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Dynamics of ORF1ab and E Gene in Covid-19 positive patients from Travnik Region, Central Bosnia and Herzegovina

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1. Abstract

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is an essential diagnostic tool used to accurately identify and confirm SARS-CoV-2 infection in patients who are showing symptoms or are suspected of being infected. The WHO recommends a combination of viral-specific genes, including the Envelope (E), the RdRP/Helicase (Hel), the spike protein-encoding gene (S), and the ORF1ab gene, as molecular targets for detection. Among the possible PCR targets, the E gene of SARS-CoV 2 is considered to be the least specific and it shows significant sequence homology to other common coronaviruses.

The present study aimed at investigating the dynamics of ORF1ab and E gene from COVID–19 positive patients considering the Ct values of both genes.

The study included population of 130 patients who showed symptoms of COVID-19 between November 2021 and February 2022 in Central Bosnia and Herzegovina. Out of these patients, 86 tested positive for the virus. For molecular confirmation of SARS-CoV-2, the RT-qPCR protocol was performed. Average Ct values were automatically generated with values ≤Ct30 reported as positive.

Average Ct value for ORF1ab was 26.43 (S.D.± 3.37) and for E gene was 27.45 (S.D.±2.29). Study also revealed that the prevalence of COVID-19 was 54.5% in males and 46.5% in females, showing that males had an increase of 8% positive cases than females.

The Average Ct value for ORF1ab is lower than for E gene which is in correlation with recent studies. Studies suggested that ORF1ab exists in higher quantities than E, thus, as patients recover, the E-gene RNA is the first to become undetectable. The current findings, as far as infectivity is concerned, indicates that men are more vulnerable than women to COVID-19.

Keywords: Ct value; SARS-CoV-2; qRT-PCR; genes

2. Introduction

A novel Coronavirus, known as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), initially originated from Wuhan, Hubei province, PRC, emerged in late 2019, causing a pandemic Coronavirus disease-2019 (COVID-19) (Wang et al., 2020; Hu et al., 2021). World Health Organization (WHO) has declared COVID-19 a global public health emergency in period from February 28, 2020 to May 10, 2023. According to WHO's Coronavirus Dashboard, the cumulative COVID-19 cases worldwide approach 772 million with nearly 7 million deaths from the disease have reported. Bosnia and Herzegovina reported 403.266 confirmed positive cases with 16.362 fatal cases (https://covid19.who.int/).

SARS-CoV-2 is a positive strand RNA virus with genome size of 30,000 bases in length which shares 79% and 50% genome sequence with, SARS-CoV and MERS-CoV, respectively (Abdelrahman et al., 2020). Pathogenicity and transmissibility of this member of Betacoronavirus family is characterized by a unique combination of polybasic cleavage sites (Walls et al., 2020). The structural proteins of the virus: spike (S), envelope (E), membrane (M), and nucleocapsid (N) are responsible for a particular step in viral infectivity and transmission (Durmaz et al., 2020).

Quantitative reverse transcriptase-polymerase chain reaction (qRT PCR) on respiratory samples(nasopharyngeal and/or oropharyngeal swabs) is a gold standard for diagnosis of SARS-CoV-2 infection (Chu et al., 2020). This molecular diagnostic tool has high sensitivity, reproducibility and reduces risk of sample contamination allowing critical detection of infected individuals (La Marca et al., 2020). qRT-PCR is using different genes such as N gene, E gene, RdRp gene, S gene, HE gene, H gene and ORF group of genes as target sequences (Khailany et al., 2020; Corman et al., 2020). In order to avoid SARS-CoV-2 genetic drift or cross reaction with other betacoronaviruses it is recommendable to use two molecular target strategy (Tang et al., 2020).

Real-time PCR cycle threshold (Ct) values represent the number of amplification cycles required for the target gene to exceed a threshold level and are therefore inversely related to viral load. Ct values can provide an indirect method of quantifying the copy number of viral RNA in the sample (Rao et al., 2022); however, the use of Ct values as a proxy of viral load is influenced by the RT-PCR assay itself and sample matrix factors that can affect amplification efficiency (Bustin et al., 2005). Viral dynamics knowledge is important for designing COVID-19 treatment and control strategies of (Chen et al., 2020). Chen et al. found that that the viral load in oropharyngeal saliva samples was highest during the first week after the onset of symptoms, and then decreased over time. Viral dynamics studies of COVID-19 showed that Ct value of a target gene is inversely proportional to viral loads regardless of patient's conditions; however several technical problems need to be considered from sample collection to RT-PCR assay (Xu et al., 2020). According to Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 9), a sample with cycle threshold values >35 for both open reading frames 1ab (ORF1ab) and nucleocapsid (N) genes by two consecutive tests (with at least 24 hours in between) is the laboratory criterion for hospital discharge of the patient (Zhang 2022).

The present study aimed at investigating the dynamics of ORF1ab and E gene from COVID–19 positive patients considering the Ct values of both genes.

3. Materials and methods

3.1. Study design

The present study was carried out to characterize the dynamics of ORF1ab and E gene in COVID-19 positive patients at Medical Centar Travnik. The study included population of 130 patients who showed symptoms of COVID-19 between November 2021 and February 2022 in Central Bosnia and Herzegovina. Each patient had typical COVID-19 symptoms: cough, fever, muscle pain and anosmia. Out of these patients, 86 tested positive for the virus. Gender of the patients was also considered in this study. Any other parameters like source of infection, patient conditions, age, treatment regime and other comorbidities were not considered for this study. Specifically, RNA derived from the E and ORF1ab genes of SARS-CoV-2 was detected. Ct values based on amplification from detected RNA were used as indicators of the copy number of SARS-CoV-2 RNA in specimens. Average Ct values were automatically generated with values ≤Ct30 reported as positive. Additionally, general and clinical information of the patients were collected, including sex, age, and underlying medical comorbidities.

3.2. Sample colection, processing and RNA extraction

The authorities of reported health complexes collected nasopharyngeal and oropharyngeal swabs from the suspected individuals by following the WHO guidelines (https://covid19.who.int). Immediately after collection of samples, proper cold chain was preserved and sent to the PCR lab. In compliance with the manufactory instructions given in the kit, Viral RNA was extracted from the patient's derived specimens using a Bosphore Extract RNA Solution. 400 μL of Solution was pipetted into the tube that contains the dry swab. Samples were vortexed for 20 seconds in order to transfer the sample into the liquid. 25 μL of the sample was pipetted into a 1.5 mL DNAse RNAse free EP (Eppendorf) tube. It was added 1 μL of internal control directly into the sample, and samples were incubated in thermal block for 8 minutes on 95°C. The samples were added directly into the PCR reaction.

3.3. RT- qPCR analysis

A real-time RT-PCR method detected the presence of SARS-CoV-2 in the respiratory specimens. RNAs were analyzed for SARS-CoV-2 identification by RT-PCR (Agilent AriaMx) using the Bosphore Novel Coronavirus (2019-nCoV) Detection kit V2 (Anatolia geneworks). Thus kit employs multiplex PCR and internal control is incorporated into the system in order to control the isolation procedure, to check for possible PCR inhibition and application errors. The reaction is performed in one PCR tube with PCR Master Mix.As described in the product manual, technical methods and interpretations of the findings have been evaluated. PCR qualitative identification of the E gene (Cy5 channel) and ORF1ab region (FAM channel) of SARS-CoV-2 was identified. The human RNA gene (HEX channel) had been used as an internal control to regulate inhibition of PCR. Real-time RT-PCR was run on AriaMx system by Agilent. One positive control and one negative control were both

carried out as quality monitoring steps to verify the detecting process. Average Ct values were automatically generated with values ≤Ct30 reported as positive (Figure 1; Figure 2)

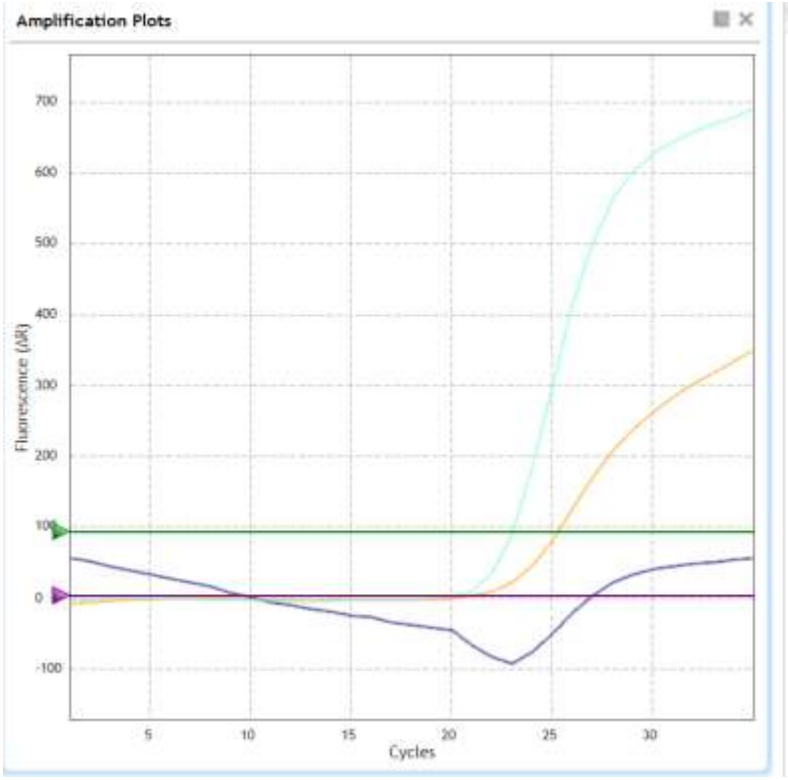

Figure 1.

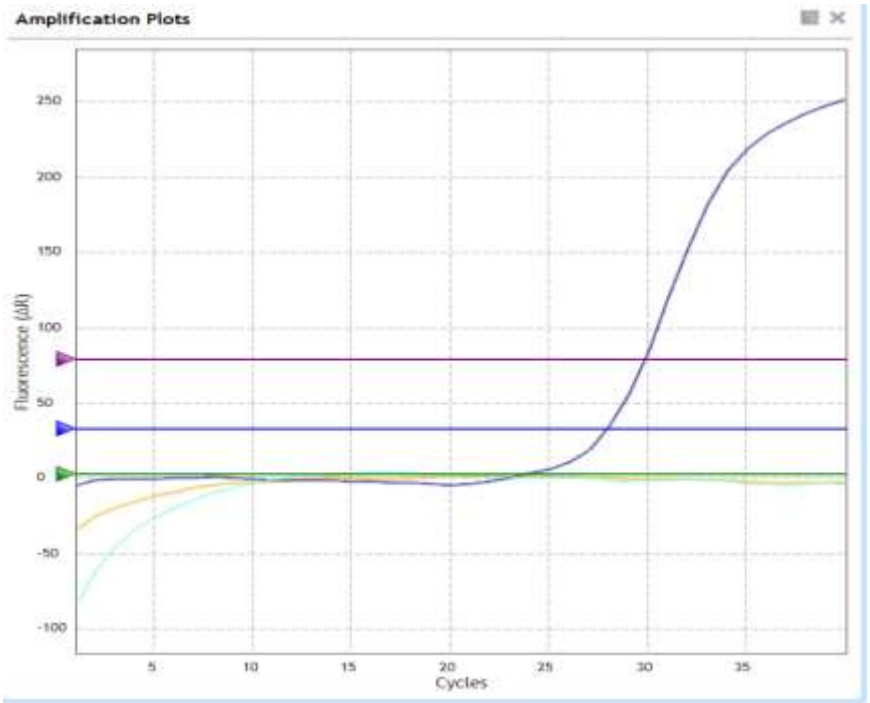

Figure 2.

3.4. Data analysis

By the end of the thermal protocol, the Real Time PCR insturment software automatically calculates the baseline codes and the thresholdes. All field derived data, including sex, age, and residence were recorded into a Microsoft Excel 2010 spreadsheet and sorted for a better presentation of outcomes. Clinical data were expressed as mean, median, mode, SD and variation. Descriptive statistics such as frequency and percentage was used.

4. Results and Discussion

In the current study, we investigated 130 patients with symptoms specific for SARS COV2 through RT-PCR in Medical Centar, Travnik. Our study has revealed that 86 patients (66,16%) tested positive for the virus. Among the 86 symptomatic adults who were SARS-CoV-2 RNA positive, the most commonly reported symptoms were headache (61,2%), cough (51.8%), muscle or body aches (50.6%), and fever (48.1%) (Figure 3.) As expected, loss of taste or smell, chills, fever, cough, aches, headache, fatigue and nasal congestion were associated with positive test for SARS-CoV-2 which is in corelation with other studies from this field (Wohl et al., 2021).

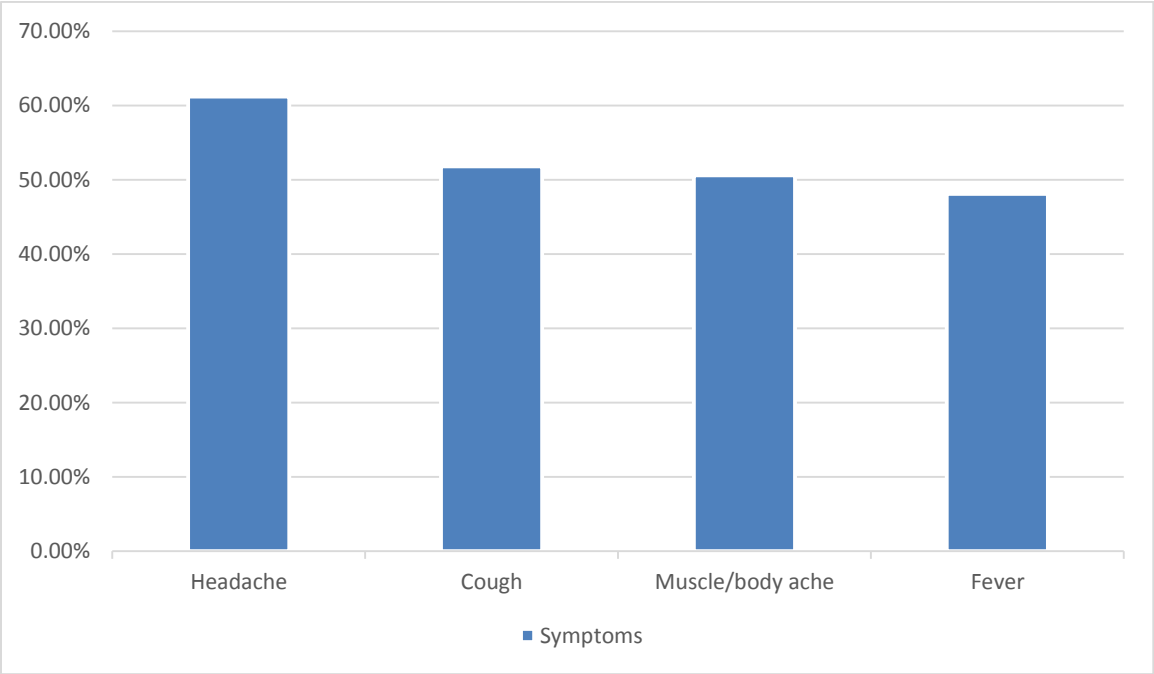

Figure 3.

Within the SARS-related viral genomes, there are three regions that exhibit conserved sequences. These regions include the RdRp gene (RNA-dependent RNA polymerase gene) located in the ORF1ab region, the E gene, and the N gene. Notably, both the RdRp and E genes demonstrate high analytical sensitivity for detection purposes. As a result, the COVID-19 testing assay is designed as a two-target system. The E gene serves to detect universal SARS coronaviruses, while a confirmatory testing approach utilizes a second set of primers (targeting either the ORF1ab gene or RdRp gene) to specifically detect SARS-CoV-2 (Corman et al., 2020). In our study average Ct value for ORF1ab was 26.43 (S.D.± 3.37) and for E gene was 27.45 (S.D.±2.29). Average Ct values were automatically generated with

values ≤Ct30 reported as positive. These results are in corelation with those from UC Davis Health where they had some results with, an ORF1ab positive, but E-gene negative and where result is still considered positive for SARS-CoV-2 RNA. It has been observed that ORF1ab positive/E-gene negative result combinations occur most frequently when patients are convalescing and present with very high cycle threshold (Ct) values suggestive of low viral loads. This phenomenon is due differing ratios of ORF1ab, and E genes produced during SARS-CoV-2 replication. Studies suggest ORF1ab and N sub-genomic material exist in higher quantities than E, thus, as patients recover, the E-gene RNA is the first to become undetectable (Tran et al., 2021).

The rRT-PCR Ct value estimates the SARS-CoV-2 RNA copy numbers, with lower cycle threshold values suggesting higher viral copy numbers and vice versa (Wang et al., 2020). A significant correlation between Ct value and successful isolation of SARS-CoV-2 virus in cell culture has been reported, where samples with Ct values of 13–17 led to positive virus culture whereas those with Ct values equivalent or above 34 lacked infectious viral particles (La Scola et al., 2020). It is crucial to acknowledge that direct comparisons of Ct values cannot be made across different laboratories, or even for the same patient in different PCR runs. This is because factors such as the quality and volume of nasal samples can vary significantly between two collection instances by the same person, leading to variations in Ct values. Additionally, differences in viral RNA extraction, RT-PCR methods, and kits sourced from various vendors can also impact the test results (Rabaan et al., 2021).

According to a study, the prevalence of COVID-19 was found to be 54.5% in males and 46.5% in females. This indicates that males had a higher percentage of positive cases by 8% compared to females. These findings align with the results of various studies conducted in Wuhan, Italy, Oman, USA, and Bangladesh, which have also shown that men are more susceptible to contracting COVID-19. (Pan et al.,2020; Khamis et al., 2020; Jin et al., 2020;Cummings et al., 2020; Ali et al., 2021). According to Zhu et al., (2020) men could be most likely at the onset of the outbreak to be exposed to the virus, for social or cultural reasons, because of the fact that women's reduction in susceptibility to viral infections may benefit from the conservation of X chromosomes and sex hormones that are of significant importance to immunity (Zhu et al., 2020). The current findings, as far as infectivity is concerned, indicates that men are more vulnerable than women to COVID-19. Additional findings indicate that men have a higher prevalence, including 56.3%, 58.1% and 67% respectively in New York and China (Yang et al., 2020; Zhu et al., 2020; Jehi et al., 2020). The differences in infection rates between males and females may be attributed to variations in physiological factors, including the expression of certain virus receptors, metabolic differences, and distinct behavioral patterns. Notably, studies conducted at Cleveland Clinics in Ohio and Florida have also found that males face a higher risk of testing positive for COVID-19 (Jehi et al., 2020). Although most studies have reported higher prevalence in males, some studies have also shown roughly similar sex distributions (Pan et al., 2020; Jin Zhang et al., 2020)

5. Conclusions

In conclusion, we identified several symptoms including new onset of loss of taste and/or smell, chills, and fever were associated with detection of SARS-CoV-2 virus. These findings can be used to inform strategies to identify those most likely to be infected with SARS-CoV-2 and allocate testing and direct preventative and therapeutic interventions.

If we talk about dynamics of ORF1ab and E gene the Average Ct value for ORF1ab is lower than for E gene which is in correlation with recent studies. Studies suggested that ORF1ab exists in higher quantities than E, thus, as patients recover, the E-gene RNA is the first to become undetectable. But it is important to add that correct interpretation of Ct value is influenced by several factors including pre-analytical conditions (timing and storage of the samples before analysis, specimen quality, type of sample) and analytical conditions (different targets, sample volumes varying according to the manufacturers). We conclude that SARS-CoV-2 Ct values of rRT-PCR alone does not have a role in aiding severity stratification among patients with COVID-19 since the viral dynamics and Ct value may vary due to the emerging variants that occur in different waves of the pandemic. The current findings, as far as infectivity is concerned, indicates that men are more vulnerable than women to COVID-19.

6. Acknowledgements

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7. Declaration of conflict of interest

There is no any conflict of interest, financial and personal relationships including employment, consultancies, stock ownership, paid expert testimony, patent applications/registrations, and grants or other funding. All authors have seen and approved the manuscript being submitted. The corresponding author is responsible for the submission of the manuscript, on behalf of all co-authors. All listed authors have contributed significantly, have read the manuscript and that research submitted is not under consideration elsewhere.

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10. Figures

Figure 1: Amplification plot with Ct value for COVI-19 positive patient

Figure 2: Amplification plot with Ct value for Covid-19 negative patient

Figure 3: Symptoms distribution for Covid-19 positive patients