleotide molecules must be reverse transcribed (thus the RT) to DNA nucleotide molecules because RNA itself cannot be amplified by DNA polymerases.

In RT-quantitative PCR (RT-qPCR) cycling usually is conducted to 40 or 45 cycles, resulting in an amplification kinetic curve. Due to non-specific, off-target amplification, this curve may be generated whether there are viral RNA molecules in a patient's sample or not. The study of the entire amplification curve is necessary to make the presence/absence call. This is because patients with SARS-CoV-2 infections vary in their viral load – and their clinical swab samples vary arbitrarily in the amount of viral RNA present. Therefore RT-qPCR test kits, such as those in use for the Monkeypox virus, use positive controls – targets of known sequence and known quantity, and baseline varies from individual sample to individual sample.

As the polymerase chain reaction progresses following reverse transcription, two amplification curves are generated: one for the clinical sample, and one for the internal positive control. The comparison of the curves (using methods such as delta delta Ct; Rao et al., 2013) allows a relative quantitative determination of the amount of viral material present by providing an empirical determination of a cycle threshold (Ct) that corresponds to the exponential part of the curve derived for the positive control. Further, if the positive control fails, a false negative can also be avoided.

If the positive controls are not used, or even if they are used but the information is not used to determine a sample-specific Ct, and an arbitrary Ct value is used for clinical samples instead, the

number of true positives and false positives found by nonQ-RT-PCR tests will be primarily a function of the cycle cutoff used for a kit. When prevalence is low, a high rate of false positives among the positive samples is expected if the Ct cutoff is too high. The higher the arbitrary Ct threshold for a kit, the higher the number of false positive results expected among the positive results. The percentage of clinical samples that are identified falsely as positive for the SARS-CoV-2 virus among all samples that test positive is known as the false discovery rate.[1]

In diagnostic pathology, it is well-known that screening tests with false positives can pose a serious risk to patients, especially in cancer diagnosis. The rate of false positives, commonly referred to as the "false positive rate", is commonly used for evaluating a new test against a gold standard test or the ability of a pathologist in diagnosing cancers correctly. This false positive rate does not vary with the prevalence of cancer in the patient population being screened. It is the function of the ability of a specific test or a specific person in cancer diagnosis.

In SARS-CoV-2 molecular tests, the issue of high false discovery rates for nonQ-RT-PCR, or RT-qPCR, screening was first reported by Basile et al. (2020) but was also noted by Wernicke et al. (2021) and others. A study of Marine recruits failed to produce sequences for about 36% of the non-quantitative RT-PCR (Letizia et al., 2020), and yet inexplicably concluded that mass SARS-CoV-2 screening should be conducted more frequently.

When SARS-CoV-2 tests were being evaluated for emergency use authorization, a few vocal scientists, myself being among them, warned FDA and CDC that the use of non-quantitative RT-PCR results alone would yield false positive results – reports that the viral genome target was present when in fact it was not – unless the amplicon population was sequenced. The target nucleotide sequence, of course, should match the known genetic sequences of the target. (In the case of COVID-19, the amplicons should match the genetic sequence of the virus announced by the Chinese Center for Disease Control and Prevention on January 9, 2020.)

When virus is not present, and amplification occurs anyway, the dominant amplicon should match some other nucleotide source, such as the patient's genome. Further, If the dominant amplicons were routinely sequenced, the identities of the off-target amplicons in the false positive results could be known. For example, Lee (2021) reported that a published forward primer designed to detect the SARS-CoV-2 N gene via RT-PCR has 100% match with at least 3 genomic sequences which are not those of SARS-CoV-2. In spite of the absence of evidence of 100% specificity, PCR tests have been used to diagnose "COVID‑19" based on a positive PCR test result alone, and without confirmation that a viable viral sequence was present in the clinical samples.

FDA refused to consider Sanger sequencing as a primary clinical test for COVID-19, placing the entire population at risk of a test with a high false positive rate and a resulting high false discovery rate. Yet studies have shown that the virus can only rarely be cultured in samples called positive using Ct > 35. Singanayagam et al. (2020), for example,

[1] The False Discovery Rate, or FDR = FP/(TP+FP). This is not to be confused with the False Positive Rate, aka, the probability of false alarm or FPR = FP/(FP+TN). Because both share the same numerator, however, comparison across situations will show increases in FDR will show a tendency in increases in FPR, sharing about 81% variation with every point increase in FDR leading to 0.9-point increase in FPR, all else being equal. Among pathologists evaluating one test against the standard, FDR is often referred to as "The False Positive Rate Among the Total Test Positive Samples" sometimes leading to confusion with the FPR commonly used in epidemiology.

found that only 6/35 samples that reached a detection threshold only after 35 cycles could be cultivated in the lab. This corresponds to a 91% false discovery rate. Their data, converted to FDR, are presented in Figure 1; the curve-wide FDR for the use of nonQ-RT-PCR implied by their data is 58.6%.

The inability to culture viruses from samples that require high Ct values to call the virus "present" has been acknowledged as problematic by Dr. Anthony Fauci of the US NIAID (Racaniello et al., 2021).

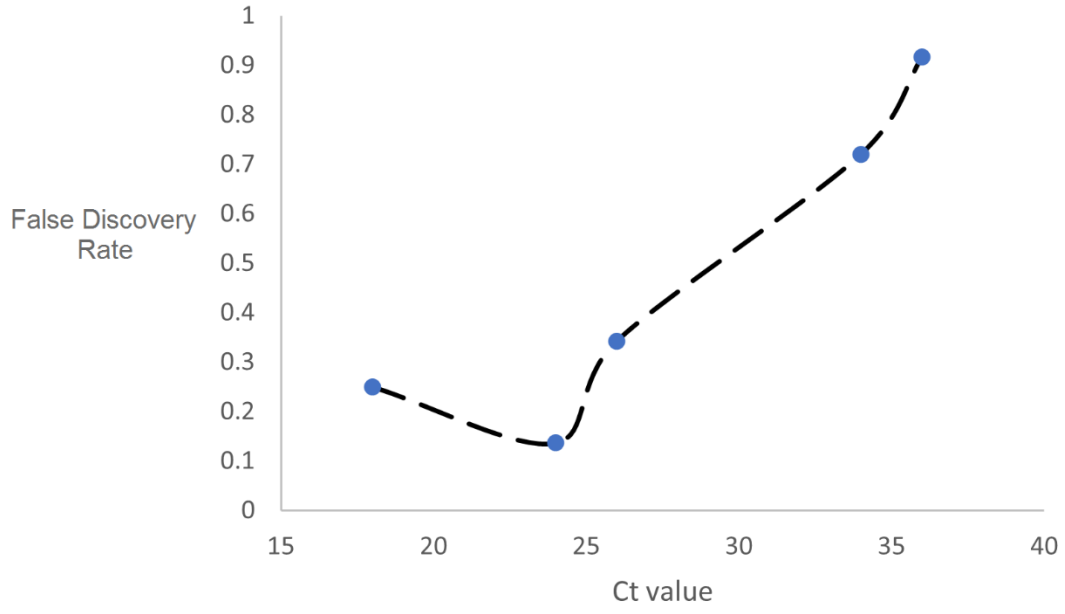
This issue has been raised by US media (*New York Times*, 2020), which reported that as many as 90% of positive test results might not indicate "contagious" COVID-19. Indeed, CDC issued a guidance for submitting samples for sequencing to be restricted to vaccinated persons only if their Ct < 27. Obviously, this would cause the false impression that new variants that cause PCR failure emerged among the unvaccinated due to a lack of sequence data from the matching vaccinated population of unvaccinated patients with Ct ≥ 27.

Even the World Health Organization has issued alerts acknowledging the problem of high false positive rates among the positive test results as an elevated risk:

> "WHO reminds IVD users that disease prevalence alters the predictive value of test results; as disease prevalence decreases, the risk of false positive increases (2). This means that the probability that a person who has a positive result (SARS-CoV-2 detected) is truly infected with SARS-CoV-2 decreases as prevalence decreases, irrespective of the claimed specificity."
> (WHO, 2021)

Their citation is of Altman, 1994. This issue has been known for nearly 30 years. Why this aspect of screening with tests with false positives at low prevalence was ignored – and in many cases denied – by members of the public health community in the US is a mystery.

Figure 1. False Discovery Rate calculated from Singanayagam et al., "Duration of Infectiousness and Correlation with RT-PCR Cycle Threshold Values in Cases of COVID-19…"



In another guidance document, the WHO acknowledged that PCR tests can "produce false signals at high Ct values."

Evolution changes in the SARS-CoV-2 viral mRNA sequence at PCR primer sites have also caused primer failure at the target site, as would be expected due to early practice of quarantine of PCR-positive persons and the use of 2-out of-3 primer pair results. If one primer pair failed due to mutations at the primer target site, the expected false negative rate would be 50% for that population of the virus, and the lineage missed by the test would spread – at least until the fault in the test was discovered. We have seen reports of S-gene drop-out now occurring twice associated with the emergence of new lineages; inexplicably, the FDA has concluded that such a loss of information would not lead to a loss in sensitivity. Until the S-gene drop-out was discovered, the tests using ⅔ primer set positive would have lost sensitivity by 50% in those patients carrying the variants that led to the S-gene drop-out.

Now, Lee (2022, this volume) has confirmed that sample reference sets themselves contain false positives, meaning the validation of specific individual non-quantitative RT-PCR kits is in question. This is the second time he has reported false positive results in gold-standard sample reference sets (see also Lee, 2021). Further, his results also show that sequencing will result in the much-desired "zero false positive test" called for by CDC Director Rochelle Walensky et al. (Paltiel et al., 2020), an evaluation performance characteristic that sequencing does not share with nonQ-RT-PCR.

Note that both the Lee study and the Basile et al study focus on samples drawn from a population of "cases" previously screened as "positives" for confirmatory testing, and both authors have used the term false positive rate "loosely" to quantify the percentage of screening errors – a common acceptable practice in diagnostic pathology; their use should not be conflated with the population-wide false positive rate, which of course will also be increased by any factor that causes an increase the number of false positives.

The current state of knowledge of the effects of non-Q-RT-PCR with arbitrarily high threshold is at direct odds with a scientifically based public health policy for COVID-19. Not everyone who tests positive via RT-PCR has "COVID-19", and not everyone who tests positive "for COVID" via a positive RT-PCR who dies has died "from COVID-19". This is also true if they have a true positive COVID-19 test when the infection is not a contributing factor to their death.

Since the emergence of the SARS-CoV-2 virus, large social media companies have been responsive to demands from "official" experts controlling the narrative in the public square to ensure that only the CDC's, NIH's (including NIAID), and the FDA's positions on these matters are understood by the public. Many scientists and physicians have been blocked and censored for sharing this type of information. The rapid dissemination of accurate information is key to public health, and this cannot occur when the results of objective peer-reviewed studies are prevented from becoming public knowledge.

The few scientists who warned the FDA and CDC about these issues in early 2020 were ignored. Yet in 2003, CDC shut down SARS transmission using what FDA has acknowledged for other tests to be the gold standard assay: Sanger sequencing. Sanger sequencing is recognized by the FDA as the gold standard for PCR-based detection of Enterovirus. One cannot help but wonder whether we could have prevented such a massive negative impact and cost of COVID-19 on our society if Sanger sequencing had been adopted from the beginning, preventing hundreds of millions of unnecessary rounds of 10-day isolation.

There is also an unmeasured clinical cost of COVID-19 false positives in terms of morbidity and mortality: the missed diagnoses of other respiratory ailments. Influenza cases dropped to near zero for a while in 2020/2021; further, the conflation of bacterial pneumonia or fungal infections with COVID-19 can be fatal. Patients and the families of patients whose life-saving surgeries have been delayed due to COVID-19 false positives are also among those who suffered needlessly.

The actual number of false positives since PCR testing was adopted is hard to know, in part because prevalence estimates have depended on nonQ-RT-PCR and estimating the FDR and FPR requires knowledge of the prevalence. However, at very low prevalence, the FDR problem becomes more amplified. Clearly, the issue of the cost of the false positives outnumbering true positives when the prevalence is low must be fully addressed, and a massive course correction must be undertaken; indeed, the entire concept of COVID-19, and past case number and death number estimates, must be revised and updated based on sequence-confirmed data and rate adjustments.

The WHO recommends restricting PCR testing to only symptomatic patients, urging "careful interpretation of weak positive results" and a focus on increasing the positivity in the tested population via restricting testing to those who most likely need it. While helpful, this tactic would decrease the false discovery rate among tested patients, because COVID-19 would be enriched among the patients with respiratory symptoms. It would not, however, not prevent primer drop-out and false negative results because the primer strategy for RT-PCR is not nested or hemi-nested. Studies are needed on diagnostic algorithms that consider (1) the exposure to a sequence-confirmed COVID-19 case, (2) symptoms, (3) antigen tests (which can be non-specific), and (4) the vaccination status of patients. "Sequencing" does not entail the determination of the entire genome of the virus; rather, overlapping "hemi-nested" primer sets of the type designed and published by Lee (2022) should be adopted by hospitals for internal suspected COVID-19 case confirmation.

It follows that the efficacy outcomes of vaccine studies and treatment studies that have been conducted using PCR tests with arbitrarily high Ct thresholds as the basis for the diagnosis of COVID-19 must now similarly be re-assessed.

We must return to the use of Sanger sequencing as the Gold Standard nucleic acid assay for infectious diagnosis. Dr. Lee has generously provided – for unrestricted use – the primer sets that any hospital can use to confirm or rule out a suspected case of SARS-CoV-2 infection and reduce the disease burden caused by inaccurate COVID-19 diagnoses.

The policies and practice of equating the positive test with COVID-19 disease and the automatic assignment of COVID-19 as a cause of death due to positive PCR results alone also remain problematic.

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