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Short report

Aging correlates with lower threshold cycle values for *RdRP/RdRP+S* genes during molecular detection of SARS-CoV-2

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ABSTRACT

RT-qPCR is the most reliable molecular method for the detection of severe acute respiratory syndrome *coronavirus* 2 (SARS-CoV-2). Here, we analyzed results of RT-qPCR obtained for 3044 patients diagnosed as SARS-CoV-2-positive using four different molecular tests utilizing five RNA sequences. The analysis showed that patients' age inversely correlates with *threshold* cycle (*Ct*) values for *RdRP* gene (LightMix® Modular Wuhan CoV RdRP-gene by Roche Diagnostics) and *RdRP+S* genes (MutaPLEX® Coronavirus RT-PCR kit by Immundiagnostic). At the same time, there was no correlation between age and Ct values for *E*, *N*, and *ORF1ab* genes. When patients were grouped by age, mean Ct values for *RdRP* gene in older patients were significantly lower compared with younger individuals. Collectively, our report indicates that older SARS-CoV-2-infected individuals exhibit higher viremia at diagnosis than younger patients, which may reflect impaired functioning of their immune response and predispose to more severe disease and worse prognosis.

1. Introduction

Laboratory medicine aimed at acute respiratory syndrome *coronavirus* 2 (SARS-CoV-2) diagnosis allows identifying three separate indicators of the infection: viral RNA, antigens, and specific antibodies produced by immune cells. Due to the highest sensitivity of *nucleic acid amplification* tests (NAATs), the real-time (RT)-quantitative (q)PCR directed on the identification of SARS-CoV-2 RNA is commonly treated as the gold-standard in COVID19 diagnostics (Yuce et al., 2020).

During the RT-qPCR, the presence of SARS-CoV-2 nucleic acid is shown as the exponential (S-shaped) curve of amplification, arising from the point where the amplification curve crosses a threshold line. This intersection point, called a threshold cycle (Ct), is a unique feature of a given sample and thus it can be treated as a relative measure of a target quantity. Considering that the number of a template copies doubles with each cycle of amplification, lower Ct values indicate higher number of target copies and vice versa. In case of virologic diagnostics, when RT-qPCR is conducted under reproducible conditions, i.e. the same reagents, reaction volumes, and instruments, Ct values may indirectly reflect the magnitude of viral load (Tom and Mina, 2020).

From the beginning of the pandemic, older people were at a relatively high risk of severe COVID19 and bad prognosis. In fact, aged patients are the group with the highest mortality among all infected patients (Kang and Jung, 2020). Most frequently, it is related to the presence of various age-related diseases, but it cannot be ruled out that an impaired functionality of their immune system, a natural feature of organismal aging (Montecino-Rodriguez et al., 2013), also plays a role.

In this study, we analyzed retrospectively results of RT-qPCR reactions for more than three thousand SARS-CoV-2-positive patients to verify if there exists any relationship between their age and Ct values at which the viral RNA started to generate a diagnostically relevant fluorescence.

2. Materials and methods

2.1. Patients

Nasopharyngeal swabs were taken from 3044 symptomatic and asymptomatic patients living in the Greater Poland Voivodeship, between May and October 2020 (Table 1). The samples were analyzed towards SARS-CoV-2 infection in the Poznan University of Medical Sciences Coronavirus Laboratory using RT-qPCR. Informations regarding patients do not include their clinical status as this parameter was not evaluated at the stage of diagnosis and the laboratory in which the RT-qPCR was conducted has no access to those data.

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Table 1
Characteristics of patients.

	All	Men	Women
Number	3044	1428	1616
Age (mean ± SD; range)	44.8 ± 18.5; 1–98	43.2 ± 18.5; 1–94	46.3 ± 18.4; 1–98
LightMix® Modular Wuhan CoV RdRP-gene/SARS and Wuhan CoV E-gene	658	312	346
Vitassay qPCR SARS-CoV-2	264	108	156
MutaPLEX® Coronavirus RT-PCR kit	1933	919	1014
2019-nCoV Triplex RT-qPCR Detection Kit	189	89	100

2.2. RNA isolation and RT-qPCR

Viral RNA was extracted using MGISP-960 (MGI Tech Co., Ltd., Shenzhen, China), Chemagic 360 (PerkinElmer, Waltham, MA, USA), and Maxwell ® RSC (Promega, Madison, WI, USA). The material was subjected to RT-qPCR using LightCycler® 480 (Roche Diagnostics, Indianapolis, IN, USA). The distribution of samples between three RNA extractors was random and depended on laboratory needs on a given day, whereas their distribution among tests was related to their availability on the market. Four different molecular tests approved and validated for SARS-CoV-2 diagnostics were used strictly in line with manufacturers' instructions (Table 2). RT-qPCR reactions were conducted up to 45th cycle (Fig. 1). All consumables (e.g. tips, plates, strips) were consistent with requirements of each assay and mostly provided by tests' manufacturers.

2.3. Statistics

Statistical analysis was performed using GraphPad Prism™ v.9.0 (GraphPad Software, San Diego, USA). The correlations were analyzed using the Spearman test. The means were compared with the Kruskal-Wallis test and Dunns test (comparison of all pairs of groups) as the post hoc. Statistical significance was acknowledged when the P value was less than 0.05.

The means were additionally corrected for multiple comparisons by controlling the False Discovery Rate on the basis of two-stage step-up method of Benjamin, Krieger and Yekutieli test with a desired false-discovery rate Q less than 0.05 as the criterion.

Table 2
Characteristics of RT-qPCR tests used in the study.

Feature/test	Test 1	Test 2	Test 3	Test 4
Test name	LightMix® Modular Wuhan CoV RdRP-gene/ SARS and Wuhan CoV E-gene	Vitassay qPCR SARS-CoV-2	MutaPLEX® Coronavirus RT-PCR kit	2019-nCoV Triplex RT-qPCR Detection Kit
Manufacturer	TIB MOLBIOL[a]	Vitassay	Immundiagnostic	Vazyme
City/Country	Berlin, Germany	Huesca, Spain	Bensheim, Germany	Nanjing, China
Catalog number	53-0777-96 and 53-0776-96	7081046	KG192696	CD302-02
Gene 1	*RdRP*	*ORF1ab*	*RdRP* + *S*	*ORF1ab* (*RdRP* region)
Gene 1 channel	FAM	FAM	FAM	FAM
Gene 2	*E*	*N*	*E*	*N*
Gene 2 channel	FAM	ROX	Cy5	ROX
Sensitivity	≤10 copies	≥10 copies	≤10 copies	4 copies

[a] Distributed by Roche.

3. Results and discussion

Molecular diagnostic of SARS-CoV-2 using RT-qPCR is based on the detection of at least two targets – viral RNA sequences – of which, ideally, one region is specific and one is conserved to avoid cross-reactivity with other endemic coronaviruses as well as potential genetic drift (Tang et al., 2020b). Indeed, coronaviruses contain several genes that have been identified and employed as targets in SARS-CoV-2 diagnostics. They belong to two categories, that is replication-driving genes and those coding for structural proteins. The first group includes RNA-dependent RNA polymerase (*RdRP*), hemagglutinin-esterase (*HE*), and open reading frame 1a (*ORF1a*) and *ORF1b*, whereas envelope glycoproteins spike (*S*), envelope (*E*), transmembrane (*M*), helicase (*Hel*), and nucleocapsid (*N*) belong to the second group (Mathuria et al., 2020).

In this study we analyzed results of RT-qPCR obtained for SARS-CoV-2-positive patients using assays from four manufacturers, in which four pairs of genes were utilized: *RdRP/E*, *ORF1ab/N*, *RdRP+S/E* (a trigenic test), and *ORF1ab* (*RdRP* region)*/N*. Correlative analysis revealed that Ct values obtained for *RdRP* gene (LightMix® Modular Wuhan CoV RdRP-gene by Roche Diagnostics) and *RdRP+S* pair of genes (MutaPLEX® Coronavirus RT-PCR kit by Immundiagnostic) remain in an inverse relationship with patients' age. The older the patients were, the lower were the Ct values. In case of remaining genes and assays, any correlation between Ct values and age was absent (Table 3). In order to find out, which age values particularly determine this relationship, patients were assigned according to their ages into ten decade-wide groups. The comparison of means showed that Ct values for RdRP gene in patients 41–50, 51–60, and 91–100 years old were significantly lower compared with patients 21–30 and 0–10 years old. In case of *RdRP+S* pair statistically significant differences were found between Ct values established for 91–100 years old and 0–10 and 21–30 years old patients. Noteworthy, the statistical power of the differences obtained was also confirmed by additional analysis of the False Discovery Rate, in which the determined Q values confirmed the truth of the discoveries, eliminating the risk of a false negative result (Table 4). On the other hand, discrepancies in the size of some age groups were responsible for the lack of statistically significant differences between them although the average Ct values would seem to indicate otherwise. However, this situation was unavoidable because of the random distribution of patients at different ages.

Taking into account that Ct values may indirectly reflect the viral load in a given patient, our results suggest that older SARS-CoV-2-infected individuals have higher viremia at diagnosis (Tom and Mina, 2020), which may at least partly explain more severe course of the disease and worse prognosis (Karahasan Yagci et al., 2020). A relationship between lower Ct values and higher clinical severity has already been found years ago in patients with various upper respiratory tract infections, caused by adenovirus, human metapneumovirus, and parainfluenza virus (Fuller et al., 2013).

Significantly lower Ct values for aged patients (80–89 years old) were previously described by Buchan et al., albeit six important differences with respect to our study should be emphasized. The first is that they analyzed three times smaller cohort of patients, other genes (*ORF1* and *E*), employed only one molecular test, did not include a correlative analysis, and – last but not least – analyzed the differences between means in a quite unique way, that is by comparing of average Ct values for each age group with the mean of all Ct values (Buchan et al., 2020). At the same time, our findings challenge those by Heald-Sargent et al., who found that young children (<5 years old) have higher viral load than adults (18–65), but made this observation using far lower group of patients that we did (145 vs. 3044) and did not perform correlative tests (Heald-Sargent et al., 2020).

As per potential reasons of higher viral load in aged individuals and specific course of the disease in this group, one may speculate that they may be linked with age-associated changes in a functioning of immune

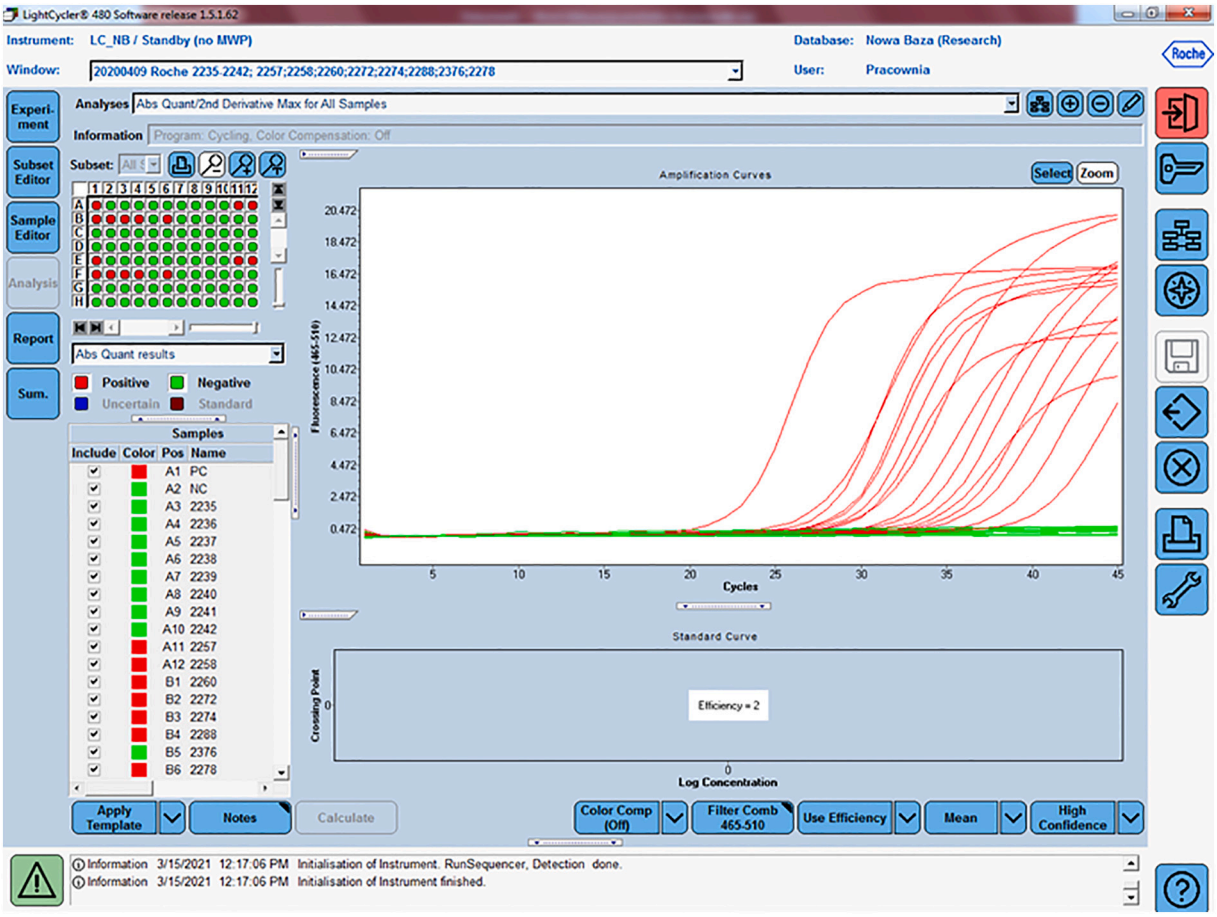

Fig. 1. Exemplary RT-qPCR reactions demonstrating the amplification of SARS-CoV-2 genes in different patients.

Table 3
Relationship between Ct values and SARS-CoV-2-positive patients' age.

Test/gene	*RdRP*	*E*	*ORF1ab*	*N*	*RdRP+S*	*ORF1ab* (*RdRP* region)
LightMix® Modular Wuhan CoV RdRP-gene/SARS and Wuhan CoV E-gene	r = −0.7466 P = 0.0002	r = −0.1107 P > 0.05	– –	– –	– –	– –
Vitassay qPCR SARS-CoV-2	– –	– –	r = 0.008644 P > 0.05	r = 0.04204 P > 0.05	– –	– –
MutaPLEX® Coronavirus RT-PCR kit	– –	r = −0.04011 P > 0.05	– –	– –	r = −0.8345 P = 0.0188	– –
2019-nCoV Triplex RT-qPCR Detection Kit	– –	– –	– –	r = 0.08708 P > 0.05	– –	r = 0.07420 P > 0.05

system. These changes include declined adaptive immunity (*immunosenescence*) coupled with activated innate immunity (*inflamm-aging*) (Salminen et al., 2008). Major features of immunosenescence include regression of thymus, decreased numbers of pro- and pre-B cells, reduced ability of hematopoietic stem cells to repopulate, diminished RAG expression, impaired cytotoxicity of NK cells, reduced phagocytic activity of neutrophils, reduced responsiveness of naïve T cells to interleukin 2 (IL-2), and decreased naïve to memory T cell ratio. All these changes contribute to a progressive deterioration of the ability of aged people to respond to infections (Aw et al., 2007). At the same time, one may not exclude that the worsening of immune response in elderly individuals may also depend on some specific features of SARS-CoV-2 itself, including its plausible ability to modulate some regulatory mechanisms in the host (e.g. miRNAs) that facilitate the virus expansion (Bartoszewski et al., 2020). On the other hand, at the level of tissues and organs, mostly due to the accumulation of senescent cells, several pro-inflammatory cytokines, chemokines, growth factors, and extracellular matrix remodeling molecules are overproduced generating a microenvironment that promotes various pathological changes, leading to increased morbidity and mortality of aged individuals (Xia et al., 2016). Taking into account this dichotomic pattern of age-associated changes in the immunologic status one may suppose that the higher viremic load in this group of patients may be closely linked with immunosenescence phenomenon, whereas the inflamm-aging phenotype may synergize with cytokine storm recognized as the prime determinant of high mortality of COVID19 patients. Interleukin-6 (IL-6) is the best example of this plausible relationship, as its elevated level is one of the most reliable markers of inflamm-aging (Maggio et al., 2006), and – at the same time – its concentration in severely ill COVID19 patients is significantly higher than in those who experience moderate symptoms of the disease (Hojyo et al., 2020; Tang et al., 2020a). In this regard we can, however, only speculate that cytokines overproduced by senescent cells constitute an undetermined part of the total amount of proinflammatory cytokines in aged patients.

Table 4
Ct values estimated for tested genes in different age groups.

Test/gene	Gene	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
LightMix® Modular Wuhan CoV RdRP-gene/SARS and Wuhan CoV E-gene	*RdRP*	38.8 ± 1.3	n.a.	38.7 ± 1.3	37.2 ± 4.8	37.0 ± 1.2[a]	36.7 ± 1.6[a,b]	37.4 ± 4.3	36.8 ± 5.3	36.8 ± 3.8	33.3 ± 1.1[a,b]
	E	33.5 ± 3.5	n.a.	33.7 ± 3.2	33.2 ± 4.4	32.5 ± 4.4	32.2 ± 4.6	33.4 ± 4.8	32.9 ± 5.4	32.6 ± 5.0	32.8 ± 2.7
Vitassay qPCR SARS-CoV-2	*ORF1ab*	30.5 ± 5.4	n.a.	31.4 ± 2.9	30.7 ± 4.2	32.1 ± 4.1	31.5 ± 4.1	32.6 ± 2.9	29.9 ± 4.6	28.7 ± 6.8	30.5 ± 4.2
	N	30.3 ± 5.7	n.a.	31.6 ± 3.7	30.9 ± 3.8	31.9 ± 3.5	32.1 ± 3.6	32.6 ± 2.8	30.9 ± 4.1	29.4 ± 6.2	31.6 ± 3.1
MutaPLEX® Coronavirus RT-QPCR kit	*RdRP+S*	32.6 ± 1.9	28.1 ± 4.4	32.1 ± 2.1	32.5 ± 5.1	32.5 ± 4.9	31.6 ± 5.5	31.8 ± 5.2	31.8 ± 5.3	31.0 ± 4.7	27.2 ± 1.2[c,d]
	E	28.9 ± 5.0	24.5 ± 4.4	28.4 ± 5.0	28.9 ± 5.3	28.8 ± 5.0	28.1 ± 5.5	28.2 ± 5.2	28.3 ± 5.4	27.3 ± 4.8	25.8 ± 4.3
2019-nCoV Triplex RT-qPCR Detection Kit	*ORF1ab* (*RdRP* region)	25.0 ± 7.1	29.3 ± 2.9	27.4 ± 7.1	27.7 ± 6.1	28.8 ± 6.5	29.4 ± 6.6	29.0 ± 5.5	27.2 ± 7.8	27.9 ± 5.6	n.a.
	N	24.2 ± 5.9	31.5 ± 0.7	28.1 ± 6.5	28.9 ± 5.1	30.1 ± 4.2	30.1 ± 5.4	29.6 ± 5.7	28.9 ± 7.4	29.1 ± 3.4	n.a.

Results are expressed as means ± SDs.
[a] $P < 0.05$ vs. 21–30 ($Q = 0.0194$, 0.0174 and 0.0162 for 41–50 vs. 21–30; 51–60 vs. 21–30, and 91–100 vs. 21–30, respectively).
[b] $P < 0.05$ vs. 0–10 ($Q = 0.0189$ and 0.0178 for 51–60 vs. 0–10 and 91–100 vs. 0–10, respectively).
[c] $P < 0.05$ vs. 21–30 ($Q = 0.0191$ for 91–100 vs. 21–30).
[d] $P < 0.05$ vs. 0–10 ($Q = 0.0163$ for 91–100 vs. 0–10).

Taken together, results provided in this report show that some molecular tests, utilizing RT-qPCR detection of *RdRP/RdRP+S* gene, inform about relatively high SARS-CoV-2 viremia in aged individuals at diagnosis, which may directly translate to severity of the disease and its outcome on the basis of several age-associated changes in immunological status of these patients. Taking into account a well-established knowledge about mechanisms of inflamm-aging development, it seems reasonable to ask whether the use of senolytics that eliminate senescent (read: cytokine overproducing) cells from the organism (Hickson et al., 2019) could be a barrier to the cytokine storm in elderly coronavirus-infected people, thus reducing the risk of severe disease progression and death.

CRediT authorship contribution statement

Conceptualization – KK; Investigation – JMP; Methodology – KK, Writing-original draft – JMP, KK; Writing – review & editing – JMP, KK.

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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