Seasonal Respiratory Virus Circulation in a Tertiary Care Hospital in Greece

Antonia Mourtzikou, Marilena Stamouli, Elpida Toka, Panagiotis Koumpounos, Georgia Kalliara, Christina Seitopoulou, and Maria Kimouli

ABSTRACT

Background: The COVID-19 pandemic caused by the novel SARS-CoV-2 virus affected health care systems and public health worldwide dramatically. Several measures were applied in order to prevent or stop the rapid transmission of the virus and the subsequent disease, such as lockdowns, physical distancing, strictly hygiene, along with travel restrictions. Global population after vaccination programs against COVID-19 were carried out, is facing a “tripledemic” situation threat, with the co-existence of SARS-CoV-2, influenza and RSV. The aim of the present study was to evaluate the co-existence of SARS-CoV-2, influenza and RSV, as well as the correlation with gender, age, Cts and vaccination doses.

Methods: A total of 302 patients were included in the study. All patients were admitted to the emergency department of General Hospital Nikiao, Piraeus with common upper respiratory tract symptoms and were suspected for COVID-19 disease, between March to July 2022. Patients’ age, gender, vaccination doses, and results from RT-PCR detection for SARS-CoV-2, RSV and Influenza viruses were recorded.

Results: 139 were male and 163 female, aged between 18-94 years. Out of the patients included in the study, 206 were vaccinated and 96 were not vaccinated. Among vaccinated patients 97 were male and 109 were female. A percentage of 3.3% had received one vaccination dose, 16.9% two and 47.7% three. Moreover, 88 patients presented infection symptoms; 81 patients had a positive rapid test result. We detected 15 cases of co-infection of SaRS-CoV-2 and RSV and only one case, with co-infection of SaRS-CoV-2 with influenza virus.

Conclusions: The majority of patients admitted to the emergency department of GHNP with common upper respiratory tract clinical manifestations were female. A significant lower rate on co-infection with SARS-CoV-2 and RSV was detected in patients having received 2 vaccination doses, compared to patients having received 3 out of 3 vaccination doses or up to 1 vaccination dose. Ct values for SARS-CoV-2 and RSV pathogens were between 10-17. Co-infection with SARS-CoV-2 and Influenza was detected in only 1 patient.

Keywords: COVID-19, co-infections, Ct, Influenza viruses, RSV, SARS-CoV-2, vaccines.

I. INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in December 2019 in Wuhan, China and rapidly spread worldwide leading to a pandemic level in early March 2020 [1], [2]. Therefore, there was a high need to diagnose suspected cases rapidly and accurately, in order to prevent or control the spread of SARS-CoV-2 infection. The major technique to achieve that purpose was using real-time reverse transcription polymerase chain reaction (rRT-PCR) [3], [4]. Moreover, wearing masks in all daily human activities, handwashing, physical distancing, travel restrictions and several lockdowns were applied, to stop or partially block the highly infectious virus transmission among the population. Those measures critically affected the human-virus relations, thus preventing the occurrence of other types of respiratory diseases from circulating among worldwide population [5]–[9].

There are several respiratory diseases, caused mainly by influenza A and B viruses (IAV and IBV) and respiratory syncytial virus (RSV). These viral infections cause and share mild to severe symptoms such as cough, fever, headache, muscle ache and pneumonia, similar to COVID-19 disease [10], [11]. During December 2019 until mid-2022, the highly increased existence of COVID-19 disease and the measures taken to prevent spreading among human population, led to a parallel dramatic decrease of IAV, IBV and RSV presence.
As vaccination against COVID-19 increased over the last 2 years of pandemic status, those measures were partially lifted off or stopped, resulting the increased presence of those respiratory viruses in addition to SARS-CoV-2 virus. The simultaneously presence of SARS-CoV-2, influenza and RSV viruses described as a “tripledemic” situation [15, 16]. This surge of “tripledemic” situation have risen the need of rapidly and accurately diagnose the respiratory virus infection and treat disease appropriately. The objective of the study was to examine the presence of influenza, RSV and SARS-CoV-2 viruses on mid-March 2022- mid-July 2022 (prior winter surge of “tripledemic”) on 302 patients that were admitted to Nikaia General Hospital “Agios Panteleimon”, Piraeus, Greece, a tertiary care hospital and possibly predict the winter burst of hospitalized patients.

II. MATERIALS AND METHODS

Our study was performed following the guidelines of the Helsinki Declaration of ethical principles for medical research that include human subjects. The study included 302 symptomatic patients admitted to tertiary General Hospital of Nikaia “Agios Panteleimon”, between March and July 2022. Clinical specimens were collected by hospital professional medical staff. Nasopharyngeal sampling method and storage was in accordance with the guidelines of the Disposable Virus Sampling Tube Kit. Collected specimens were randomly numbered by the Director of the Emergency Department and then stored at 4°C, for no more than 48 hours, before they were tested. All samples were tested with Boshphore SARS-CoV-2 Respiratory Pathogens Panel kit v1. using Boshphore SARS-CoV-2/ Respiratory Pathogens Panel kit v1 and Unio Viral DNA-RNA Extraction Kit 600, manufactured by Anatolia Geneworks, as presented in Table 1. The PCR testing of the specimens took place at the Molecular Department of General Hospital of Nikaia “Agios Panteleimon”. Statistical analysis was performed with MINITAB 17 statistical package.

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Boshphore SARS-CoV-2/ Respiratory Pathogens Panel kit v1</th>
<th>Unio Viral DNA-RNA Extraction Kit 600</th>
<th>Disposable Virus Sampling Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>REF No Storage conditions</td>
<td>ABSCR3</td>
<td>UVD600</td>
<td>30 TUBE/KIT</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>+25 °C</td>
<td>2°C ~35°C</td>
</tr>
<tr>
<td>Company</td>
<td>Anatolia Geneworks</td>
<td>Anatolia Geneworks</td>
<td>BioBaseBiodiary (Shandong) Co., Ltd</td>
</tr>
</tbody>
</table>

*After resuspension PK and Carrier RNA were stored at +4°C and -20°C respectively.

A. Testing Procedure

After specimen collection, the RNA extraction from the samples was performed. For the RNA extraction and purification, we used Unio B2448 Extraction System and Unio Viral DNA-RNA Extraction Kit 600, both manufactured by Anatolia Geneworks, which is based on magnetic bead method. The kit consists of cartridges prefilled with lysis and wash buffers, as well as the magnetic beads. Disposable tips and rods are included in the cartridge. According to the manual of the kit, Proteinase K (PK), Carrier RNA and sample need to be inserted into the lysis well of each cartridge as presented in Table II.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase K</td>
<td>20 μL</td>
</tr>
<tr>
<td>Carrier RNA</td>
<td>10 μL</td>
</tr>
<tr>
<td>Sample</td>
<td>600 μL</td>
</tr>
</tbody>
</table>

All samples were handled and tested separately (no batch testing was performed) and the preparation of the cartridge was completed in the laminar of our lab. For more accurate results, all samples were vortexed for 30 seconds, before their addition into the lysis well of the cartridge. After all cartridges were placed into Unio B24 Extraction System, the following settings were selected according to manufacturer’s guidelines (Table III):

<table>
<thead>
<tr>
<th>Kit Selection</th>
<th>VDR600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit Control and Sample Number</td>
<td>1-24 Samples</td>
</tr>
<tr>
<td>Sample Volume and Position</td>
<td>600 μL - Directly in well</td>
</tr>
<tr>
<td>Elution Volume and Position</td>
<td>60 μL - Directly in well</td>
</tr>
</tbody>
</table>

Until the extraction and purification of the sample was completed, PCR Master Mix preparation was performed. For the SARS-CoV-2 detection we used Boshphore SARS-CoV-2/ Respiratory Pathogens Panel kit v1. The PCR Master Mix was prepared according to the manufacturer’s instructions, by mixing PCR Master Mix 1 and RT Mix. The final volume of the PCR Master Mix was calculated in accordance with the number of samples tested in each run+ 10% (Table IV).

| PCR Master Mix 1 | 25.6 μl |
| RT Mix | 0.4 μl |
| Sample | 14 μl |
| Test Master Mix volume | 26 μl |

After mixing and smoothly shaking the PCR Master Mix we divided into the 0.2 mL PCR 8strips by inserting 26 μl of master mix into each strip. As soon as the extraction step was completed, 14μl of the purified sample RNA was added into each strip well containing the PCR Master Mix. Finally, the PCR 8 strips were sealed and placed into Montania 4896 thermocycler manufactured by Anatolia Geneworks. For each PCR run, a positive and negative control sample were tested. Positive control tube contains synthetic DNA for SARS-CoV-2, Influenza A/B, RSV A/B and Human RNase P gene region.

Boshphore SARS-CoV-2/ Respiratory Pathogens Panel kit v1 detect target genes ORF1ab and N for SARS-CoV-2 at FAM channel, mp2 gene for FluA and NS1 gene for FluB at Cy5 channel and Np gene for RSV A&B at HEX channel. Human endogenous nucleic acid sequence RNase P is used as an endogenous internal control (IC). The analytical sensitivity of the kit for nasopharyngeal swab samples is as 100 copies/ml for SARS-CoV-2, 504 copies/ml for Influenza A, 672 copies/ml for Influenza B, 504 copies/ml for the RSV A, 1176 copies/ml for RSV B. The applied thermal protocol is presented in Table V.

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B. Result Interpretation

As the thermal protocol is completed, SLAN 8.3.2 software automatically calculates the baseline cycles and the threshold to export the interpretation of the results. The Threshold Value Ct for the positive control is determined ≤30 and ≤32 for the internal control (IC) (Table VI).

III. RESULTS

The study included 302 patients, 139 (46.0%) were male and 163 (54.0%) were female. Patient ages ranged from 14 to 99 years. Age of male patients ranged from 18 to 94 years (mean value 51.35 years). Age of female patients ranged from 14 to 99 years (mean value 50.45 years). The median value was equal to 49.00 years in both gender groups. There is not a statistically significant difference of the mean age value between the two groups (p-value = 0.707; 95% CI for difference: (-5.60; 3.80). Gender distribution is presented in Fig. 1.

Out of the patients included in the study 206 (68.2%) were vaccinated against COVID-19 and 96 (31.8%) were not vaccinated. Among vaccinated patients 97 were male and 109 were female. A percentage of 3.3% (5 female and 5 male) of the patients had received one vaccination dose, 16.9% (33 female and 18 male) had received two vaccination doses, 47.7% (70 female and 74 male) had received three vaccination doses and 0.3% (1 female patient) had received four vaccination doses. Vaccination rates and vaccination doses are presented in Fig. 2 and 3.

Moreover, 88 patients (36 male and 52 female) presented infection symptoms, while 214 patients (103 male and 111 female) did not present any infection symptoms. Infection rates in the patient group is presented in Fig. 4.

We performed a rapid test for Sars-CoV-2 in all patients included in the study. 221 patients (73.2%; 105 males and 116 females) had a negative rapid test result, while 81 (26.8%; 34 males and 47 females) had a positive rapid test result. Rapid test results are presented in Fig. 5.

We performed RCR tests for the detection of Sars-CoV-2 virus, Influenza virus and Respiratory Syncytial Virus (RSV) to all patients included in the study. 213 patients (70.5%; 100 males and 113 females) had a Sars-CoV-2 negative result and 89 (29.5%; 39 males and 50 females) had a Sars-CoV-2 positive result. PCR results for Sars-CoV-2 are presented in Fig. 6.
288 patients (95.4%; 133 males and 155 females) had an Influenza negative result and 14 patients (4.6%; 6 males and 8 females) had an Influenza positive result. PCR results for influenza are presented in Fig. 7.

288 patients (94.4%; 132 males and 153 females) had an RSV negative result and 17 patients (5.6%; 7 males and 10 females) had an RSV positive result. PCR results for RSV are presented in Fig. 8.

We detected 15 cases of co-infection (7 males and 8 females) of SARS-CoV-2 and RSV (4.97%) and only one case, a male patient, with co-infection of SARS-CoV-2 with influenza (0.33%) (Fig. 9).

We performed a chi-square test of independence to observe all the possible associations between the following parameters: gender, vaccination, infection, rapid antigen test result, SARS-CoV-2 result, Influenza result and RSV result. The results and the respective p-values are presented in Table VIII.

We observed significant associations between infection and rapid test results, between infection and PCR result for SARS-CoV-2, between rapid test results and PCR results for all viruses and between SARS-CoV-2, Influenza and RSV (p-value <0.05). The p-value for the association between Influenza virus and rapid test was marginal.

A logistic regression model was fitted, with response variable the infection (yes, no) and explanatory variables: gender, age, vaccination, vaccination doses, rapid test result, SARS-CoV-2, Influenza and RSV, (Table IX). The estimated model was the following:

Regression Equation

\[ P(YES) = \exp(Y)/(1 + \exp(Y)) \]

\[ Y = -0.087 - 0.01067 \text{AGE} + 1.605 \text{VACCINATION DOSES} + 0.0 \text{GENDER}_F + 0.004 \text{GENDER}_M + 0.0 \text{VACCINATION}_NO - 4.86 \text{VACCINATION}_YES + 0.0 \text{RAPID TEST}_NEG + 0.010 \text{RAPID TEST}_POS + 0.0 \text{SARS-COV}_NEG + 0.493 \text{SARS-COV}_POS + 0.0 \text{INFLUENZA}_NEG - 1.72 \text{INFLUENZA}_POS + 0.0 \text{RSV}_NEG - 0.647 \text{RSV}_POS \]
TABLE VIII: CH-SQUARE TEST RESULTS AND P-VALUES

<table>
<thead>
<tr>
<th></th>
<th>Gender</th>
<th>Vaccination</th>
<th>Infection</th>
<th>Rapid Test</th>
<th>Sars-CoV-2</th>
<th>Influenza</th>
<th>RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.388</td>
<td>0.251</td>
<td>0.392</td>
<td>0.619</td>
<td>0.807</td>
<td>0.680</td>
</tr>
<tr>
<td>Vaccination</td>
<td>0.068</td>
<td></td>
<td></td>
<td>0.147</td>
<td>0.125</td>
<td>0.123</td>
<td>0.751</td>
</tr>
<tr>
<td>Infection RSV</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>0.093</td>
<td>0.593</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sars-CoV-2 RSV</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td>0.055</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza RSV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.034</td>
<td>0.000</td>
<td>0.876</td>
</tr>
</tbody>
</table>

IV. DISCUSSION AND CONCLUSION

The results of this study revealed a higher admission to the Emergency Department of Nikaia hospital for female patients (54%) with common upper respiratory tract clinical manifestations, than male (46%). Similar results are described in the literature [17]–[20], [28]. However, there are studies conducted in India, which conclude the opposite results about hospital admission regarding gender [21], [22]. This fact arises the question whether females in Western countries are more susceptible to upper respiratory symptoms appearance or they have a different health awareness compared to men and Eastern populations [23].

Most of the patients in our study were vaccinated (68.2%); 47.7% had received 3 vaccination doses and 16.9% only 2 vaccination doses. According to the study of Hefdal et al., 2022 patients who received 2 out of 3 doses of a COVID-19 vaccine have a lower risk of breakthrough infections with SARS-CoV-2 [24]. In agreement with that, we noticed a significant lower rate on co-infection with SARS-CoV-2 and RSV in patients having received 2 vaccination doses (5.9%), compared to patients having received 3 vaccination doses (52.9%) or up to 1 vaccination dose (41.2%). Co-infection with SARS-CoV-2 and Influenza was found only in 1 patient (0.33%), which is in absolute accordance with the findings of Acuña-Zegarra, et al., 2021 [25,27]. These findings are based on real-time PCR testing of nasopharyngeal samples from symptomatic patients, where Ct values for both pathogens (SARS-CoV-2 and RSV) were between 10 and 17 [25]. Moreover, our findings indicate a significant relation between 2 out of 3 vaccination doses and PCR positivity in men for SARS-CoV-2. In detail, only 10% of PCR positive men have received 2 doses of vaccination while 42.2% and 47.5% have received 0, 1 and 3 doses of vaccination respectively. Additionally, 2 doses vaccination seems to be more effective in men than women, with a total 10% PCR positivity in men and 28.6% PCR positivity in women. Veerapu et al. [21] studying vaccination effectiveness of Indian vaccines found also a higher vaccination effectiveness in males than females [21]. In the present study, Ct values range was between 10 and 19, indicating that symptomatic patients have a high viral load [26]. The limitations of our study lay on the small number of patient samples, absence of data about illness stage and severity.

CONFLICT OF INTEREST

The authors declared no conflicts of interest.

REFERENCES


