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COVID or no COVID: Interpreting inconclusive SARS-CoV-2 qPCR results in different populations and platforms

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A B S T R A C T

Introduction: High cycle threshold values (Ct) value) results for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may be true infections or false-positive results. Misinterpretation of results has negative consequences. Goal of this study was to evaluate quantitative real-time polymerase chain reaction (qPCR) results with high Ct-values, to reach a point where a correct interpretation can be given.

Methods: High Ct-value results of SARS-CoV-2 in respiratory samples taken between April 2020 and January 2021 were analysed. Three different SARS-CoV-2 qPCR assays (in-house, Alinity M and Xpert Xpress) were used for screening patients and healthcare workers (HCW). High Ct-value results were defined as "inconclusive". The Ct-value cut-off for the interpretation of the test as "positive" and "inconclusive" were based on quality assurance panel results and manufacturers' instructions.

Results: Out of totally 50.295 samples tested for SARS-CoV-2, the in-house and Alinity M qPCR together yielded 379 inconclusive results. A second sample existed for 217 samples, allowing dynamics of the PCR in time. Of these, 187 were negative (86%), 11 again inconclusive (5%) and 19 positive (9%). Sixteen out of 19 persons with a positive result were HCW, 14 (74%) had a link to a SARS-CoV-2 infected person. The majority of inconclusive results detected with the Xpert Xpress (n=45 of 3603), were related to individuals with a known history of SARS-CoV-2 infection (n=28, 62%).

Conclusion: This study shows the importance of re-testing inconclusive SARS-CoV-2 qPCR results. Only then, the correct (true or false) interpretation can be given, leading to the right measures.

1. Introduction

Since the beginning of the severe acute respiratory corona virus 2 (SARS-CoV-2) pandemic, large numbers of detection tests have been carried out by most diagnostic virology laboratories. The most reliable and accurate method is by quantitative real-time polymerase chain reaction (qPCR). Besides being negative or positive, the test results in a cycle threshold (Ct) value, reflecting the viral load; with lower Ct-values reflecting higher viral loads. A correlation between Ct-value and infectivity exists, which is usually determined by successful culture of the virus [1].

Both positive and negative tests have ramifications for every tested individual with regards to isolation and contact tracing. Reliable results are therefore essential in all contexts. False-negative results may lead to an infectious person not being adequately isolated and therefore permitting the continuous spread of the virus. False-positive results have adverse consequences especially in hospital settings; these patients may be admitted to COVID-19 units and cohorted with other positive patient. Moreover, medical procedures and treatments may be postponed, depending on local protocols. False-positive results in healthcare workers (HCW) may lead to inflated staff shortages. False-positive results have made headline news during the pandemic, with Olympic athletes being banned from competing and professional sports teams having to cancel games. These incidents have been used by critics to sow distrust in the medical profession and public handling of the pandemic [2]

It has been shown that at the beginning of a SARS-CoV-2 infection, the Ct-value decreases very quickly from negative to peak viral load within 48 hours [3,4]. False-positive results may result from errors at any point in the pre-analytical, analytical and post-analytical phase [5]. While errors in the pre-analytical phase and the post-analytical phase may lead to false-positive results with any Ct-value, errors in the analytical phase result from nonspecific signals from sample material and cross contamination from other samples within some analytical platforms, usually yielding results in the lower range of detection with high Ct-values.

High Ct-values may cause interpretative difficulties. They may represent the detection of small quantities viral RNA at the beginning of an infection, or the end of an infection with persistence of viral RNA. It may also represent a false-positive result where no viral RNA is present in the sample. An additional test, carried out the next day can often help distinguish early infections, which will most likely have a higher viral load and lower Ct-value the next day, from a past infection, which will most likely have the same of a lower viral load the next day. False-positives which did not detect true infections are most likely negative the next day. The predictive value of a test is dependent on the tested population (pre-test probability; symptomatic or asymptomatic).

In this retrospective observational study, we evaluate the outcomes of SARS-CoV-2 PCR results with inconclusive results, i.e. high Ct-values, around the lower limit of detection. To determine the likelihood that inconclusive results represent true or false results we evaluated 239

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follow-up samples of patients and HCW after an initial inconclusive result tested from April 2020 to January 2021.

2. Materials and methods

Between April 2020 and January 2021, the medical microbiology laboratory of the University Medical centre Groningen routinely tested their patients as well as their personnel. Patients were screened before admission, before surgery and in case of symptoms. HCW were tested in case of symptoms and in case of close physical contact with an infected individual irrespective of the setting of this contact.

For retrospective observational analysis of inconclusive high Ct-values, we extracted all our SARS-CoV-2 qPCR results (only respiratory tract samples) from April 2020 till February 2021 from patients and HCW. Within this observation period, all inconclusive test were selected, and outcomes of follow-up samples – when present – were evaluated. For the descriptive statistics, SPSS version 28 was used.

2.1. Targets and interpretation per assay

Results of three qPCR assays were analysed for purpose of this study: the in-house developed SARS-CoV-2 qPCR which was used during the entire period, the Xpert Xpress SARS-CoV-2 (Cepheid) which was used from June 2020 onward and the Alinity M (Abbott) assay which was used from October 2020 onward. The in-house and Alinity M assays were used for both patients and HCW. The Xpert Xpress was mainly used for patients. Cut-off values for positive, negative and inconclusive results were determined using quality assurance panels and trend analyses.

2.2. In-house SARS-CoV-2 qPCR

The in-house SARS-CoV-2 qPCR targets the envelope (E) gene, based on Corman et. al [6]. It requires pipetting steps which were carried out in laminar flow cabinets. Materials were handled one by one. Only when primers and probes were added by the pipetting robot, all samples were open and accessible at the same time, resulting in a limited potential for cross-contamination. In patients without a known history of a SARS-CoV-2 infection, results with a sigmoidal amplification curve and a cycle threshold (Ct) value of ≤33 were considered positive, Ct-values between 34 and 40 inconclusive, and over Ct 40 were considered negative.

2.3. Alinity M real-time qPCR (Abbott)

The Alinity M instrument performs automatic sample preparation, PCR assembly, amplification and detection of SARS-CoV-2, targeting the RNA dependent RNA polymerase (RdRp) gene and N gene [7]. Materials are pipetted in laminar flow cabinets and handled one by one, while several can be open at the same time within the instrument that is closed by a lid, representing a limited potential for cross-contamination. In patients without a known history of a SARS-CoV-2 infection, results with a sigmoidal amplification curve and a Ct-value of ≤37 were considered as positive, Ct-values between 37 and 41 as inconclusive, above Ct 41 as negative.

2.4. Xpert Xpress SARS-CoV-2 monoplex (Cepheid)

The Xpert Xpress SARS-CoV-2 is a rapid automated qPCR, targeting the E gene and nucleocapsid (N2) gene [8]. Every material is handled one by one in a laminar flow cabinet, with the further PCR being performed in a closed procedure, representing virtually no potential for cross-contamination. This test reports a positive result when the N2 gene alone or both genes (E and N2 gene) are detected and a presumptive positive test when the E gene is detected. Based upon our quality assurance process, we adjusted these automatically generated results as follows: irrespective of the N gene, in patients with an unknown history of a SARS-CoV-2 infection, results with E gene of Ct ≤33 are considered as positive, Ct-values between 34 and 45 as inconclusive, above Ct 45 as negative. All N gene results without the E gene being detected, were considered as inconclusive.

2.5. Inconclusive results, reporting and follow-up

All inconclusive results were reported as indeterminate with the information that the result either fits a non-specific reaction, past infection or the beginning of an infection with SARS-CoV-2. In patients with a known history of a SARS-CoV-2 infection, we reported every PCR result yielding a Ct value as positive. In absence of a known history of SARS-CoV-infection, a new sample was requested for the next day to determine the definite outcome of the qPCR result, preferably after 12–24 hours. For the purpose of this study, we selected patients and HCW of whom a follow-up sample was obtained after an initial inconclusive result. The time between the two samples was categorized: <12 hours, 12–24 hours, 24–72 hours and >72 hours. Since the Xpert Xpress is a closed system, with virtually no change of cross-contamination between samples in the apparatus, these inconclusive results were separately analysed from the in-house and Alinity M qPCR platforms.

3. Results

3.1. Overview of total and inconclusive samples in-house and Alinity M qPCR

A total of 50.295 SARS-CoV-2 qPCR results from patients and HCW were extracted; n=38.522 samples were tested in the in-house qPCR, n=8170 in the Alinity M and n=3603 in the Xpert assay. A total of n=30.118 samples were from patients and n=20.177 from HCW. An overview of the total and inconclusive samples from the in-house and Alinity M platforms is shown in Table 1. The number of tested samples varied greatly in time, showing the different pandemic waves in the country (see https://coronadashboard.government.nl/ for numbers and graphs of the different parameters delineating the epidemiology of SARS-CoV-2 in the Netherlands over time).

Of the 50.295 SARS-CoV-2 results, a total of 379 (0.8%) were inconclusive results; the in-house method and the Alinity M platform, both having (limited) potential for cross-contamination, generated n=339 (0.9%) and n=40 (0.5%) inconclusive results respectively. Of these 379 inconclusive teste, 217 were followed by a second test, allowing observations in Ct-value dynamics of the PCR test in time. The majority of follow-up tests yielded a negative result (187/217; 86%), thereby demonstrating that the first test was most likely false-positive. Results of the follow-up samples are presented in Fig. 1. Within the inconclusive range of Ct-values, the precise Ct-value of the index test did not predict the outcome of the second test (data not shown).

3.2. Positive follow-up samples

Only 19 out of 217 (9%) persons with an inconclusive test result using the in-house and Alinity M platforms, tested positive in the follow-up test sample. Of these, 16 were from HCW and 3 were from patients. See Table 2 for characteristics. December had the highest number of persons with a positive follow-up sample, which coincides with the second wave of the pandemic in our country. Interestingly, 14 out of the 19 (74%) with a positive follow-up test, had been tested after having close contact with a known infected person. The majority of the individuals (11/14) also had symptoms compatible with an early SARS-CoV-2 infection. Fig. 2 shows the course of Ct-values in time, for the 19 positive-by-follow-up, after an initial inconclusive result. The figure shows that for most persons (n=11 of 19), time between an inconclusive result and the follow-up sample which produced a positive result was ≤24 hours. The Ct-value decreased from the initial inconclusive result (median 37, interquartile range 35–39) to the positive result (median 28, interquartile

Table 1
Overview of numbers of SARS-CoV-2 test results by in-house and Alinity M qPCR from April 2020-January 2021.

Month	Inconclusive test results in-house qPCR/number of tested samples (%)	Inconclusive test results Alinity M / number of tested samples (%)	Total inconclusive test results in-house qPCR+Alinity M/total number of tested samples (%)
April 2020	48/3343 (1.5%)	NA	48/3343 (1.4%)
May 2020	30/2771 (1.1%)	NA	30/2771 (1.1%)
June 2020	62/3487 (1.8%)	NA	62/3487 (1.8%)
July 2020	34/2437 (1.4%)	NA	34/2437 (1.4%)
August 2020	29/3034 (1%)	NA	29/3034 (1%)
September 2020	21/5592 (0.4%)	NA	21/5592 (0.4%)
October 2020	21/5902 (0.4%)	1/183 (0.5%)	22/6085 (0.4%)
November 2020	31/4131 (0.8%)	5/858 (0.6%)	36/4989 (0.7%)
December 2020	49/4376 (1.1%)	17/3568 (0.5%)	66/7944 (0.8%)
January 2021	14/3449 (0.4%)	17/3561 (0.5%)	31/7010 (0.4%)
Total	**339/38,522 (0.9%)**	**40/8170 (0.5%)**	**379/46,692 (0.8%)**

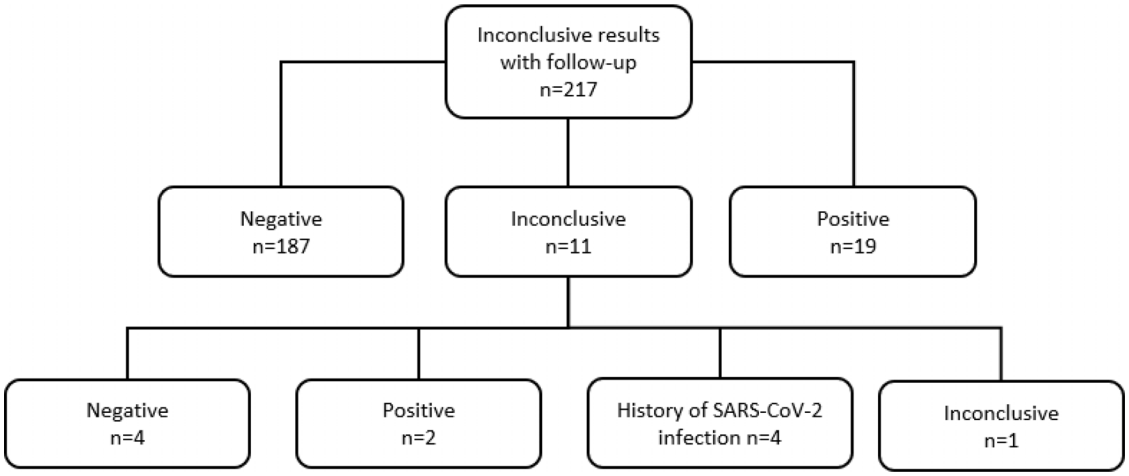

Fig. 1. Characteristics of follow-up samples of 217 SARS-CoV-2 inconclusive test results using the in-house and Alinity M platforms.

Table 2
Characteristics of 19 persons with a positive SARS-CoV-2 follow-up sample after an initial inconclusive test result using the in-house and Alinity M qPCR platforms.

	n= (%)
HCW / patient	16 (84) / 3 (16)
Month*	
April 2020	1
September 2020	2
October 2020	1
November 2020	3
December 2020	9
January 2021	3
Reason for testing	
Contact with known infected person	3
Contact with known infected person + complaints	11
Contact tracing after outbreak hospital ward	2
Complaints	1
Unknown	2
History of infection with SARS-CoV-2	1
Ct-value of inconclusive result	
Ct 34	3
Ct 35	3
Ct 36	2
Ct 37	2
Ct 38	3
Ct 39	3
Ct 40	1
Ct 41	2
Time in hours between index test and positive test, median (IQR)	22 (20–54)
Test type in-house/Alinity M	7/12

n = number, Ct = cycle threshold, IQR = interquartile range.
*In the months May, June, July and August of 2020, there were no positive follow-up samples.

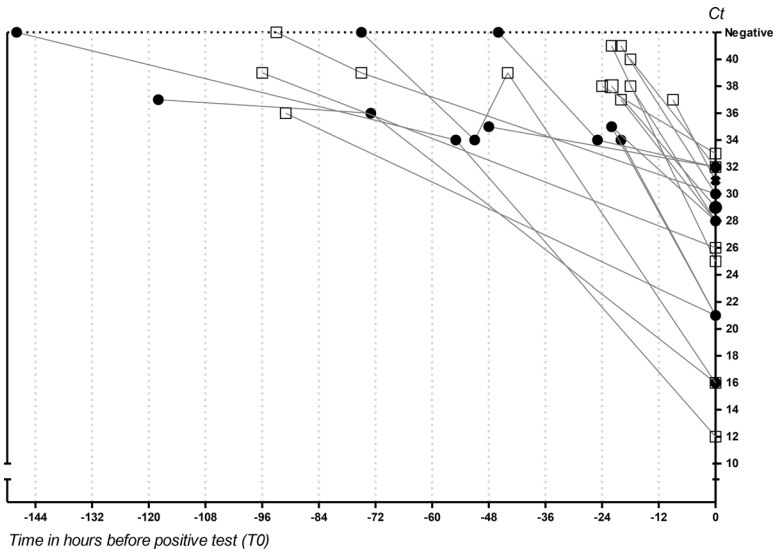

Fig. 2. Course in time of Ct-values of samples of 19 persons who initially tested inconclusive (using the in-house and Alinity M platforms) and eventually were found positive for SARS-CoV-2.
T0 = moment of positive test, black circle: in-house qPCR, clear square: Alinity M, black X: Xpert Xpress.

range 21–31) with a median of 10 Ct-value points (interquartile range 7–15).

3.3. Inconclusive follow-up samples

Follow-up samples of 11 persons (5%, 4 patients and 7 HCW) were once again inconclusive on testing the second sample. A third sample was obtained from six persons. Two persons (both HCW) tested positive in the third sample, obtained 2 and 5 days after the index test. Both had been exposed to a known infected person. The other four tested negative. Four persons with an inconclusive second sample had a history of SARS-CoV-2 infection one to two months prior to the index test. Only one person with two inconclusive results did not receive a third test and no history of a SARS-CoV-2 infection was documented.

In conclusion, we have found n=187 true negative results, n=11 inconclusive results and n=19 true positive results out of the initially 217 inconclusive results.

3.4. Results of Xpert Xpress

A number of 45 inconclusive test results were identified among a total of 3603 tests (1.2%). For 23 results, no follow-up sample was obtained. In 20 of 23 cases (87%), a SARS-CoV-2 infection had been diagnosed in recent history, indicating that the inconclusive results were likely attributable to persistent viral RNA.

3.5. A follow-up sample was obtained for 22 tests with inconclusive results

Nine out of 22 (41%) follow-up samples were negative, indicating the index test may have been false-positive. Five of these nine had a known history of recent SARS-CoV-2, for one person this was suspected and for the other three persons it could not be confirmed.

Seven of 22 (32%) follow-up samples tested inconclusive again. One of these tested positive with a low Ct-value in a third test. Two of 7 with inconclusive follow-up tests had a known history of SARS-CoV-2. Patient history of one of these individuals with repeated inconclusive test results was compatible with a past SARS-CoV-2 infection. For the remaining 3 persons, a history of SARS-CoV-2 infection was not noted.

Six of 22 (27%) follow-up samples tested positive, indicating the index test was a true (early) positive.

In conclusion, we have found n=9 true negative results, n=6 inconclusive results and n=7 true positive results out of the initially 22 inconclusive results. This was found statistically significant ($p<0.05$) compared the in-house and Alinity M qPCR method.

4. Discussion

Rapid and correct detection of SARS-CoV-2 for patients as well as hospital staff is of paramount importance in a hospital under pressure during an ongoing pandemic. It is therefore essential to differentiate inconclusive results into true or false positive results. Different laboratories use different cut-off Ct-values for positivity, and definitions may vary. Boeckmans et al. define high Ct-value results as “borderline”, showing that these results occur in 0.66% of total tests carried out [9]. In this present study we aimed to investigate if the inconclusive test was true or false positive, with true positive signifying either an early or a recently past infection. Inconclusive results occurred in 424 of 53.898 samples (0.8%) tested for SARS-CoV-2 in patients and HCW using three different platforms. Two hundred thirty-nine follow-up samples were available for analysis, showing that the inconclusive index test had been most likely detecting either an early infection with subsequent positive test in 7.7% (n=26) of cases, or false positive with subsequent negative test in 87.3% (n= 296) of cases, or still inconclusive after subsequent tests 5% (n=17)

Re-testing in case of inconclusive results has been advised by some authors [9,10] since repeated testing allows a definite conclusion of the test result in most cases. In our study, the majority (86%) of samples with an inconclusive result in our in-house and Alinity M qPCR had a negative follow-up sample, meaning that the index test was most likely false positive, although it is still possible that some of these index tests detected a small amount of remnant RNA. Only a small percentage of the initial inconclusive results resulted in eventual true positive results (9%), of which the majority (74%) reported exposure to a person with a known or suspected infection with SARS-CoV-2. Because the screening algorithm of our hospital mandated testing of large numbers of asymptomatic patients and HCW, many of these inconclusive tests were in people with low-pre-test probability for an actual infection. The high probability of a negative follow-up test has also been recognised by others [11,12]. Persons who have had a repeated inconclusive result, often reported a history of SARS-CoV-2 infection. In our study this was the case in four of the 239 inconclusive index tests. Inconclusive test results in the Xpert Xpress, which was only used in patients and not in HCW, were often indicative of a past infection. This was the case in at least 27 of 45 (60%) cases, many of which were not tested a second time, because the patient reported a recent infection. It is known that

the complete clearance of SARS-CoV-2 in a part of persons can take up to several weeks or even months [13–15].

We show notable differences between the platforms analysed in this study. Inconclusive results, with negative follow-up tests, were more likely in PCR platforms where moments are created in which the samples are open. In our study we showed that negative follow-up tests happened in 86% of inconclusive initial tests when tested in the open systems, whereas a negative second tests occurred in 41% of the inconclusive GX tests ($p<0.05$). We hypothesize that cross contamination from between samples can occur by aerosol forming procedures when using an open platform, although the chance of this happening is still very low, as is demonstrated from the low overall percentage of inconclusive tests (0.8%).

Limitations of our study include possibly missing information in some cases, such as undocumented past infections with SARS-CoV-2. Because of the retrospective nature of our study, we were dependent on the information that was recorded. Furthermore, timing of obtaining a repeat sample was not harmonized. Follow-up samples were only available in 217 out of 379 (57%) samples. It is likely that individuals with a clear history of recent infection did not always receive a second test. Our data suggest that this was the case, as 20 of 23 patients with an initial inconclusive GX test without follow-up sample had a documented recent infection.

In conclusion, our study shows the importance of reporting inconclusive results and the need of re-testing these persons. The probability whether the second test being negative, positive or once more inconclusive, the timing of the test within the different waves of the pandemic and the platform used. By reporting and re-testing inconclusive results, definitive true positive and true negative results in the fast majority of cases and a diagnosis can be given. The delay in definite result is vastly superior to potentially reporting false positive results as SARS-CoV-2 infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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