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Distribution of cycle threshold values in RT-qPCR tests during the autumn 2020 peak of the COVID-19 pandemic in the Czech Republic

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Abstract

Reverse-transcription quantitative PCR (RT-qPCR) is currently the most sensitive method to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19). We analysed 1927 samples collected in a local public hospital during the autumn 2020 peak of the pandemic in the Czech Republic. The tests were performed using the Seegene Allplex 2019-nCov assay, which simultaneously detects three SARS-CoV-2 genes. In all samples analysed, 44.5% were negative for all three genes, and 37.6% were undoubtedly positive, with all three viral genes being amplified. A high degree of correlation between C_t values among the genes confirmed the internal consistency of testing. Most of the positive samples were detected between the 15th and 35th cycles. We also registered a small number of samples with only one (13.2%) or two (4.7%) amplified genes, which may have originated from either freshly infected or already recovering patients. In addition, we did not detect any potentially false-positive samples from low-prevalence settings. Our results document that PCR testing represents a reliable and robust method for routine diagnostic detection of SARS-CoV-2.

INTRODUCTION

With the emergence of the coronavirus disease 2019 (COVID-19) pandemic, it became apparent that one of the main tasks was to identify individuals who were carrying the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus [1]. The gold standard to do this is the reverse-transcription quantitative PCR (RT-qPCR) technique, based on reverse transcription (RT) of viral RNA into cDNA, which is then monitored during quantitative polymerase chain reaction (qPCR) amplification. Since the publication of the first RT-qPCR protocol, shortly after the outbreak [2], a wide variety of comparably performing commercial tests have been developed [3–5]. Two major points of concern have arisen regarding the interpretation of RT-qPCR results. Firstly, when the prevalence of an infection is very low, even a low false-positive rate (FPR) can result in a considerable number of false positives [6, 7]. Secondly, due to the high sensitivity of RT-qPCR, even trace amounts of viral RNA [reflected by a high cycle threshold (C_t)] can be detected, but they do not necessarily indicate an acute infection or whether the person is still infectious [7, 8].

The first case of COVID-19 in the Czech Republic was registered on 1 March 2020. Due to rapidly introduced anti-pandemic measures, the Czech Republic avoided the spread of the infection during the first pandemic wave in spring 2020. With almost no new positive cases, most of the restrictions were relaxed on 25 May 2020. In September 2020, the number of SARS-CoV-2 cases started to increase. Facing an increasing number of COVID-19 patients, the Czech government announced a general lockdown on 14 October 2020. During the peak of the pandemic, at the end of October and the beginning of November, the Czech Republic became one of the most seriously affected countries in the European Union. The situation improved later in November as the number of new cases started to decline.

Due to the limited testing capacity of hospitals, several Czech academic institutes offered facilities to perform RT-qPCR tests. Our department at the Institute of Microbiology of the Czech Academy of Sciences in Třeboň assisted a local public hospital from 15 October 2020 until 21 November 2020. During this period the incidence in the studied district reached over 200 new daily cases per 100 000 citizens, and over 1200 active cases per 100 000 citizens (Fig. 1). We used

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Abbreviations: COVID-19, coronavirus disease 19; C_t, cycle threshold; RT-qPCR, reverse transcription quantitative PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA.

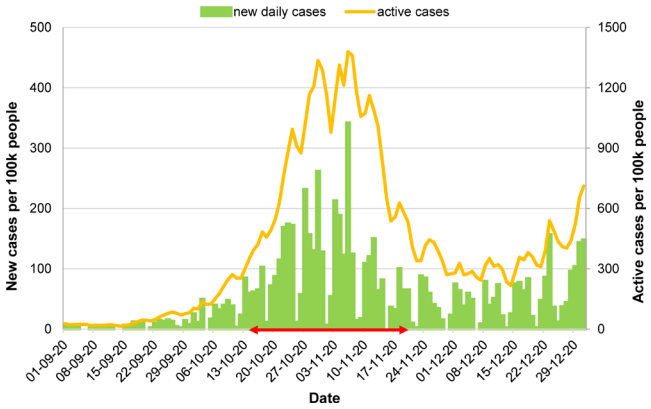

Fig. 1. Development of the autumn 2020 wave of COVID-19 in the studied district (data obtained from the Czech Ministry of Health, https://onemocneni-aktualne.mzcr.cz/api/v2/covid-19). The red bar represents the period reported in this study.

the obtained dataset to analyse the distribution of the registered C_t values, and compared the C_t values obtained for the same sample using different probes to verify the consistency of the results. Finally, we discuss the probability of false-positive results occurring.

METHODS

Samples were collected from patients with COVID-19 symptoms, as well as from reported (asymptomatic) contacts using nasopharyngeal swabs. The swabs were immediately inactivated by immersing them directly into DNA/RNA Shield (Zymoresearch, Irvine, CA, USA). Viral RNA was then extracted from the inactivated samples using the MagPurix Viral Nucleic Acid Extraction kit (Zinexts, Taipei, Taiwan, ROC) with an automatic purifier ZiXpress 32 (Zinexts, Taipei, Taiwan, ROC). RNA was isolated from 200 µl of the sample containing an internal standard using the manufacturer's isolation set-up, with the only difference being elution of the samples into 200 µl of buffer. Increasing the elution volume alleviated issues with RT-qPCR inhibition that were experienced at lower elution volumes. For the detection of SARS-CoV-2, the commercially available kit Allplex 2019-nCov was used (SeeGene, Inc., Seoul, Republic of Korea) in line with manufacturer's instructions. This kit performs the reverse transcription (RT) and subsequent qPCR reaction in one protocol. Each RT-qPCR reaction consisted of 5 µl of 2019-nCoV master-mix (MOM), 5 µl of PCR water, 5 µl of 5x Real-time One-step Buffer, 2 µl of Real-time One-step Enzyme and 8 µl of template. The RT and subsequent qPCR was run as follows: RT step 20 min at 50 °C; initial denaturation 15 min at 95 °C; 45 cycles of 15 s at 94 °C and 30 s at 58 °C. The presence of the virus was detected using three different SARS-nCoV-2 genes: gene E for the capsid protein, gene N for nucleoprotein and the RdRP gene for RNA-dependant RNA polymerase. The kit also contains an internal standard, which was used to verify the process of RNA isolation and the ensuing RT-qPCR reaction. All tests were performed using a CFX96 qPCR cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), where 96-well plates were used to hold the samples. The course of the qPCR reactions was detected for all genes, including the internal standard, using four fluorescent channels (FAM for the E gene, CalRed 610 for the RdRP gene, Quasar 670 for the N gene and HEX for the internal standard). The obtained signals were processed using Bio-Rad's CFX Maestro software, which automatically computed the C_t values.

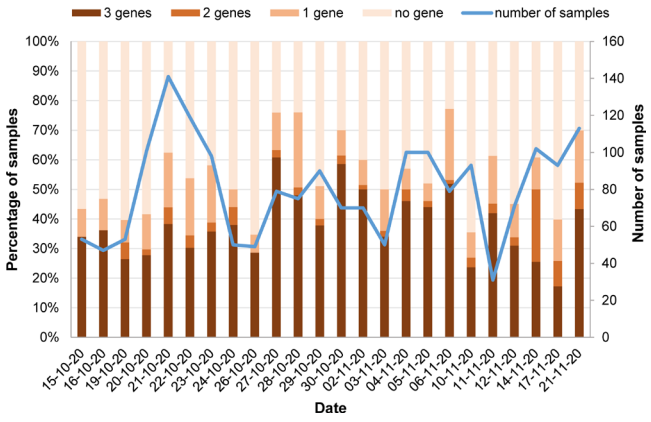

Fig. 2. Results of RT-qPCR tests performed during October and November of 2020. On the *y*-axis is the percentage of samples where no (pink), one (pale orange), two (orange), or three (brown) viral genes (brown) were detected.

According to the manufacturer's directions [9], a sample is assumed to be negative should there be no viral genes detected in it (or the sample's C_t value is above 40). A sample is reported as being positive if any viral genes are amplified within the range of 10–40 cycles.

RESULTS

The RT-qPCR tests were conducted during the peak of the COVID-19 pandemic in October and November 2020 (Fig. 1). Over the course of 4 weeks, we analysed 1927 samples, 44.5% of which were negative. Three genes were detected in 37.6% of the samples. Interestingly, a small number (4.7%) of samples had two amplified genes. Amplification of one gene only (in most of the cases it was the N gene) was registered in 13.2% of samples (Fig. 2). Based on the instructions of the manufacturer [9], these samples were to be considered as SARS-CoV-2-positive, which resulted in 55.5% samples being deemed positive. The number of samples with fewer than three detected genes was variable within the testing period (Fig. 2). No trend in time and no correlation with the number of samples per week was detected, indicating no systematic source for these incomplete results. Furthermore, no exceptionably high number of positive results for only one or two genes was recorded for any of the weeks, which would have indicated a possible singular contamination event.

The C_t value obtained from the PCR reaction is indirectly proportional to the concentration of the amplified targeted

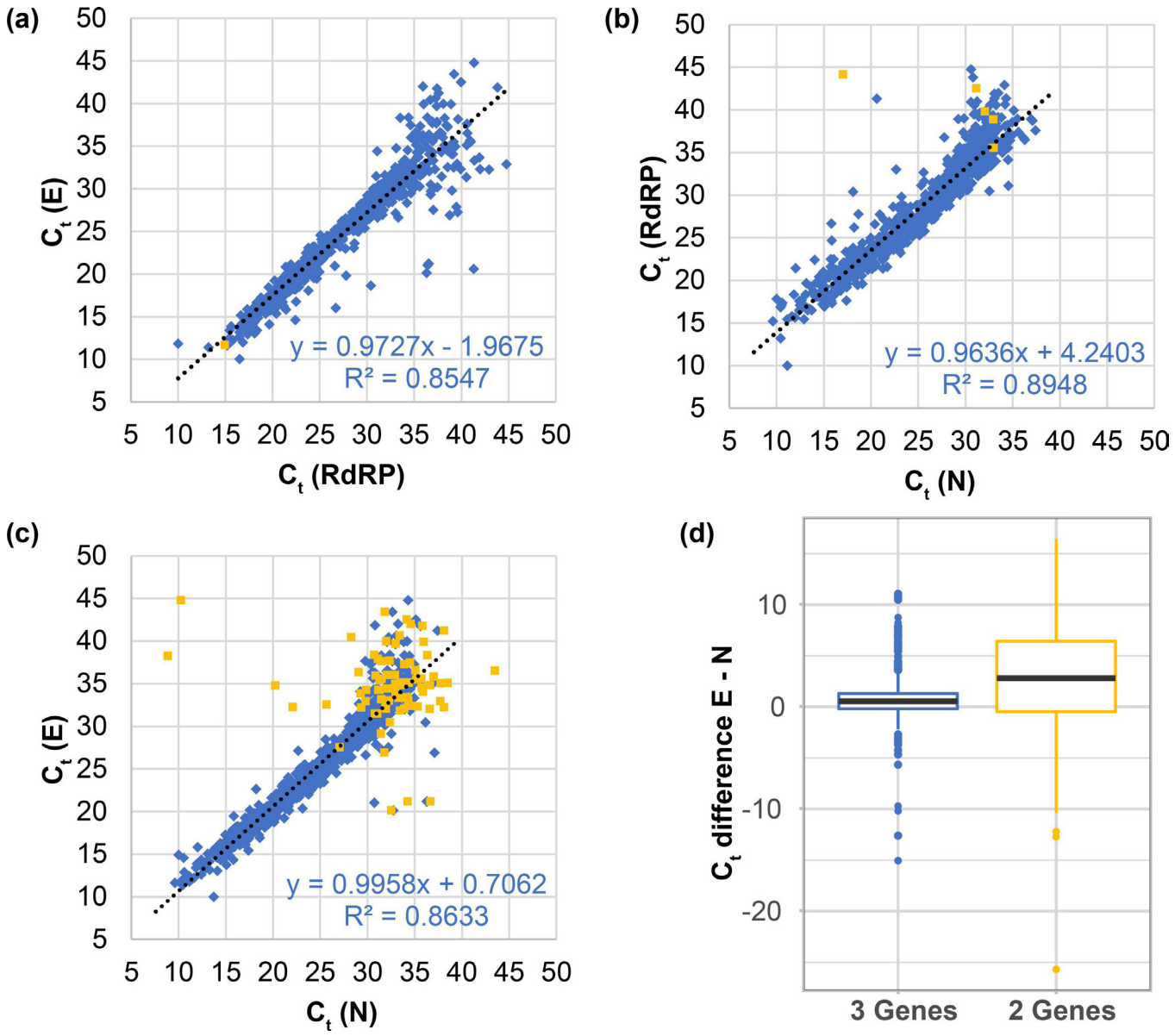

Fig. 3. Correlation between C_t values in the detected genes. (a) Correlation of C_t values between E and RdRP genes. (b) Correlation of C_t values between RdRP and N genes. (c) Correlation of C_t values between E and N genes. (d) Difference in C_t values between E and N genes for samples with three or two detected genes. The black horizontal lines represent median values. Blue labelled samples tested positive for all three SARS-CoV-2 genes. The samples with only two amplified genes are shown in orange. The linear regression (dotted line) corresponds to samples with all three genes detected (blue dots). Respective regression equations and R^2 are shown.

gene. Since the virus contains one copy of each targeted gene, the three C_t values from an individual sample should correspond appropriately. Thus, to verify the internal consistency of our analyses, we analysed the relationship of the C_t values from the individual tests. Good correlation was found between C_t values for the genes RdRP and E (slope=0.9728, R^2=0.8547, n=748; Fig. 3a) and he genes N and RdRP (slope=0.9636, R^2=0.898, n=748; Fig. 3b). We also found a very strong relationship between C_t values for the genes N and E (slope=0.9958, R^2=0.86, n=748; Fig. 3c), which is also reflected in the average deviation between C_t values for genes E and N, being only 0.54 cycles. This shows that the detected virus particle was complete, the primers used in the kit were well designed and that no background was detected. However, for samples with only two genes detected (usually N and E) C_t values are far more dispersed (Fig. 3d).

Distribution of the detected C_t values was not homogenous among the samples. Most of the unambiguously positive samples (all three genes detected) were detected between the 15th and 35th qPCR cycle (Fig. 4). This corresponds with previously published results [10]. In the cases where only one

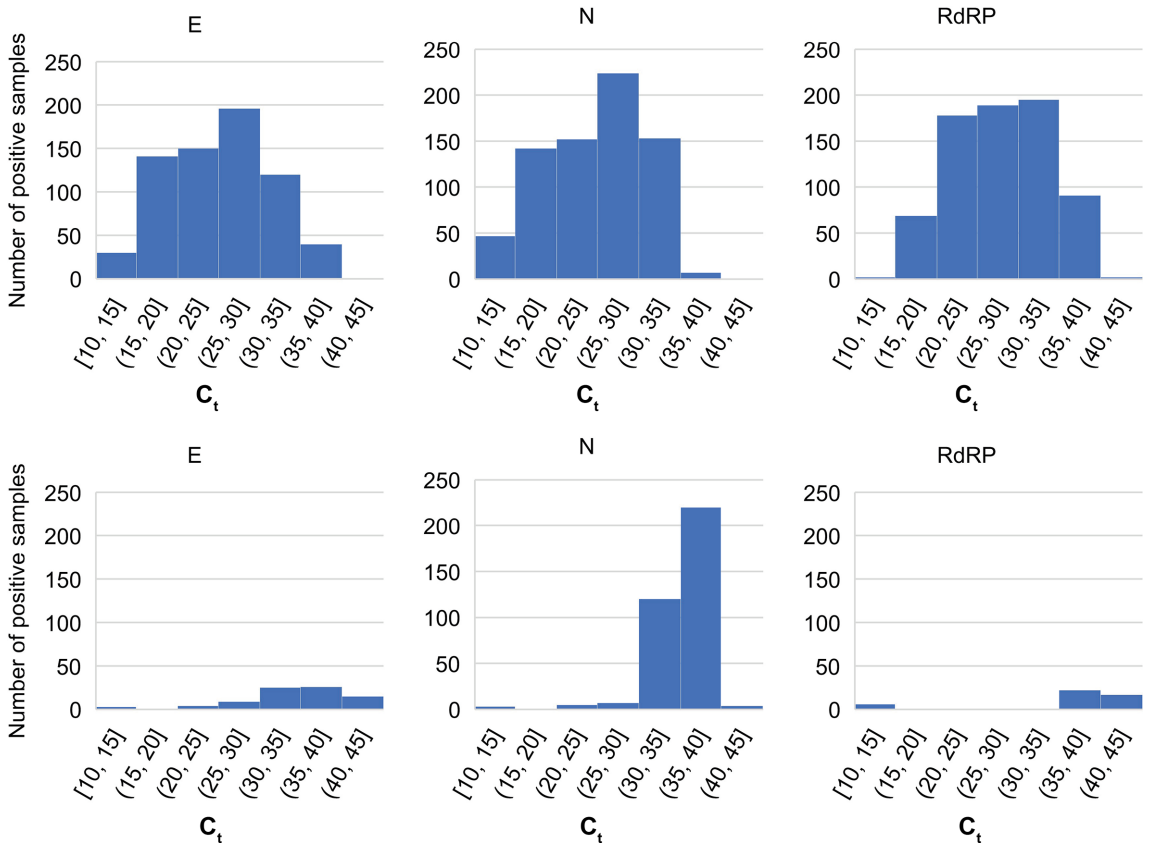

Fig. 4. Distribution of C_t values of analysed samples from autumn 2020. In the first row are the samples in which all three viral genes were detected for SARS-CoV-2 and which were therefore unquestionably considered to be positive. The second row shows samples in which only one or two viral genes were detected.

or two of the viral genes were seen, the number of PCR cycles tended to be above 30. This suggests that these samples only contained a small amount of viral RNA.

A commonly discussed issue is the number of false positives in the analyses, in particular when the prevalence is expected to be very low. We conducted the first tests in May 2020, when the number of infected individuals in the analysed district was minimal (<5 per 100000). We performed a total of 124 tests, which were all negative for all 3 tested genes. The same result was obtained during the compulsory weekly testing of all employees introduced by the Czech government in April 2021. Here we conducted 663 tests for our department employees. All of them were negative. This indicates that the number of tests that could be false positives for this given method is minimal (<<1%), which is consistent with other reports [11].

DISCUSSION

The observed incompletely detected samples could have two different origins. Some of these samples could have originated from patients in the early stages of infection. The quantity of viral RNA in these samples is small, and part of the detected RNA could originate from infected human cells. The replication cycle of SARS-CoV-2 within the host cell includes the generation of subgenomic (sg)RNAs that serve as templates for translation of structural components of the virus particle. The sgRNA for the nucleoprotein (N) gene is the most abundant, while no sgRNA is produced for the RdRP gene [12]. This could explain why the N gene was detected in almost all of the inconclusive samples, while the RdRP gene was detected in the fewest. Other samples could have originated from patients who had been infected and had recovered, with the RT- qPCR only detecting degradation products of viral RNA.

Our assumption that the incompletely detected samples represent two different groups of patients is consistent with the study of Drew and colleagues [13]. Using the same Allplex 2019-nCov assay, they documented that the first group were patients who had experienced symptoms for more than 7 days. According to the authors of the study, this indicates viral RNA degradation. The second group were patients who were tested on the first or second day after infection. This could suggest a low viral load. In another study, it was shown that the probability of culturing the virus declines to 8% in samples with >35 and to 6% 10 days after symptom onset [14]. Furthermore, in a study focused on the correlation between detectable viral RNA and culturable virus in 60 clinical samples, the importance of viral

genome integrity for potential infectivity of the sample was demonstrated [15, 16].

Our results document that RT-qPCR is a reliable and robust method to detect SARS-CoV-2 with a very low probability of false-positive results. In settings where the prevalence was expected to be very low, only clearly negative samples were recorded. As demonstrated for the high-prevalence setting, the majority of positive samples had amplification cycles in the range of 15 to 35 cycles and had highly consistent C_t values between the tested genes. However, what should be considered is to report samples with only one positive gene or two positive genes with inconsistent C_t values in a special category (e.g. inconclusive or borderline), differentiating them from clearly negative or clearly positive cases. Further assessment of these cases should be then undertaken by a physician based on the overall clinical situation of the patient and retesting should be performed.

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Author contributions
M.K., conceptualization, data analysis, wrote the paper; D.H., data analysis, wrote the paper; K.K., performed analyses; J.T., data analysis, wrote the paper; K.B., performed analyses.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The Ethical Committee of the Institute of Microbiology Czech Acad. Sci. approved this work

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