SUMMARY

Purpose: To describe clinical characteristics of patients positive simultaneously for RdRp, N and E genes of SARS-CoV-2 and to analyse dynamics of symptoms and Ct values in the interval from day 0 to day 10 after symptoms onset.

Materials and methods: Retrospective analysis of data obtained on the day of specimen collection from 1044 individuals was carried out. Detection of RdRp, E and N genes of SARS-CoV-2 was conducted by RT-PCR. Only patients positive for all three genes were included.

Results: Out of all patients, 47.8% were female, and 52.2% were male with a median age of 60 years, and the elder (>50 years) population constituted 71.5%. The most common comorbidities were cardiovascular disease (44.5%), diabetes mellitus (11.0%), and chronic pulmonary disease (8.6%). The prevalent symptoms at the time of specimen collection were fever (74.7%), cough (67.1%), and dyspnea (39.6%). Fever and cough were leading symptoms in the time interval between zero and the tenth day since the onset of COVID-19. A linear trend for dry cough and dyspnea, with a daily increase of 2.2% and 2.3%, respectively, was observed. Dry cough was the primary discriminator in distinguishing mild and moderate infection. Gradual increase of Ct values of all genes was observed from day 0 to day 10, and day 3 was essential for separating two time intervals.

Conclusions: Flu-like symptoms were leading over the study period. Male sex, older age, cardiovascular disease, diabetes mellitus, and chronic pulmonary disease were risk factors for infection. Cough, age over 65 years and male sex in the group 33-63 years were predictors of more severe disease.

Keywords: COVID-19, symptoms, dynamics, RT-PCR, Ct values,

INTRODUCTION

SARS-CoV-2 infection can manifest in various clinical forms, ranging from asymptomatic or mild infection with nonspecific symptoms to severe acute respiratory distress syndrome [1]. The most common symptoms reported since the onset of the pandemic include fever, fatigue, and dry cough. The less common symptoms are headache, nausea or vomiting, diarrhea, loss of smell or taste, myalgia, arthralgia, sore throat, rhinorrhea, and chest tightness [2]. As the clinical characteristics of COVID-19 infection are similar to those of other respiratory diseases, they need to be studied in more detail, especially about the onset of symptoms [3]. Proper recognition of specific COVID-19 symptoms would direct the clinician to request an early SARS-CoV-2 RT-PCR test, thus enabling timely initiation of treatment and limiting the spread of the virus.

Currently, real-time polymerase chain reaction (RT-PCR) is the primary method for detecting SARS-CoV-2. The cycle threshold (Ct) values of essential viral genes are the principal criterion in interpreting the result as positive or negative. Measurement of viral load by Ct values was used in numerous studies to monitor clinical progression and outcome of COVID-19 infection, but most of them have focused on hospitalised patients [4], and only a limited number have revealed viral kinetics in patients with symptoms of mild to moderate infection [5]. Further research is required to find relationships between clinical symptoms dynamics and variations in viral load during early COVID-19 infection.

This retrospective study aimed to describe clinical characteristics of 1044 patients who were positive simultaneously for three essential SARS-CoV-2 genes and to analyse dynamics of various symptoms and changes in Ct values in the interval from day zero to the tenth day after the first onset of symptoms suggestive of COVID-19 infection.
MATERIALS AND METHODS

Data collection and definitions

In this descriptive study, we retrospectively analysed data obtained on the day of specimen collection from 1044 patients from the Central-North region of Bulgaria, who tested positive for SARS-CoV-2 between May 4, 2020, and November 30, 2020. Our criteria to enroll a patient in this study group were: the presence of COVID-19 symptoms and positive result for RdRp, N and E genes upon first RT-PCR testing. All data were collected during sampling in the emergency office following the SARS-CoV-2 guidelines of the National Center for Infectious and Parasitic Diseases containing information about patients No. demographic characteristics, clinical symptoms, comorbidities, epidemiological data, the onset of the first symptoms, and sampling date. Based on the symptoms, patients were classified into two groups of clinical severity. The first group, “mild infection,” included cases with “flu-like” symptoms and other symptoms associated with COVID-19, without pneumonia and dyspnea. The second group, “moderate infection”, consisted of patients with clinical signs of pneumonia (fever, cough, dyspnea) with or without chest CT or x-ray findings, according to the WHO interim guidance [6].

Ethics statement

The study was approved by the Ethics Committee at the Medical University of Pleven (protocol number 673) according to the Declaration of Helsinki.

Nasopharyngeal samples

Both nasal and oropharyngeal swab specimens were obtained from all patients by qualified medical staff. Then the swabs were placed and maintained in a viral-transport medium and kept at +4ºC to avoid contamination and virus degradation.

Isolation of viral RNA

Viral RNA was obtained by manual (NucleoSpin Dx Virus, 740895.50, Macherey-Nagel, Germany) or automated isolation (MagCore viral nucleic acid extraction kit, MVN400-03, RBC Bioscience, Taiwan) according to the manufacturers’ instructions. For automated extraction MagCorePlus II Automated Nucleic Acid Extractor (RBC Bioscience, Taiwan) was used. Upon isolation, viral RNA was used for RT-PCR and then stored at -30ºC for further analysis.

RT-PCR analysis

Detection of RdRp, N and E genes was carried out with GeneFinder COVID-19 PlusRealAmp kit (IFMR-45, OSANG Healthcare Co, Korea) according to the manufacturers’ instructions. Detection of SAR-CoV-2 genes was run and analysed using CFX96 Real-time PCR (1854095-IVD, Bio-Rad). The amplification curves and Ct values for each gene were analysed within 45 cycles. The quality of RNA was confirmed by amplification of internal control.

Statistical analysis

We analysed the studied variables using descriptive statistics: means, medians, standard deviation and inter-quartile range. Categorical variables were given as whole numbers and percentages. The potential associations between categorical variables have been evaluated through cross-tab generation, Chi-square test and Fisher exact test. All p-values were reported as two-sided with a significance level of 0.05. Statistical tests were performed in SPSS version 26.0 (IBM, NY, USA) and SigmaPlot 11 program (Systat Software Inc, USA). Variation analysis was used to study the dynamics of symptoms: one-way analysis of variance (ANOVA) and non-parametric Kruskal–Wallis (one-way ANOVA on ranks) were selected according to Levene’s test. Dunn’s post-hoc test was performed for multiple comparisons between the percentage of manifested symptoms on different days after the onset of infection. A decision tree model for distinguishing mild and moderate infection was built with CHAID (Chi-Square Automatic Interaction Detector) algorithm available in IBM-SPSS23. CHAID chooses the independent (predictor) variable with the most robust interaction with the dependent variable at each step.

RESULTS

Of the total population positive for the RdRp, N, and E genes, 481 (46.1%) were with symptoms of mild upper respiratory infection, and 563 (53.9%) had moderate infection with clinical signs of pneumonia (fever, cough, dyspnea) on the day of sample collection (table 1). The median age of all patients was 60 (range: 48-72 years), and the elder (>50 years) population constituted 71.5%. In the group with a mild infection, the median age was 57 (range: 45-70 years)(p<0.0001), and in the moderate infection group, it was significantly higher, 64 (range: 53-74 years) (p<0.0001).

Table 1. Demographics and basic characteristics of patients who were positive for RdRp, N and E genes of SARS-CoV-2.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients (n=1044)</th>
<th>Patients with mild disease (n=481)</th>
<th>Patients with moderate disease (n=563)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>60(48-72)</td>
<td>57(45-70)</td>
<td>64(53-74)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0-18</td>
<td>20(1.9%)</td>
<td>14(2.9%)</td>
<td>6(1.1%)</td>
<td></td>
</tr>
<tr>
<td>19-49</td>
<td>277(26.5%)</td>
<td>162(33.7%)</td>
<td>115(20.4%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>50-64</td>
<td>346(33.1%)</td>
<td>167(34.7%)</td>
<td>179(31.8%)</td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>401(38.4%)</td>
<td>138(28.7%)</td>
<td>263(46.7%)</td>
<td></td>
</tr>
</tbody>
</table>
Of the 1044 patients, 545 (52.2%) were male, and 499 (47.8%) were female. Males dominated (58.6%) in the moderate infection group, whereas females were prevalent (55.3%) in the mild infection group (p<0.0001). In this cohort, little more than half of the patients (53.0%) had at least one comorbidity. The most common underlying comorbidity was cardiovascular disease (44.5%), diabetes mellitus (11.0%), and chronic pulmonary disease (8.6%). The proportion of these three underlying conditions (53.1%, 13.1%, 11.2%, respectively) was significantly higher in the group of patients with moderate infection vs. patients with mild infection (34.5%, 8.5%, 5.6%) (p<0.0001, p=0.018, p<0.001). Less common symptoms were loss of taste/smell (8.5%), fatigue (7.0%), muscle pain (4.7%), headache (4.1%) and sore throat (2.7%). In contrast, these symptoms were more commonly reported in patients with mild infection (p<0.0001).

Figure 1 presents the dynamics of symptoms of COVID-19 infection in the interval from the onset of disease to the day of sample collection. Between zero and the tenth day since the onset of COVID-19, fever and cough were leading symptoms. The proportion of patients with temperature varied between 61% (day 1) and 85% (day 10), but there was no statistically significant trend. Dry cough was reported from 54.0% of patients on day 1, 52.0% on day 2, and 84.0% on day 10. There was a linear trend (p=0.011, R squared=0.53), and a daily increase was 2.2%. Dyspnea on day one and day ten was observed in 20% and 65.6% of patients, respectively (p=0.016, R squared=0.49), and the daily increase was 2.3%.
Fig. 1. Dynamics of COVID-19 symptoms in the time interval from the onset of disease to the day of sample collection.

(A) fever, (B) dry cough, (C) dyspnea, (D) loss of taste/smell, (E) fatigue, (F) muscle pain, (G) headache, (H) sore throat, (I) nausea and vomiting, (J) diarrhea. A statistically significant trend in dynamics of fever was not observed in the whole time interval, but cough and dyspnea showed a linear trend with a daily increase of 2.2% and 2.3%, respectively. Due to the small number of patients that reported symptoms from (D) to (J) on individual days of the time interval, statistical analysis was not relevant.
The main symptoms of COVID-19 in patients during the early (0-3) and late (4-10) days of sample collection are shown in figure 2. There was no statistically significant difference in the proportion of patients with fever between the early and late sample collection groups (p>0.05). Concerning dyspnea and cough, a significantly higher proportion of positive patients was observed in the late sample collection group compared to the early group (cough 78% vs. 59%, p=0.004; dyspnea 53% vs. 28%, p=0.024).

Fig. 2. Common symptoms of COVID-19 that were reported by patients during the early (0-3) and late (4-10) days of sample collection.

The difference in the proportion of patients with fever in the early and late days of sample collection was statistically non-significant. The proportion of patients with cough and dyspnea was significantly higher in the late sample collection group compared to the early group (p=0.004 and p=0.024, respectively).

The less common symptoms of COVID-19 infection during the two time intervals are presented in table 2.

Differences in the proportion of patients with loss of taste/smell, fatigue, muscle pain, headache, sore throat, nausea and vomiting were not significant during the early and late days of sample collection. Diarrhea was an exception, and it was observed only during the late days of sample collection.

Table 2. The Proportion of less common symptoms of COVID-19 during the early (0-3) and late (4-10) days of sample collection.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Day 0-3</th>
<th>Day 4-10</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of taste/smell</td>
<td>9.3±0.3</td>
<td>6.8±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Fatigue</td>
<td>7.5±0.07</td>
<td>6.4±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Muscle pain</td>
<td>5.4±0.05</td>
<td>4.2±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Headache</td>
<td>4.7±0.09</td>
<td>3.6±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Sore throat</td>
<td>3.1±0.03</td>
<td>2±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Nausea and vomiting</td>
<td>1.3±0.01</td>
<td>1.1±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0±0</td>
<td>1.4±0.02</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

We developed a decision tree model by CHAID algorithm to find essential interactions between the symptoms in our study group (figure 3). The primary discriminator in distinguishing mild and moderate infection is dry cough (p<0.0001). The cough increases the patient’s risk of developing a moderate infection by 18% (RR=1.18). Then, in this branch of the tree, age becomes a critical discriminator: 1.) Patients aged ≤ 33 years have a 48% lower risk of developing moderate infection (RR=0.52); 2.) Patients aged >33 years and those aged ≤ 63 years have 7% lower risk of developing moderate infection (RR=0.93); 3.) Patients aged > 63 years have 16% higher risk of developing moderate infection (RR=1.16). An additional discriminator for the age group 33-63 is sex: the risk of developing moderate infection increases by 16% in males (RR=1.16).

The second main branch of the tree includes patients without cough. Lack of cough decreases the chance of a patient falling into the moderate infection group by 36% (RR=0.64). The next discriminator level is loss of taste and smell. These patients have additionally decreased risk of developing a moderate infection by 65% (RR=0.35).
Fig. 3. Decision tree model for distinguishing mild and moderate infection.

It was developed using CHAID (Chi-Square Automatic Interaction Detector) algorithm available in IBM-SPSS23 to find essential interactions between the symptoms in our study group. Cough, age over 65 years and male sex in the age group 33-63 years were risk factors for developing a moderate infection. Lack of cough and loss of taste/smell decreased the risk of developing moderate COVID-19 infection.

Correlation between the expression of essential SARS-CoV-2 genes represented by their Ct values and the days after onset of the first COVID-19 symptoms is displayed in figure 4. Our RT-PCR analysis revealed a trend connecting the decreasing gene expression to the increasing time interval after the onset of the first symptoms of COVID-19 infection (figure 4a). Despite the weaker expression of the RdRp gene, there was no significant difference between the initial Ct values of each of the three investigated genes (RdRp, N, and E gene). The data showed a gradual increase of the Ct value of each gene from day 0 to day 10. The values of RdRp, N, and E genes on day 0 were 26.68±1.71; 24.91±2.45 and 23.98±2.72, respectively, whereas on day 10, these values increased to 31.14±1.55; 29.36±2.11, and 28.59±2.44, respectively. According to the mean Ct values of all three genes, we were able to separate the results from day 0 to day 10 in two time intervals, namely initial (day 0-3) and subsequent (day 4–10) after the onset of symptoms (figure 4b).

Our data demonstrated no significant change in Ct values within the two marked time intervals except the comparison between day 0 (25.19±2.04) and day 3 (27.61±3.10). We also found that Ct values in the initial time interval were significantly different from the subsequent one. Ct values on day 0 were significantly lower compared to days 3-10. Similarly, Ct values on day 1 (25.51±3.25) and day 2 (26.21±2.84) were lower than days 4–10. Surprisingly, Ct values on day 3 (27.61±3.10) were significantly different only to days 8 (29.35±1.92), 9 (29.40±2.83), and 10 (29.70±1.84).
Fig. 4. Correlation between SARS-CoV-2 gene expression in the time interval from day 0 to 10th day after the first onset of COVID-19 symptoms.

(A) Gradual increase of the Ct RT-PCR values during the whole time interval. (B) A significant difference between SARS-CoV-2 gene expression on days 0 to 10 with higher viral load in the initial (day 0-3) than in the subsequent (day 4-10) time interval. Data represent mean Ct values ± SD, n ≥ 50; * p<0.05.

Despite the timely implementation of PCR testing of all individuals with flu-like symptoms in Bulgaria, most patients of our cohort sought medical attention 2-6 days after the onset of the first symptoms. In the meta-analysis by Li et al. of 212 studies from 11 countries/regions involving 281461 patients, the meantime from the onset of symptoms to hospitalisation was 5.48 days, with significant differences between regions which may be related to their healthcare resources [8].

The clinical presentation of patients with mild and moderate infection in our study involves mainly fever (74.7%), dry cough (67.1%), and dyspnea (39.6%). The proportion of other symptoms associated with COVID-19, such as loss of taste/smell, fatigue, sore throat, nausea and vomiting, and diarrhea, is between 8.5% and less than 1%. Our results were consistent with data from other studies showing that fever, cough, and dyspnea are the most prevalent symptoms [7]. Meta-analysis performed by Cao et al., including 31 articles and 46959 patients, showed that fever (87.3%), cough (58.1%), dyspnea (38.3%), muscle soreness or fatigue (35.5%), and chest distress (31.2%) were the most common clinical manifestations [2]. However, our results were different from those reported by other investigators. Lechien et al. found headache (70.3%), loss of smell (70.2%), and nasal obstruction (67.8%) as the prevalent symptoms in 1420 patients from 18 European hospitals with mild-to-moderate infection [9]. Myalgia (54.6% %), headache (51.2%), and non-productive cough (49.3%) were the most common presenting symptoms at disease onset in 1564 individuals positive for SARS-CoV-3 in Iceland [10]. The reasons for those discrepancies are probably multifactorial. We assume that the appearance and spread of numerous variants of SARS-CoV-2 were responsible for reported differences in the clinical presentation of COVID-19.

In this study, the proportion of patients with loss of smell or taste was 8.5%, however, it was significantly higher among those with mild disease (14.0%) than in individuals with moderate disease (3.9%). Olfactory and gustatory dysfunctions in patients with COVID-19 were published worldwide, but the data reported were heterogeneous [11]. Olfactory and gustatory dysfunction was documented in 47 (10.8%) of 435 patients with confirmed COVID-19 infection [12]. Lechien et al. found a higher proportion of olfactory (73.7%) and gustatory (46.8%) dysfunction among 2579 patients with various clinical presentations of COVID-19 infection [13]. Both dysfunctions were more common in individuals with mild infection than in those with moderate and severe infection, and they were more prevalent in females than in males. Von Bartheld et al. analysed data of 104 studies, including 38198 patients, and found almost equal rates of olfactory and taste dysfunction rates (43.0% and 44.6%, respectively), and chemosensory dysfunction showed significant differences between Caucasian (54.82%) and Asian (17.72%) ethnic populations [14]. They suggested that the geographic and ethnic population differences likely resulted from mutations of SARS-CoV-2 and ethnicity-specific host factors.

DISCUSSION

Our results showed that older age and comorbidities, especially cardiovascular diseases, diabetes mellitus, and chronic pulmonary diseases, were risk factors for COVID-19 infection. These factors and male sex were associated with a more severe course of infection with the development of pneumonia and dyspnea. Overall, these findings are in concordance with other published studies on comorbidities in patients with COVID-19 [7]. Although there were some variations in the proportion of circulatory and cardiovascular diseases in different studies, they remained the main comorbidities.

As expected, we observed a longer median time since symptom onset to PCR testing in patients with pneumonia compared to those with mild infection. Obviously,
In our study, fever and cough were the most common symptoms from disease onset to the day of sampling (0-10 days). We did not find a statistically significant trend in the proportion of patients with fever in this time interval, but those with cough and dyspnea showed a daily increase of 2.2% and 2.3%, respectively. This increase can be explained by the infection progression in the lower respiratory tract. Cough (78.2%) and fever (62.1%) were also reported as the initial presenting symptoms in 821 outpatients in Michigan [7]. Fever, then cough, nausea/vomiting, and diarrhea were identified as the most likely order of COVID-19 symptoms [3].

Based on the distribution of differences in initial symptoms between patients with mild and moderate infection, we elaborated the Decision Tree Model. According to this model, cough is a major discriminatory factor between mild and moderate infection. The presence of cough, age over 65 years, and male sex in the age group 33-63 years increases the risk of developing a moderate infection. Lack of cough and loss of taste and smell significantly reduce this risk. Loss of smell was discussed by other investigators as a clinical marker for the severity of COVID-19 infection and as a predictive factor for a milder course of infection and better prognosis [15].

Our RT-PCR results showed a continuous reduction of the viral load in nasopharyngeal samples over the tested period, measured by its inverse correlation with the Ct values. This finding could be explained by the spread of the virus from the upper to the lower respiratory tract and the limitation of sample collection for SARS-CoV-2 only to the upper parts of the respiratory system. This is consistent with other studies that also demonstrated a continuous decrease of the viral load after the onset of symptoms [16] and that active replication of SARS-CoV-2 in the throat occurs during the first five days after the onset of symptoms [17].

Our study revealed a common trend for RdRp-gene, indicating its lower expression compared to the other genes that encode viral structural proteins. This is not consistent with the findings of Sangineto et al. [18], who found a constant decrease of the viral load over time with a more pronounced trend for N gene profile than for RdRp and E genes. Based on the common knowledge for SARS-CoV-2 [19], we speculate that our result concerning the low level of the enzyme RNA-dependent RNA polymerase is due to the function and the stability of the enzyme, which is essential for the assembly of the replicase-transcriptase complex, pivotal for the replication of the viral RNA.

Analysis of the RT-PCR data for all three essential genes allowed us to separate two-time intervals, namely initial (day 0-3) and subsequent (day 4–10) after the onset of the first symptoms. This differentiation goes in line with the common knowledge for SAR-CoV-2 progression, defining the disease as mild with symptoms like fever, cough, sore throat, loss of smell, headache, and body aches occurring in the first days upon infection, and severe, which involves the lower respiratory tract resulting in bronchitis, bronchiolitis, and pneumonia [20]. We found that day three was separating the two time intervals since it differed significantly from day 0 and days 8-10, and thus, it most likely marks the time of virus relocation to the lower respiratory tract. This result is in accordance with our findings on the dynamics of cough and dyspnea, showing an increase after the fourth day of the onset of the first symptoms. However, due to the limitation of the nasopharyngeal sampling, this suggestion needs to be further confirmed.

Altogether, based on our RT-PCR data, we can conclude that a higher viral load can be detected in the first four days (from day 0-3) after the onset of symptoms. The lower viral load during days 4-10, on the one hand, might reflect the migration of the virus towards the lower respiratory tract, resulting in an escalation of the infection from mild to moderate or severe, or, on the other hand, the drop in viral load might be due to the successful patient recovery.

CONCLUSIONS
Flu-like symptoms, like fever and cough, were leading in the majority of individuals positive for SARS-CoV-2 in the interval from day 0 to day 10 after the onset of symptoms. The data on the dynamics of common COVID-19 symptoms and RT-PCR data allowed us to separate two time intervals, initial (day 0-3) and subsequent (day 4-10). We found a continuous reduction of the viral load during the interval from day 0 to day 10 after the onset of symptoms, and we determined that day 3 was essential for dividing the two time intervals. Cough, age over 65 years and male sex in the age group 33-63 years were predictors of a more severe course of infection. Lack of cough and loss of taste/smell were predictive factors for milder progression of COVID-19 infection.

REFERENCES:
5. Ra SH, Lim JS, Kim GU, Kim MJ, Jung J, Kim SH. Upper respiratory viral load in asymptomatic individuals and mildly symptomatic patients with...


DOI: https://doi.org/10.5272/jimab.2022282.4409

Received: 12/01/2022; Published online: 15/06/2022

Address for correspondence:
Mariya Petrova Sredkova,
Virology Laboratory, Institute of Science and Research, Medical University - Pleven,
1, St. Kliment Ohridski Str., Pleven, Bulgaria.
E-mail: microvir@abv.bg

J of IMAB. 2022 Apr-Jun;28(2)