Original research article

Quantitative analysis of RT-PCR test results for SARS-CoV-2 diagnostics across Poland during COVID-19 pandemic: Comparison between early stage and major pandemic waves in 2020 and 2021 with reference to SARS-CoV-2 variants

Rafał Gierczyński [a], Aleksandra Czerw [b,c], Grzegorz Juszczyk [d], Radosław Charkiewicz [e], Jacek Nikliński [f], Piotr Majewski [f], Joanna Reszeć [g], Piotr Piątyszek [h], Hubert Baniecki [h], Przemysław Biecek [h], Brandon Michael Henry [i,*]

[a] Department of Bacteriology and Biocontamination Control, National Institute of Public Health, National Institute of Hygiene, Warsaw, Poland
[b] Department of Economic and System Analyses, National Institute of Public Health, National Institute of Hygiene, Warsaw, Poland
[c] Department of Health Economics and Medical Law, Medical University of Warsaw, Warsaw, Poland
[d] Department of Public Health, Medical University of Warsaw, Warsaw, Poland
[e] Center of Experimental Medicine, Medical University of Bialystok, Bialystok, Poland
[f] Department of Clinical Molecular Biology, Medical University of Bialystok, Bialystok, Poland
[g] Department of Medical Pathomorphology, Medical University of Bialystok, Bialystok, Poland
[h] Faculty of Mathematics and Information Sciences, Warsaw University of Technology, Warsaw, Poland
[i] Clinical Laboratory, Division of Nephrology and Hypertension, Children's Hospital Medical Center, Cincinnati, OH, USA

ARTICLE INFO

ABSTRACT

Purpose: From April to September 2020, Poland was minimally affected by COVID-19 compared to other EU countries. We aimed to investigate the risks of false reverse transcription polymerase chain reaction (RT-PCR) results during the first wave (compared to later waves), that rises when cycle threshold (Ct) of positive result is close to limit of detection (LOD).

Materials/methods: We analyzed Ct values of SARS-CoV-2 positive RT-PCR results of 7726 patients in Poland from April–September 2020. SARS-CoV-2 positive RT-PCR results of 14,534 patients in the 2nd-3rd wave and 10,861 patients in the 4th-5th pandemic waves were used. Statistical analysis was based on one-way analysis of variance. To verify, 95% confidence intervals with Bonferroni correction were computed. Incidence of SARS-CoV-2 variants in Poland was analyzed using Whole Genome Sequencing from 923 (3.6%) patients.

Results: The mean Ct of RT-PCR positive test results analyzed ranged between 22.89 and 26.71 depending on the month of the results collection. The differences between months were significant (p < 0.001). Differences in Ct were observed between age groups, with younger patients displaying higher Ct values, however, major trends over time were paralleled between age groups.

Conclusions: The mean Ct of the tested RT-PCR positive test results was lower than 35 which is considered an upper borderline for reliable positive results of the assay. Therefore, most COVID-19 cases recorded in Poland from April to September 2020 were detected with minor risks of inaccuracy. Data from a single center exhibited greater consistency for both virus Ct level and SARS-CoV-2 virus variant identification.

1. Introduction

The ongoing worldwide coronavirus disease-2019 (COVID-19) pandemic has directly impacted over 546 million people causing over 6.3 million deaths as of July 3rd, 2022 [1]. In Poland, the first confirmed COVID-19 case was detected on March 4th, 2020, by the National Institute of Public Health – National Institute of Hygiene (NIPH–NIH), Warsaw, Poland, using a reverse transcription polymerase chain reaction

(RT-PCR) protocol developed by the World Health Organization (WHO) collaborating laboratory, the Institute of Virology, Charite, Berlin, Germany [2]. Over the next three weeks, 1389 additional COVID-19 cases were recorded across Poland [3] despite the early introduction of strategies to prevent the spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) within the country [4]. From the first case in March to December 6th, 2020, over 1 million confirmed cases (n = 1,054, 273) and almost 20 thousand deaths (n = 19,861) were recorded in Poland [5]. However, the aforementioned rapid introduction of strong mitigating measures, including "lock-downs", likely protected Poland from a massive excess of infections and deaths when compared to some other EU countries in spring 2020. The average daily incidence of new COVID-19 infections in Poland from March 2020 to end of June 2020 was about 287 cases that resulted in a relatively low average 14-day cumulative number of new confirmed cases (about 10 per 100,000 inhabitants). According to data from The European Surveillance System (TESSy) [5], the highest 14-day cumulative number of new confirmed cases per 100,000 inhabitants in Poland during the abovementioned period reached 15.83. At the same time (i.e. week 10–25 of the year 2020), the average positivity test rate in Poland was 2.67% based on the average test number of 213.71 tests per week per 100,000 inhabitants [6]. These relatively low values, when compared to other EU countries, raised questions as to the accuracy and quality of COVID-19 molecular diagnostics in Poland.

The NIPH–NIH is responsible at the national level for verification of diagnostic test results for SARS-CoV-2 performed by routine laboratories testing for COVID-19. Since March 2020, in accordance with initial WHO recommendations, each diagnostic laboratory in Poland conducting RT-PCR testing for SARS-CoV-2 was obliged to send the first 10 negative and first 5 positive samples for confirmation testing at the central COVID-19 laboratory at the NIPH–NIH [7]. Although internal results of the verification suggested a high quality of SARS-CoV-2 diagnostics and did not arouse suspicion of poor accuracy or quality, other assessments were also needed to analyze SARS-CoV-2 laboratory detection for COVID-19 surveillance across Poland.

The use of RT-PCR detecting two distinct fragments of the SARS-CoV-2 genome was recommended by the WHO for laboratory confirmation of COVID-19 cases [7]. Each RT-PCR test for *in vitro* diagnostics requires validation to evaluate its sensitivity and specificity. Most RT-PCR tests for SARS-CoV-2 are qualitative, however, real-time PCR by its nature allows quantitative results analysis [12]. Generally, RT-PCR tests enable 45 cycles of DNA amplification (cycle threshold, Ct). However, reliable and reproducible positive test results are limited up to 36–42 Ct in the majority of commercially available tests. Because the Ct required to detect a positive RT-PCR signal correlates with the amount of the viral nucleic acid in the tested sample, lower Ct values suggested a higher viral load in a tested patient [13]. Consequently, positive results with Ct below 35 are considered more reliable than results with Ct nearer to the limit of detection (LOD) with a high false positive risk. Therefore, statistical analysis of Ct values of positive test results for SARS-CoV-2 may help to estimate cumulative risk of false positive/negative RT-PCR results and its influence on reliability of COVID-19 diagnostics. There are now a multitude of clinical RT-PCR assays for SARS-CoV-2 available on the commercial market, however, their clinical accuracy has not yet been fully and independently evaluated [9]. Therefore, we decided to collect quantitative RT-PCR data from diagnostic laboratories and perform a statistical analysis to retrospectively estimate how reliable SARS-CoV-2 molecular diagnostic testing was in Poland before the second wave of the pandemic.

Since the COVID-19 incidence before the second pandemic wave was relatively low in Poland, positive test results could have been affected by a low probability of truly positive results (low positive predictive value). Thus, we included the Ct data collected during the 2nd-3rd and the 4th-5th waves of COVID-19 pandemic in Poland, when the incidence of SARS-CoV-2 infections was much higher. The aim was to better evaluate the 1st wave diagnostics accuracy, thus data on Ct collected in spring 2020 were analyzed against Ct data from further pandemic waves collected in a single laboratory to limit testing methodology bias.

2. Material and methods

Two sets of data were used in this study. The first set was collected during the first epidemic wave of COVID-19 in different laboratories of different locations in Poland. These laboratories used different tests including tests detecting single or multiple (2 or 3) targets in the SARS-CoV-2 genome. The second set of data was collected during the 2nd-3rd (November 2020–June 2021) and 4th-5th (September 2021–February 2022) epidemic waves in a single laboratory. We termed this later dataset as the reference, since samples were collected and tested in the optimal conditions (high COVID-19 prevalence and no inter-laboratory sample processing diversities).

In the first epidemic wave we analyzed Ct values of SARS-CoV-2 positive RT-PCR results from 7726 patients across Poland who received clinically-indicated testing via nasopharyngeal swab. Samples were processed and tested at local laboratories in 6 of the 16 voivodeships (provinces) of Poland: Lower Silesian (dolnośląskie), Łódź (łódzkie), Lesser Poland/Małopolska (małopolskie), Masovian (mazowieckie), Pomeranian (pomorskie), and Silesian (śląskie). Analyzed test results were collected from April to September of 2020. RT-PCR was performed at each participating laboratory in accordance with manufacturer's instructions. The RT-PCR assays employed were confirmed for accuracy prior to widespread use for clinical testing purposes by the Central COVID-19 Laboratory of the NIPH–NIH as described above.

The second dataset was collected from a single diagnostic laboratory at the Medical University of Bialystok, Poland. A total number of 25,395 positively tested patients were included in the analysis. From November 2020 to June 2021, 14,534 positive test results were collected and from September 2021 to February 2022, 10,861 were collected. The majority of positively tested samples were collected from patients living in Bialystok city and neighboring locations in the Podlaskie voivodeship, Poland. The most predominant age group in the reference cohort was 25–50 years old (over 38%) with small subset of patients below 15 years old (5%) and patients above 80 years old (7.8%). Fraction of females was slightly higher than males (56%–44%). The diagnostic laboratory of the Medical University of Bialystok used only two diagnostic tests during the study period; 21,159 positive tests were identified using Allplex 2019-nCoV Assay (Seegene Inc., Seoul, South Korea) and 4236 positive tests were identified using COVID-19 Real Time Multiplex RT-PCR Kit (Labsystems Diagnostics Oy, Vantaa, Finland) that were cross-validated in the laboratory to enable results conformity and reproducibility.

During this reference time period, sequencing was performed for 923 samples from patients whose genetic material was collected at the same center (which accounted for 3.6% of the samples with material collected at this center). Incidence of SARS-CoV-2 variants in Poland were analyzed using Whole Genome Sequencing. All samples were analyzed with the same bioinformatics pipeline using TruSeq Custom Amplicon Library Preparation kits and Illumina panels. All samples were sequenced on an Illumina MiSeq platform using a 300-cycle MiSeq Reagent Kit v2 to obtain paired-end reads of 150 bp. Reads were preprocessed with SAMTools and variant calling was performed with the Genome Analysis Toolkit (GATK) variant caller. Consensus genome sequences were generated using the majority threshold criterion. Only sequences with a coverage level above depth 10 for more than 95% of genome were considered for the analyses. SARS-CoV-2 variants were identified with the Pangolin tool, while Nextstrain was used to identify the GISAID clades and amino acid (aa) substitutions.

Descriptive data was presented as frequency (%) or mean. Statistical analysis was based on one-way analysis of variance applied to compare mean values of Ct acquired in consecutive months from April till September 2020. The analysis was conducted on the total sample and in 6 age subgroups: <15 years, 15–24 years, 25–49 years, 50–64 years, 65–79 years, and ≥80 years. In order to verify which mean Ct values differed

from each other, 95% confidence intervals (95%CI) with Bonferroni correction were computed. In the analysis conducted in the age sub-groups, repeated contrast tests were used.

2.1. Ethical issues

This study was performed in full accordance with ethical standards for research, under the rules and regulations of the Republic of Poland, the policies of the NIPH–NIH, and in compliance with the 1964 Declaration of Helsinki with its later amendments. This study was carried out as part of routine NIPH–NIH public health surveillance procedures and data was transferred between local/regional clinical laboratories and the NIPH–NIH in Warsaw under pre-existing agreements as part of routine public health operational protocols. Data of the test results of all the patients was anonymized by the local laboratory prior to transmission to the NIPH–NIH. Only information on gender, age and voivodeship of sample collection were the only patient characteristics collected. The unique sample ID did not allow for patient identification.

3. Results

Age groups and distribution by voivodeship (province) of patients with positive RT-PCR results for SARS-CoV-2 in the study cohort are presented in Table 1. Among the 7726 test results analyzed, 3515 (45.5%) were from females. Those aged 25–49 years made up the largest proportion of the sample, accounting for 4040 (52.3%) of all analyzed test results. Those ≥80 years old accounted for only 256 test results (3.3%), while children (<15 years old) accounted for only 404 test results (5.2%). The largest number of test results were from the Lesser Poland/Małopolska voivodeship, which is where Poland's second largest city (Krakow) is located, contributing 4217 (54.6%) of the analyzed results, followed by Lower Silesia which contributed 1341 (17.3%) of the analyzed results. The highest number of test results were collected in August 2020 (n = 2161 tests, 28.0%), followed by September 2020 (n = 1676; 21.7%), in line with a rising number of cases across Poland during those months (Table 2). The lowest number of test results were collected in April 2020 at the beginning of the pandemic (n = 294, 3.8%).

The mean Ct values in particular months for RT-PCR results of all

Table 2
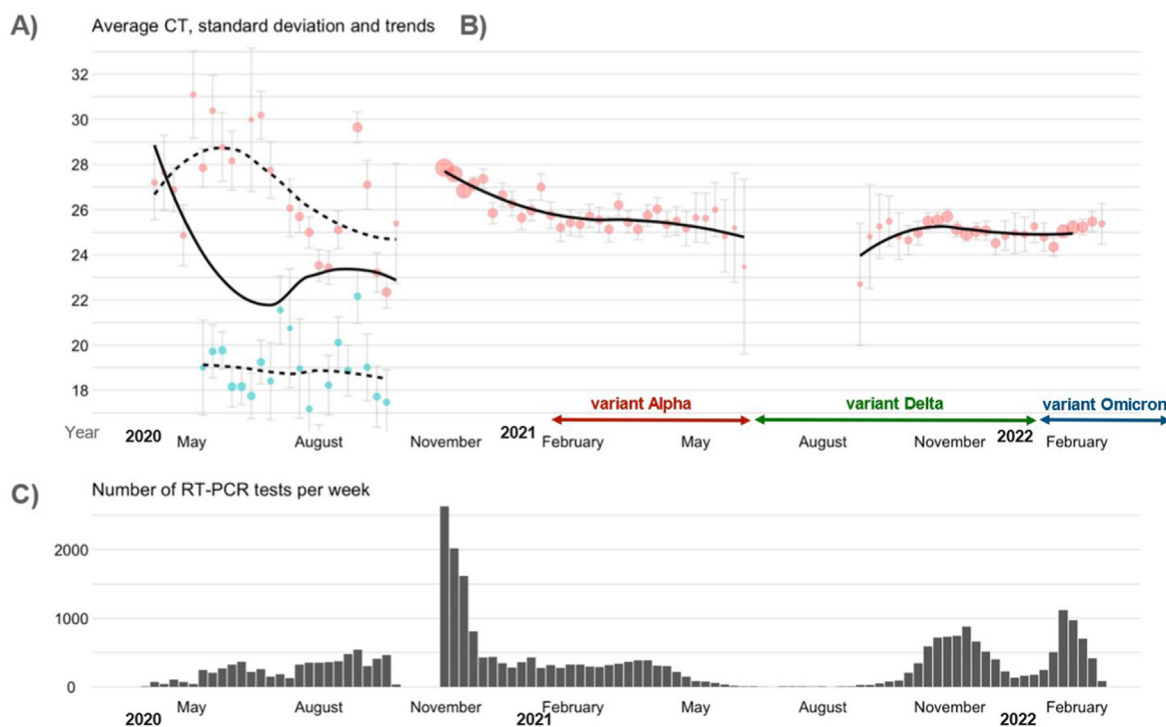Total number of study patients analyzed in particular month in 2020 during the study period and their incidence.

Month	Number	Percentage (%)
April	294	3.8
May	1000	12.9
June	1293	16.7
July	1302	16.9
August	2161	28.0
September	1676	21.7
Total	7726	100

tested patients are shown in Fig. 1A. The differences between months were statistically significant (p < 0.001). The 95% CIs were calculated with Bonferroni's coefficient. The weekly average Ct of all positive tests ranged from 18.87 up to 30.85 and the monthly average Ct of all positive tests ranged from 21.09 up to 27.85, being much lower than 35, which is generally considered as an upper borderline for reliable positive results independent of the type of commercial RT-PCR test used in SARS-CoV-2 diagnostics. In Table 3, we present the summary of Ct measurements in weekly aggregation for both the study cohort (March–September 2020) and the reference cohort (October 2020–February 2022). The mean Ct per week is shown as well as the standard deviation (SD) and the number of measurements per week.

The average Ct values of tested patients in particular age groups revealed only minor diversity with respect to change over time (data not shown). Observed Ct patterns for age groups were insufficient to make any valuable analysis and we decided not to show this data to prevent highly speculative conclusions.

RT-PCR assays with different properties were used during the first wave of the virus. When the average positive test results Ct was analyzed separately for single or multiple target assays, the lowest range of average Ct was noted for the single target assays (weekly average from 15.02 to 22. 17), while for the multiple target assays the weekly average Ct oscillated from 22.34 to 31.09. Samples collected in subsequent waves, were tested under homogenous laboratory conditions at the Medical University of Bialystok. The three-targets assays were used with primers for genes N + E + RdRP (Seegene Inc., Seoul, South Korea) or N + E +

Table 1
Age groups and distribution by voivodeship (province) of patients with positive RT-PCR results for SARS-CoV-2 in the study cohort.

Voivodeship	Gender	Age (years)														
		<15		15–24		25–49		50–64		65–79		≥80		Total	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%
Lower Silesian (dolnośląskie)	Female	21	44.7	73	52.5	297	50.9	235	65.3	82	60.3	56	73.7	764	57
	Male	26	55.3	66	47.5	286	49.1	125	34.7	54	39.7	20	26.3	577	43
	Total	47	100	139	100	583	100	360	100	136	100	76	100	1341	100
Łódź (łódzkie)	Female	7	38.9	14	45.2	68	46.3	27	55.1	11	50	10	83.3	137	49.1
	Male	11	61.1	17	54.8	79	53.7	22	44.9	11	50	2	16.7	142	50.9
	Total	18	100	31	100	147	100	49	100	22	100	12	100	279	100
Lesser Poland/Małopolska (małopolskie)	Female	119	45.8	205	46	796	34.1	365	47	168	57.1	64	61	1717	40.7
	Male	141	54.2	241	54	1540	65.9	411	53	126	42.9	41	39	2500	59.3
	Total	260	100	446	100	2336	100	776	100	294	100	105	100	4217	100
Masovian (mazowieckie)	Female	25	48.1	59	51.3	241	48.1	113	57.9	34	53.1	11	78.6	483	51.3
	Male	27	51.9	56	48.7	260	51.9	82	42.1	30	46.9	3	21.4	458	48.7
	Total	52	100	115	100	501	100	195	100	64	100	14	100	941	100
Pomeranian (pomorskie)	Female	8	61.5	16	44.4	85	48.6	37	50.7	19	43.2	11	73.3	176	49.4
	Male	5	38.5	20	55.6	90	51.4	36	49.3	25	56.8	4	26.7	180	50.6
	Total	13	100	36	100	175	100	73	100	44	100	15	100	356	100
Silesian (śląskie)	Female	8	57.1	20	34.5	106	35.6	57	44.9	28	45.9	19	55.9	238	40.2
	Male	6	42.9	38	65.5	192	64.4	70	55.1	33	54.1	15	44.1	354	59.8
	Total	14	100	58	100	298	100	127	100	61	100	34	100	592	100
Total	Female	188	46.5	387	46.9	1593	39.4	834	52.8	342	55.1	171	66.8	3515	45.5
	Male	216	53.5	438	53.1	2447	60.6	746	47.2	279	44.9	85	33.2	4211	54.5
	Total	404	100	825	100	4040	100	1580	100	621	100	256	100	7726	100

Fig. 1. Evolution of average Ct level in Poland based on data from positive RT-PCR tests. Panel (A) shows results from the first six months, the first wave of the virus in Poland. Ct values of tests detecting 2 or 3 targets in SARS-CoV-2 genome are shown in red. Ct values of the single gene targeting tests are shown in blue. Dashes are averaged results for single gene tests and multiple gene tests. The solid line represents the average of single and multiple gene tests. Panel (B) shows average Ct values of tests from single laboratory and their correspondence to SARS-CoV-2 virus variants. Panel (C) shows the number of RT-PCR assays analyzed in a specific week. The size of the dot corresponds to the number of tests analyzed in a given week.

ORF1ab (Labsystems Diagnostics Oy, Vantaa, Finland). At the beginning of the second wave in November 2020, the average Ct level exceeded 30, but it quickly decreased and became stable around 26 (Fig. 1B).

The incidence of SARS-CoV-2 variants in Poland was analyzed to assess potential alterations in COVID-19 RT-PCR diagnostics and average Ct value due to mutations in the virus genome. Fig. 2 shows changes in the number and percentage of SARS-CoV-2 variants and sub-lineages in time (weeks and months).

In Poland, the wave of COVID-19 associated with the Alpha variant lasted from February to June 2021, followed by the Delta variant from June 2021 to January 2022, which overlapped with the new wave of the Omicron variant. Samples sequences at the Medical University of Bialystok were collected over a period from October 2021 till February 2022 (Fig. 2A and B). The Delta variant was present in a high number of more than 100 subvariants (Fig. 2C). During the Omicron wave, the most frequent variant was BA.1, but an increasing presence of subvariant BA.2. was also observed (Fig. 2D). Data on the incidence of variants was analyzed against the reference average Ct of RT-PCR results collected in the laboratory of the Medical University of Bialystok. No significant changes in the average Ct values of positive RT-PCR tests were observed among periods dominated by particular virus variants and sub-lineages. No average Ct distortion was noted in the periods of the variants conversion (pre-Alpha to Alpha and Delta to Omicron).

4. Discussion

In order to analyze SARS-CoV-2 diagnostics during the first wave of COVID-19 in Poland, when a relatively low number of cases was recorded when compared with subsequent waves, it was necessary to combine diagnostic data from a variety of clinical laboratories and different diagnostic tests. During four subsequent waves with a high number of cases, we collected diagnostic data from a single regional laboratory (Medical University of Bialystok) in Poland which tested a large number

of samples using only two diagnostic RT-PCR tests, reducing data bias resulting from testing methodology. These data were the reference standard for the first wave "historical" study data assessment. In addition, we examined whether SARS-CoV-2 variants may affect the testing quality.

In the first wave, we found that the average Ct values of positive test results varied slightly (over 22 up to near 31) over the 6 months of this study. Nevertheless, these Ct values are typical of those observed in symptomatic patients with high and detectable viral loads [10,13]. These results confirm that the majority of positive RT-PCR test results were based on Ct values significantly lower than the LOD and thus carry a relatively low risk of false positive results, despite the relatively low COVID-19 incidence that reduces positive predictive value (PPV) of testing results [14,15]. Interestingly, similar averaged Ct values were reported in a study on symptomatic patients in the United Kingdom [13].

The lowest average Ct observed in June 2020 is challenging to explain. There appears no direct correlation with positivity rate or testing rate reduction in Poland during this period. Patients diagnosed as positive with elevated Ct could have been tested in an early or late phase of infection when viral load increases or decreases, respectively. Thus, reduction of Ct in June 2020 may suggest that patients were mostly diagnosed at the early to midpoint of the RT-PCR test window. When a diagnostic system is oversensitive, patients are tested early in the event of any suspicion of a potential infection. Consequently, Ct may be elevated when more pre-symptomatic patients are captured, especially when asymptomatic contacts of COVID-19 cases are frequently tested. When patients are tested a few days after the onset of disease, a lower Ct could be expected due to higher viral loads.

The presented results indicate that the average Ct values were mostly under 27 for the entire study period. During the first pandemic wave in Poland (March–June 2020), single target RT-PCR assays were commonly used. In our study group, these assays reveled lower average Ct (around 19) when compared with multiple (2–3) target assays. This phenomenon

Table 3

Statistics on Ct measurements by week for both the study cohort (March–September 2020; 27 weeks) and the reference cohort (October 2020–February 2022; 58 weeks). The first column shows the date starting the specified week; the second column shows the average Ct for the week, the standard deviation (SD) from the Ct, and the number of PCR measurements for the week. Weeks in which there were fewer than 10 measurements were removed.

| Week starting date	Average Ct for the week, (SD from the Ct, n of PCR measurements for the week)	Week starting date (cont.)	Average Ct for the week, (SD from the Ct, n of PCR measurements for the week) (cont.)	Week starting date (cont.)	Average Ct for the week, (SD from the Ct, n of PCR measurements for the week) (cont.)	Week starting date (cont.)	Average Ct for the week, (SD from the Ct, n of PCR measurements for the week) (cont.)
2020-03-28	24.6 (SD:8.5, n = 9)	2020-08-29	28 (SD:7.6, n = 540)	2021-02-27	25.13 (SD:5, n = 315)	2021-10-16	25.49 (SD:4.7, n = 593)
2020-04-04	27.19 (SD:6.8, n = 70)	2020-09-05	24.31 (SD:8.5, n = 307)	2021-03-06	26.21 (SD:4.5, n = 342)	2021-10-23	25.53 (SD:4.8, n = 719)
2020-04-11	27.63 (SD:5.5, n = 44)	2020-09-12	21.19 (SD:8, n = 411)	2021-03-13	25.46 (SD:4.7, n = 370)	2021-10-30	25.7 (SD:4.4, n = 730)
2020-04-18	26.88 (SD:5.1, n = 110)	2020-09-19	21.31 (SD:7, n = 462)	2021-03-20	25.14 (SD:4.6, n = 391)	2021-11-06	25.14 (SD:4.6, n = 748)
2020-04-25	24.87 (SD:5.8, n = 73)	2020-09-26	25.39 (SD:8, n = 36)	2021-03-27	25.75 (SD:4.7, n = 387)	2021-11-13	24.92 (SD:4.6, n = 878)
2020-05-02	30.71 (SD:6.4, n = 46)	2020-10-31	27.85 (SD:4.1, n = 2633)	2021-04-03	26.03 (SD:4.4, n = 308)	2021-11-20	25.06 (SD:4.7, n = 662)
2020-05-09	26.44 (SD:7, n = 244)	2020-11-07	27.58 (SD:4.2, n = 2019)	2021-04-10	25.37 (SD:4.8, n = 307)	2021-11-27	25.07 (SD:4.8, n = 518)
2020-05-16	23.13 (SD:8.4, n = 206)	2020-11-14	26.83 (SD:4.6, n = 1619)	2021-04-17	25.51 (SD:4.7, n = 220)	2021-12-04	24.52 (SD:5.1, n = 400)
2020-05-23	21.66 (SD:7, n = 271)	2020-11-21	27.13 (SD:4.4, n = 807)	2021-04-24	25.17 (SD:4.7, n = 147)	2021-12-11	24.85 (SD:4.9, n = 223)
2020-05-30	20.7 (SD:8, n = 326)	2020-11-28	27.36 (SD:4.5, n = 429)	2021-05-01	25.65 (SD:5.1, n = 87)	2021-12-18	24.96 (SD:5.2, n = 134)
2020-06-06	20.45 (SD:7.6, n = 365)	2020-12-05	25.85 (SD:4.9, n = 436)	2021-05-08	25.63 (SD:5, n = 82)	2021-12-25	24.91 (SD:4.9, n = 163)
2020-06-13	18.87 (SD:7.9, n = 218)	2020-12-12	26.65 (SD:4.9, n = 345)	2021-05-15	26 (SD:4.5, n = 56)	2022-01-01	25.27 (SD:4.9, n = 178)
2020-06-20	22.75 (SD:7.8, n = 262)	2020-12-19	26.27 (SD:4.6, n = 281)	2021-05-22	24.84 (SD:4.8, n = 36)	2022-01-08	24.8 (SD:4.9, n = 244)
2020-06-27	22.95 (SD:8, n = 152)	2020-12-26	25.65 (SD:5.1, n = 359)	2021-05-29	25.2 (SD:4.5, n = 14)	2022-01-15	24.35 (SD:4.6, n = 505)
2020-07-04	24.73 (SD:6.4, n = 183)	2021-01-02	25.97 (SD:4.7, n = 427)	2021-06-05	23.47 (SD:6.1, n = 10)	2022-01-22	25.04 (SD:4.3, n = 1119)
2020-07-11	24.71 (SD:7, n = 129)	2021-01-09	26.99 (SD:4.9, n = 278)	2021-08-28	22.7 (SD:7.3, n = 29)	2022-01-29	25.22 (SD:4.4, n = 974)
2020-07-18	24.44 (SD:7, n = 325)	2021-01-16	25.76 (SD:5.2, n = 316)	2021-09-04	24.81 (SD:5.9, n = 26)	2022-02-05	25.23 (SD:4.6, n = 702)
2020-07-25	23.26 (SD:6.9, n = 355)	2021-01-23	25.22 (SD:5.2, n = 274)	2021-09-11	25.26 (SD:5.1, n = 52)	2022-02-12	25.48 (SD:4.5, n = 416)
2020-08-01	20.69 (SD:7.4, n = 350)	2021-01-30	25.43 (SD:5, n = 323)	2021-09-18	25.49 (SD:5, n = 81)	2022-02-19	25.38 (SD:4.2, n = 87)
2020-08-08	21.82 (SD:6.6, n = 358)	2021-02-06	25.35 (SD:4.9, n = 323)	2021-09-25	24.83 (SD:4.9, n = 90)		
2020-08-15	23.42 (SD:6.9, n = 375)	2021-02-13	25.73 (SD:4.5, n = 295)	2021-10-02	24.65 (SD:4.6, n = 204)		
2020-08-22	24.74 (SD:8, n = 476)	2021-02-20	25.55 (SD:4.8, n = 291)	2021-10-09	24.97 (SD:4.9, n = 345)		

may be a result of strong Ct limitation for the interpretation of positive results, recommended by the test manufacturer at the very beginning of the COVID-19 diagnostics. In Poland, a commercial SARS-CoV-2 RT-PCR test targeting a single region of the virus genome was introduced early to COVID-19 diagnostics. This test met the required standards (CE) and was distributed to a number of laboratories which introduced COVID-19 diagnostics. The Ct of valuable and reproducible positive test result was limited to 33–34 according to the manufacturer's validation data. However, multiple target RT-PCR assays were then soon introduced via commonly available COVID-19 diagnostics in Poland. Manufacturers of these assays extended Ct limit for positive test results to over 35. Noteworthy, the average Ct curve of multiple target assays used from April to September 2020 resembles the average Ct curves for positive results gained in subsequent waves of COVID-19 pandemic by the triple target assays in the laboratory serving as the reference lab.

It is particularly important when assessing the reliability of molecular COVID-19 diagnostics in Poland during the early phase of the pandemic to consider the fact that the number of performed tests and their positivity rate was relatively low and ranged from 180/100,000 and 3.8% in week 16–389/100,000 and 2.6% in week 36 of 2020 [6]. When a relatively low test positivity rate is observed for a long time it may raise the suspicion that some positive cases remain undetected and the epidemic may therefore be underestimated. On the other hand, when RT-PCR tests which Ct are close to 35 are the predominant basis for positive test certificates, it may result in overestimation of positive cases. Clinical accuracy of positive results at high Ct values may be considered disputable, especially for asymptomatic patients [15].

Ct values may play an important role in disease prognostication. Rattan and Ahmad [8] suggested that the Ct values of positive COVID-19 results may be important for disease course prognosis, as well as for estimation of infection transmission risk posed. Zacharioudakis et al. [16] reported that low Ct values were significantly associated with poor disease progression in hospitalized patients. Bullard et al. [17] demonstrated that children who tested positive for SARS-CoV-2 by nasopharyngeal swabs displayed a lower likelihood to generate viral growth in culture, had higher Ct values and therefore a lower viral load compared to adults.

It is difficult to elucidate the reasons of the average Ct variability over time. In our present study, we deeply analyzed the average Ct of positive RT-PCR results in optimal conditions (single laboratory, high COVID-19
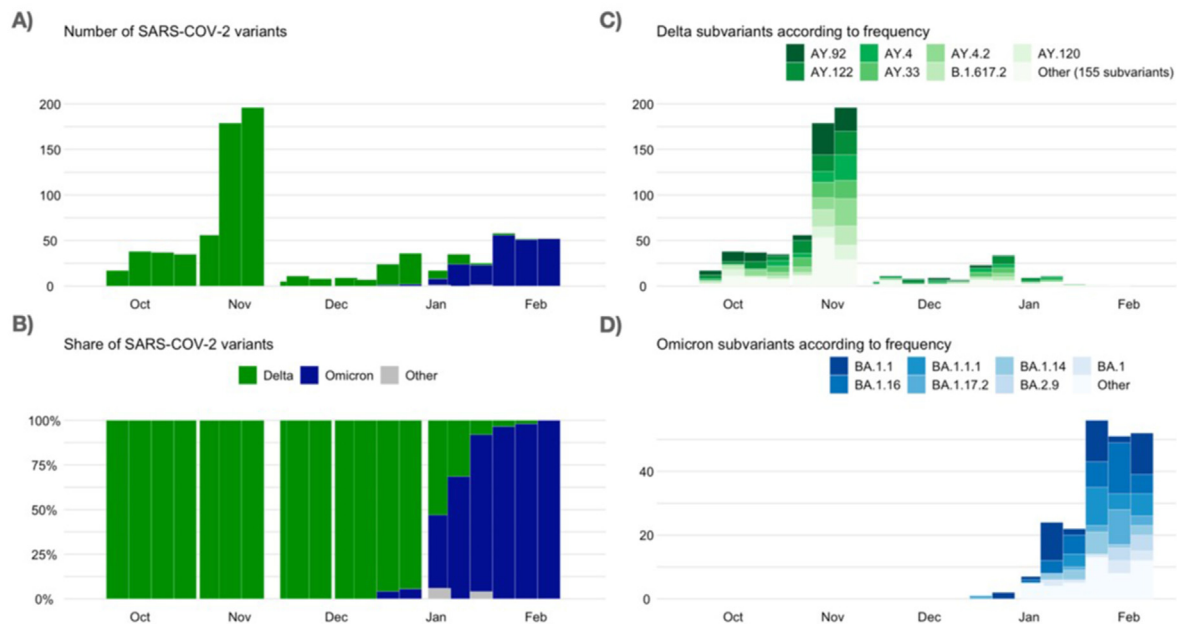
Fig. 2. Evolution of SARS-CoV-2 virus variants in Poland based on Next Generation Sequencing (NGS) data. Panel (A) shows the number of samples sequenced each week from October 2021 to the end of February 2022. The colors indicate virus variants. Panel (B) describes the percentage of each variant group in each week. Panels (C) and (D) show in detail how the proportion of each sub-variant evolved over time. The blue shades indicate the Omicron variants, i.e. Pango Lineage: B.1.1.529 and BA lineages. The green shades indicate the Delta variants, i.e. Pango Lineage: B.1.617.2 and AY lineages.

prevalence = high PPV) to determine whether SARS-CoV-2 genetic variants could affect COVID-19 diagnostics. In such optimal conditions, minor or no impact of the variant alterations on the average Ct, were observed. This may suggest that other, probably inter-laboratory factors (staff experience, different tests and manufacturers' recommendations for Ct ranges of positive results interpretation) or tested populations (symptomatic vs. asymptomatic) may more strongly affect RT-PCR COVID-19 diagnostics than minor mutations in SARS-CoV-2 genome, unless the mutation directly impacts the target regions of the assay. In Poland, according to the WHO guidelines, screening of asymptomatic persons for SARS-CoV-2 infection have not been recommended or conducted to control COVID-19 epidemic [18]. Only close contacts of confirmed cases were tested. Therefore, our results may not reflect situation in countries or populations where mass RT-PCR screening of asymptomatic patients was conducted. We, however, encourage others to quantitatively analyze records from RT-PCR COVID-19 diagnostics to extend th knowledge on effectiveness of diagnostic capacity. Broad mass testing is a key tool for controlling the COVID-19 pandemic, especially in surge situations [11]. Therefore, it would be particularly interesting to know the average Ct of positive RT-PCR results in regions where the testing and positivity rates were significantly higher than in Poland.

4.1. Limitations of the study

A few limitations should be noted, first we are unable to link the Ct of an RT-PCR result with the clinical course of the analyzed patients, as well as the presence of symptoms (or lack of thereof) at the sampling time. We are also unable to link patients for the purposes of analyzing infection transmission chain. Additionally, several different RT-PCR assays were used in the different laboratories contributing to this study, thus may have been a source of bias.

5. Conclusions

Our findings suggest that the risk of false positive/negative results due to a Ct close to the LOD was relatively low, thus likely did not impact the reliability of general SARS-CoV-2 diagnostics in Poland during the initial phase of the pandemic. Therefore, our data suggests that the vast majority of COVID-19 cases recorded in Poland from April to September 2020 were detected accurately with minor risk of false positive or negative results.

Data from a single center exhibit greater consistency for both virus Ct level and SARS-CoV-2 virus variant identification, and may therefore be particularly useful as the reference for studies assessing diagnostic results from multiple laboratories.

The author contributions

Study Design: Rafał Gierczyński, Grzegorz Juszczyk, Aleksandra Czerw, Jacek Nikliński, Radosław Charkiewicz, Przemysław Biecek, Brandon Henry.

Data Collection: Grzegorz Juszczyk, Rafał Gierczyński, Radosław Charkiewicz, Jacek Nikliński, Piotr Majewski, Joanna Reszeć, Piotr Piątyszek, Hubert Baniecki.

Statistical Analysis: Aleksandra Czerw, Hubert Baniecki, Przemysław Biecek.

Data Interpretation: Rafał Gierczyński, Aleksandra Czerw, Grzegorz Juszczyk, Radosław Charkiewicz, Jacek Nikliński, Piotr Majewski, Joanna Reszeć, Piotr Piątyszek, Hubert Baniecki, Przemysław Biecek, Brandon Michael Henry.

Manuscript Preparation: Rafał Gierczyński, Aleksandra Czerw, Grzegorz Juszczyk, Radosław Charkiewicz, Jacek Nikliński, Piotr Majewski, Joanna Reszeć, Piotr Piątyszek, Hubert Baniecki, Przemysław Biecek, Brandon Michael Henry.

Literature Search: Brandon Henry, Aleksandra Czerw, Rafał Gierczyński.

Funds Collection: Grzegorz Juszczyk, Rafał Gierczyński.

Declaration of competing interest

The authors declare no conflict of interests.

References

[1] World Health Organization. COVID-19 weekly epidemiological update. Data Rec WHO Nat Authority 6th July 2022. https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19–6-july-2022.

[2] World Health Organization. Molecular assays to diagnose COVID-19: summary table of available protocols, 24 January 2020, COVID-19: laboratory and diagnosis. https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf?sfvrsn=de3a76aa_2&download=true.

[3] Gujski M, Raciborski F, Jankowski M, Nowicka PM, Rakocy K, Pinkas J. Epidemiological analysis of the first 1389 cases of COVID-19 in Poland: a preliminary report. Med Sci Mon Int Med J Exp Clin Res: Int Med J Experiment Clinic Res 2020;26:e924702. https://doi.org/10.12659/MSM.924702.

[4] Pinkas J, Jankowski M, Szumowski Ł, Lusawa A, Zgliczyński WS, Raciborski F, Wierzba W, Gujski M. Public Health Interventions to Mitigate Early Spread of SARS-CoV-2 in Poland. Medical science monitor. Int Med J Experiment Clinic Res 2020; 26:e924730. https://doi.org/10.12659/MSM.924730.

[5] European Centre for Disease Prevention and Control (ECDC). Data on 14-day notification rate of new COVID-19 cases and deaths. https://www.ecdc.europa.eu/en/publications-data/data-national-14-day-notification-rate-covid-19; 2020.

[6] European Centre for Disease Prevention and Control (ECDC). Data on testing for COVID-19 by week and country. https://www.ecdc.europa.eu/en/publications-data/covid-19-testing; 2020.

[7] World Health Organization. Laboratory testing for coronavirus disease (COVID-19) in suspected human cases Interim guidance. https://apps.who.int/iris/rest/bitstreams/1272454/retrieve; 2020.

[8] Rattan A, Ahmad H. Can quantitative RT-PCR for SARS-CoV-2 help in better management of patients and control of coronavirus disease 2019 pandemic. Indian J Med Microbiol 2020;38(3 & 4):284–7. https://doi.org/10.4103/ijmm.IJMM_20_380.

[9] Axell-House DB, Lavingia R, Rafferty M, Clark E, Amirian ES, Chiao EY. The estimation of diagnostic accuracy of tests for COVID-19: a scoping review. J Infect 2020;81(5):681–97. https://doi.org/10.1016/j.jinf.2020.08.043.

[10] Pujadas E, Chaudhry F, McBride R, Richter F, Zhao S, Wajnberg A, Nadkarni G, Glicksberg BS, Houldsworth J, Cordon-Cardo C. SARS-CoV-2 viral load predicts COVID-19 mortality. Lancet Respir Med 2020;8(9):e70. https://doi.org/10.1016/S2213-2600(20)30354-4.

[11] Khan AA, Alahdal HM, Alotaibi RM, Sonbol HS, Almaghrabi RH, Alsofayan YM, Althunayyan SM, Alsaif FA, Almudarra SS, Alabdulkareem KI, Assiri AM, Jokhdar HA. Controlling COVID-19 pandemic: a mass screening experience in Saudi arabia. Front Public Health 2020;8:606385. https://doi.org/10.3389/fpubh.2020.606385.

[12] Afzal Adeel. Molecular diagnostic technologies for COVID-19: limitations and challenges. J Adv Res 2020;26:149–59. https://doi.org/10.1016/j.jare.2020.08.002.

[13] Singanayagam A, Patel M, Charlett A, Lopez Bernal J, Saliba V, Ellis J, Ladhani S, Zambon M, Gopal R. Duration of infectiousness and correlation with RT-PCR cycle threshold values in cases of COVID-19, England, January to May 2020. Euro Surveill 2020;25(32):2001483. https://doi.org/10.2807/1560-7917.ES.2020.25.32.2001483.

[14] European Centre for Disease Prevention and Control. Options for the use of rapid antigen tests for COVID-19 in the EU/EEA – first update, 2021. Stockholm: ECDC; 2021. https://www.ecdc.europa.eu/en/publications-data/options-use-rapid-antigen-tests-covid-19-eueea-first-update.

[15] Brendan H, Azizah K, Huria M, Ian B, Hibo A. The impact of false positive COVID-19 results in an area of low prevalence. Clin Med 2021;21(1):e54–6. https://doi.org/10.7861/clinmed.2020-0839.

[16] Zacharioudakis IM, Prasad PJ, Zervou FN, Basu A, Inglima K, Weisenberg SA, et al. Association of SARS-CoV-2 genomic load with outcomes in patients with COVID-19. Annal Am Thoracic Soc 2021;18(5):900–3. https://doi.org/10.1513/AnnalsATS.202008-931RL.

[17] Bullard J, Funk D, Dust K, Garnett L, Tran K, Bello A, et al. Infectivity of severe acute respiratory syndrome coronavirus 2 in children compared with adults. CMAJ (Can Med Assoc J) 2021;193(17):E601–6. https://doi.org/10.1503/cmaj.210263.

[18] World Health Organization. Recommendations for national SARS-CoV-2 testing strategies and diagnostic capacities. Interim guidance 2021. https://apps.who.int/iris/bitstream/handle/10665/342002/WHO-2019-nCoV-lab-testing-2021.1-eng.pdf?sequence=1&isAllowed=y.