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Orginal Article

Uncovering Gender and Age Neutrality in CT Values for *E* and *RdRp* Gene Amplification in SARS-CoV-2

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic poses a danger to public health across the world. Specific and sensitive testing is vital to tracking and containing the infected individuals. This study examines the characteristics of 705 patients based on gender and age groups, with a focus on their CT (Cycle Threshold) values associated with *E*- and *RdRp* gene amplification. Among the patient cohort, 58.87% were male, and 41.13% were female. Notably, all males and females tested positive for the E gene, achieving a 100% detection rate, whereas the RdRp gene exhibited a slightly lower positive rate of 93.4% in males and 89.6% in females. Statistical analysis through the chi-square test revealed no significant disparities between target genes and gender. Additionally, a one-way ANOVA test demonstrated no significant distinctions between target genes and age groups. This study's findings suggest that gender and age do not significantly influence CT values for E and RdRp gene amplification, and there is no preferred gene for the detection, providing valuable insights into the molecular characteristics of the studied cohort.

Keywords: COVID-19, SARS-CoV-2, *RdRp* gene, E gene, RT-PCR

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic is considered the most significant healthcare crisis around the world since the discovery in Wuhan city, China [1]. The major clinical signs of the virus are fever, cough, shortness of breath, fatigue and myalgia myalgia [2-4]. Infection may cause severe acute respiratory syndrome (SARS), pneumonia, kidney failure, diarrheal and in severe cases even death in severe cases [5,6]. Since the emergence of coronavirus disease (COVID-19) at the end of 2019, rapid tracing and isolation of confirmed cases and close contacts with restrictions social movement have played an important role in controlling onward spread of the virus [7].

Due to very quick spreading and increasing number of SARS-CoV-2 cases in the world, rapid, sensitive and very specific method is essential for the diagnosis of the virus in the laboratories and early detection of virus is important to prevent and control this pandemic [8]. Diagnosis of virus is made using clinical manifestation, laboratory and radiological features. As clinical manifestation and radiological findings of COVID-19 are non-specific, a positive test allows the clinicians and public health professionals to rapidly isolate the patient and prevent spreading of the disease. The nucleic acid amplification test is a standard method for the non-invasive detection of COVID-19. There are many methods for the diagnosis of COVID-19 virus depends on the viral nucleic acid within upper and lower respiratory samples [9]. It has previous reported that Real-time polymerase chain reaction (RTPCR) on nasopharyngeal and oropharyngeal swabs is a gold standard method for confirming the diagnosis of COVID-19 in clinical cases, since the early stages of the COVID-19 pandemic and the presence of viral RNA confirms SARS CoV2 [10].

Virus detection by RTPCR from respiratory samples is commonly used for the diagnosis and monitor SARS-CoV-2 infection, which is based on several probe primers sets for targeting SARS-CoV-2 sequences. The primer–probe sets target the different regions of SARS-CoV-2 including; membrane *(M)* envelope (*E*), nucleocapsid (*N*), glycoprotein spike (*S*), and RNA dependent RNA

polymerase (*RdRp*) sequences as the target of the RT-PCR, resulting that each of the genes has different sensitivity and specificity, and they can be used for detection and confirming of SARS-CoV-2, worldwide [11]. During early response and detection of the SARS-COV-2 outbreak, the World Health Organization (WHO) recommended using RT-PCR as the first-line screening test depend on an *E*-gene assay, followed by the *RdRp* gene, as RT-PCR assays targeting the *RdRp* gene had the highest sensitivity and specificity [12]. RT-PCR was proposed as a diagnostic tool for COVID-19. In this regard, many studies compared different diagnostic tools, such as computed tomography (CT) scan as well. It was concluded that RT-PCR is of higher sensitivity and specificity than CT scan, particularly if multiple samples are used [13]. In addition, CT scan is more laborious and more expensive than RT-PCR [13]. We believe that the shortage in RT-PCR supply and the release of patients from quarantine without diagnosis played a major role in the sharp increase in the number of COVID-19 cases and increased the spread of infection in the region [2,14]. The aim of this study is to measure amplification curves of RTPCR Ct value in SARS-CoV-2 of the E and RdRp genes and also compared the sensitivity and specificity of these genes for the detection of COVID- 19 infection

MATERIALS and METHODS

Patients and samples

In this study, a total of 1655 patients tested for SARS-Cov-2 and 705 positive patients between September 15 and December 31, 2020, were included in the present study (Figure 1). Nasopharyngeal swabs were collected from symptomatic patients admitted to a private clinical center in Duhok province, Kurdistan Region, Iraq, for the extraction of SARS-CoV-2 RNA. The collected swabs were immediately placed into the viral transport tube, which includes a universal transport medium that is stable at room temperature (W/3 mL VTM-RT, Wellkang Ltd., UK). Samples (3 ml) were kept at 4–8 °C for short-term storage and at 70 °C for the long term.

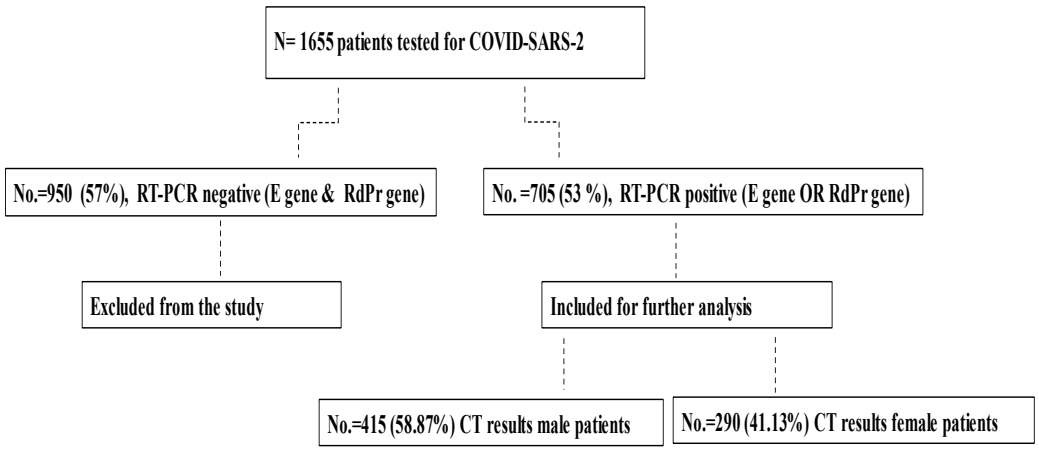

Figure 1. Study cohort structure with criteria for inclusion and exclusion of the samples.

Extraction of Viral RNA

The viral RNA extraction from nasopharyngeal samples was performed using the QIAamp Viral RNA Extraction Kit (Qiagen, Korea), following the manufacturer's instructions. Briefly, swabs were vortexed for 15 seconds, followed by extraction from 200 µl of VTM-RT, and all samples were subjected to extraction with a single elution AVE buffer volume of 60 l. RNA extracts were stored at 70 °C for further analyses. For detection of SARS-CoV-2, RT-qPCR primer-probe sets designed on the RdRp and E genes were recommended by the World Health Organization (WHO).

RT-qPCR for SARS-CoV2 detection

Sequence of primer and probe (PowerCheckTM 2019-nCoV, Kogenbiotech) used in RT-qPCR, provided by the WHO. The viral RNA sample (5 L) was mixed with the RT-PCR premix reagents (11 L) and the corresponding Rdp or E gene primers or prob mix (4 L). Additionally, internal control was used to monitor the validity of sample collection and the RT-qPCR process to avoid false-negative results and confirm the integrity of the reagents in the kit. PCR was performed at 50 °C for 30 min, 95 °C for 10 min, 95 °C for 15 s, and 60 °C for one

min for 40 cycles. The GIAGEN Rotor-Gene Q Real-Time PCR System (GIAGEN, Hilden, Germany) was used for RT-qPCR, and the cycle threshold (Ct) value of the SARS-CoV-2 target gene was ascertained (Table 1).

Table 2. The criteria for each target (E gene, RdRP, and IC) based on the manufacturer's guidelines

	E gene	RdRP gene	Internal control (IC)
Positive (+)	Ct value ≤ 37	Ct value ≤ 37	Ct value ≤ 28
Negative (-)	Ct value > 37	Ct value > 37	Ct value > 28
	or not detected	or not detected	or not detected

Ethical approval

The study protocol was approved by the scientific and ethical committee of the College of Medicine, University of Zakho. Informed written consent was obtained from all the participants before collecting samples.

Statistical analysis

Continuous variables were presented as means ± standard deviation (SD). For categorical variables were presented as counts and percentages and P value is determined using Chi-square test and One Way ANOVA. P-values less than 0.05 were considered as statistically significant. All statistical analyses were performed using GraphPad prism software, version 8.0.

RESULTS

Patients' characteristics were evaluated by gender and age group according to their CT values. The CT values for E- and RdRp gene amplification numbers are shown in Table 2. Out of the 705 patients, 415 (58.87%) and 290 (41.13%) were male and female, respectively. Also, the number of males and females positively detected by the E gene was 415/415 (100%) and 290/290 (100%), while the RdRp gene had a 388/415 (93.4%) and 260/290 (89.6%) positive rate in males and females, respectively (Table 3). According to the chi-square test, there were no significant differences between target genes and gender. We also found that there was no significant difference between target and age groups using a one-way ANOVA test (Table 2).

Table 2. The CT values for E- and RdRp gene amplification numbers according to gender and age

Variable		E gene (*Ct*)	p value	RdP gene (*Ct*)	p value
Gender	Female	24.25 ± 6.87	0.32	24.07 ± 5.85	0.82
	Male	23.76 ± 6.52		24.17 ± 6.06	
Age (Year)	< 20	22.21 ± 6.28	0.36	24.15 ± 5.78	0.95
	20-30	24.89 ± 7.11		24.49 ± 6.16	
	31-40	24.31 ± 6.52		24.29 ± 6.02	
	41-50	23.54 ± 6.71		23.96 ± 6.09	
	51-60	23.11 ± 6.61		23.83 ± 5.97	
	> 60	23.92 ± 6.21		23.89 ± 5.61	

*p value is determined using Chi-square test

Table 3. The frequency of positivity of E and RDP gene according to the gender

	E gene No. (%)		RDP gene No. (%)	
Gender	Positive	Negative	Positive	Negative
Female	290 (100)	0 (0)	260 (89.66)	30 (10.34)
Male	415 (100)	0(0)	388 (93.49)	27 (6.51)

DISCUSSION

It is well recognized that specific and sensitive detection of COVID-19 is crucial for containment and contact tracing purposes. RT-PCR has been used as a gold standard [15]. Generally, this method targets three different genes across the COVID genome: firstly, the E gene that encodes for the envelop; secondly, the RDP gene that expresses

RNA-dependent RNA polymerase; and finally, the N gene that codes for the nucleocapsid protein[12,16]. The expression of the gene is used to detect the virus, and determine the period of infection based on the viral genomic cDNA detected in a sample. In terms of the infectivity of the virus, many studies have reported higher infectivity rates in older age groups due to the prevalence of various comorbidities[17]. In this study, a similar pattern can be observed with the increase in age and the number of infections. Furthermore, statistically, there are no differences ($p = 0.95$) in the CT threshold of both genes among the same studied age groups. The absence of a significant difference between target genes and age groups suggests that the gene amplification rates were consistent across different age categories. This might imply that COVID-19 infection does not significantly vary by age in this patient cohort [18] , while other researchers used CT threshold to predict mortality and hospitalization of the patient [19]. Additionally, our study has shown no difference in relative CT values between the studied genders (males and females) for both genes, but the total positive detection rate of the E gene was higher than that of the RdRp gene. The lack of a significant difference suggests that the gene amplification rates for both E and RdRp genes are not influenced by gender in this patient population. Globally, gender differences in terms of infection rates, and outcomes are still controversial in terms of conclusive data and is an area requires intensive investigations to reveal the true factors affecting it [20]. On the other hand, as seen in the supplementary material, one noticeable point can be observed: the average CT value of positively detected samples of the E gene compared to the negative RdRp gene is nearly 35. This can be translated into different interpretations in terms of false positive or false negative results, or the E gene could be more sensitive in later stages of the infection when there is less viral genomic load to be detected. Therefore, with further studies and larger sample size, it could be concluded whether E gene is more sensitively detected than the RDP gene in later stages of the infection. However, this seems unfeasible due to the fact that the severity and hospitalization of the infection has faded globally. Additionally, the differences between the two genes could be due to a number of limitations in the testing process that include, but are not limited to, the primer and the probe, the viral load in the sample, the sample anatomical site, and time sampling[21].

The role of specific, sensitive detection methods for the surveillance of any outbreak cannot be stressed enough. However, the lack of sequence data remains one of the main issues in developing countries, especially in the Kurdistan region. Currently, there are no available reference COVID-19 genomes in the areas to compare the data with in order to track the origin of the outbreak, the polymorphism of the virus that affects Syria's infectivity and pathogenicity, and possibly the effectivity of the vaccine on it. Furthermore, the changes in the sequences could impact the detection through single gene expression RT-PCR process, which makes the gene detection rate lower and less accurate with time[22]. In general, COVID deaths are trending downward across the globe, but the issue of outbreak surveillance and its impact on social life remains an open case to be investigated and solved through cooperation[23]. Overall, the information provided suggests that, in this particular study, there were no significant gender-based or age-based differences in COVID-19 gene amplification rates. These findings can contribute to our understanding of how the virus affects different demographic groups. Consequently, proper prophylactic measures and clinical recommendations can be provided to the public accordingly to reduce surges in infection rates. Finally, the ability to diagnose the virus through other genes expression rates and their sensitivity compared to the genes used in this study is one of the limitations that could have provided the whole picture of the sensitivity and specificity of the detection rates.

CONCLUSION

In the COVID-19 outbreak, a story that is nearing its last chapter based on the WHO's decrease in the death toll across the globe, RT-PCR detection provided a rapid and sensitive method to follow the outbreak and prevent further infection through isolation and treatment procedures. The

studied genes in this project showed a similar pattern of expression in both males and females, but the RdRp gene had higher detection rates than the E gene in both males and females compared to the E gene tests. Thus, suggesting that RdRp gene could be more sensitive in later stages of the infection compared to the E gene.

Conflict of Interest

The authors declare they have no conflicting interests.

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