Studii şi Cercetări	Mai 2022	Biologie	31/2	96-104	Universitatea"Vasile Alecsandri" din Bacău

STUDY ON THE INCIDENCE OF SARS-CoV-2 VIRUS (COVID-19) IN 12 CITIES IN ROMANIA IN SEPTEMBER, OCTOBER AND NOVEMBER 2021

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Key words: *SARS-CoV-2; COVID-19; pandemic; virus*

INTRODUCTION

At the end of 2019 a pneumonia with unknown causes hit China. For political reasons, the authorities in the city of Wuhan (Hubei region) have asked doctors not to report cases in their national system. They respected the orders received and hid everything. When the situation became too serious and had already gained quite large proportions, the authorities began to react and admit that the virus was contagious. Too late though [1,2].

A few months later the new mysterious disease had spread around the world, on March 11, the pandemic was declared globally by the WHO [3].

With the help of modern science, researchers learned that the whole pandemic was due to a coronavirus. The new virus was called SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), because it pretty much resembles another one that we have hardly managed to get rid of (SARS in 2003); and the disease was called COVID-19 [1].

Because public health prevails and cannot be preserved other than by testing and isolating (quarantined) carriers and/ or sick people, antigen tests appeared and then the RT-PCR technique was adapted to detect different genes specific to the new disease (N, S, E, RdRp gene, ORF1ab, etc.) [4,5].

Due to the dynamism of this virus, it is necessary to carry out a study that has as its theme the incidence and tropism of this unseen enemy towards people. The first confirmed case of COVID-19 in Romania was registered on February 26, 2020 [6]. According to the Center for Systems Science and Engineering at the Johns Hopkins Whiting School of Engineering on November 30, 2021, Romania recorded 1622 new cases.

Since the beginning of the pandemic until now, scientists have studied the virus and made vaccines; those from Pfizer have even developed an oral antiviral called Paxlovid that is recommended for people with mild to moderate forms of COVID-19, which is a world first [7].

MATERIAL AND METHODS

1) ***Test group (study participants).*** The group of subjects was made up entirely of people who requested testing at the Psihomed Clinic based in Bacau, Romania. It is worth mentioning that at the time of the beginning of the study, the clinic had harvesting centers in 12 different cities (Bacău, Botoşani, Braşov, Bucharest, Buzău, Făgăraş, Focşani, Galați, Iași, Sibiu, Suceava and Tulcea). During the study, the clinic expanded to other cities; but throughout the entire study we kept the 12 cities as a reference. As the present study was spread over three months, the number of samples/subjects/study participants is very high considering the pandemic situation. The total number of samples analyzed reached the intimidating figure of 19 301 .

2) ***Sampling of biological material– nasopharyngeal and pharyngeal exudate***

According to the document entitled Methodology for the surveillance of acute respiratory syndrome with the new coronavirus (COVID-19) updated on 23.08.2021 and elaborated by the National Institute of Public Health in Romania (INSP); it is recommended to take biological material from the upper respiratory tract. The same document specifies that combined testing of oropharyngeal and nasopharyngeal samples is indicated because this increases the sensitivity of respiratory virus detection; this is especially the case of people who are still at the onset of the infection being asymptomatic or mild cases.

Thus, it is recommended that after sterile culture pads, made of polyester or Dacron, have been loaded with biological material (thus making two exudates: pharyngeal and nasopharyngeal) to be discharged (both) into the same transport tube [8].

Pharyngeal exudate. For the veracity of the material taken, it is preferable that sampling takes place in the morning before the toilet of the oral cavity and the ingestion of food, liquids; before smoking but also before the administration of any treatment that could distort the biological material taken and finally the result of the test (antibiotics, antivirals). The patient sits on the chair, is directed to perform a slight extension of the neck face up. The patient is asked to open the mouth to allow access to the oral cavity. Depress the tongue with the help of a spatula, insert the sterile culture pad into the oral cavity and firmly

wipe the tonsils, tonsillary piliers and the posterior wall of the pharynx, thereby loading the culture pad. At the stage of removing the tampon from the oral cavity, it is avoided to touch the tongue, soft palate and lips. Then, the loaded buffer is inserted into the special VTM tube (medium transport virus) with liquid medium that is enriched with antifungals and antibiotics [8, 9].

Nasopharyngeal exudate. From practice I can say that for a result as truthful as possible, biological material should be taken before a possible local toilet, before smoking, aerosol and the use of nasal sprays; and even before taking antibiotics or antivirals. The patient is seated on a chair where he performs a slight extension of the neck. The sterile and flexible culture pad is inserted through one of the nostrils, along the nasal floor, until we reach the posterior wall of the nasopharynx and wipe the side walls by rotating movements, thus loading the tampon. After the exudate is carried out, it is placed in the same VTM-type test tube [8, 9]. In this study we used the Biosci® medium transport (model MM-3981011- 3 ml of Inactivated Transport Medium in screw-cap tube) tubes produced by the Chinese company Shenzhen Dakewe Bio-engineering Co., Ltd. because, according to the prospectus: the liquid medium contains an increased concentration of guanidine salts that achieves a quick and light lysis of the biological specimen taken, which leads to the release of nucleic acid and at the same time inhibits the enzymatic reactions that would occur in the natural way [10].

Thus, the newly created suspension (containing: guanidine salts, remnants of the biological specimen, nucleic acid, etc.) involves a mandatory purification (stage called a little later extraction) before the determination of the nucleic acid (a stage later called amplification).

They may maintain the viable sample for analysis for a maximum period of 72 hours; however, it is preferable that, after sampling, the sample reaches the laboratory as soon as possible.

All samples that are collected in centers other than the central one (where the laboratory is located, in Bacău) are transported in accordance with all the required rules, the temperature during transport and after being between 2 and maximum 8 degrees Celsius; but factually never exceeding 5 degrees Celsius [8, 9, 10].

3) ***Reverse transcription – polymerase chain reaction (RT-PCR)***

The RT-PCR method is a very valuable technique, often used in Molecular Biology, Genetics and Biotechnologies, which allows the study of gene expression, even some genes that show a weaker activity, but which are very important (especially of viruses).

Unlike the classic PCR – which allowed reading the result at the end of the whole process; reading as "presence" or "absence" – the RT-PCR technique, which is a quantitative method, allows the quantification of DNA/ RNA in each cycle, in real time; which can be done from the exponential phase [11, 12].

The main stages of RT-PCR technique are: extraction and amplification; and then reading the results.

a) Extraction

The situation of the pandemic has imposed a large number of tests, that is why the manufacturers of biomedical materials (reagents, consumables, devices, etc.) have created kits specially designed to ease the work in the laboratory and to reduce the time required to perform the tests.

For the extraction of viral RNA, in the laboratory we use the extraction plates manufactured by Biobase Biodustry (Shandong), Co., Ltd. based in China. The kit used (Virus DNA/RNA Extraction Kit – Magnetic beads method) is identified by the catalog code KYD-01-64; having as advantages the simple and rapid way of use, the independence of rinsing and elution from centrifugation, the ability to extract viral material (be it DNA or RNA type) from samples such as blood, animal tissues, saliva, nasal fluids, exudates and even samples from the environment; and the fact that all materials (reagents) are non-toxic. This kit contains: 4 extraction plates of 16 tests (extraction positions) each (Fig. no. 1), the leaflet, 1.4 ml of Lysis 2 and 4 sets of two plastic arm systems (plastic cover for magnetic beads) [13].

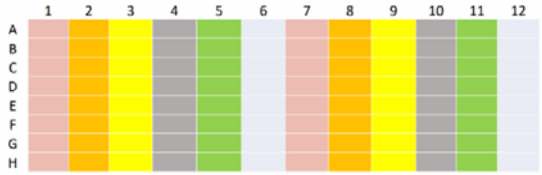

Figure 1. Schematic representation of the disposition of the BIOBASE extraction plate spaces (top view)

Way of working. According to the leaflet, after removing the kit from the refrigerator, opening the box and removing the components, we will remove all the coatings that seal the extraction plates; but not before shaking the plates slightly, gentile to homogenize both the decanted magnetic particles and the other reactants.

The plates are divided as seen in Figure 1 and as follows: in columns 1 (A1-H1) and 7 (A7-H7) each well contains 600 µl Lysis 1; in columns 2 (A2 to H2) and 8 (A8-H8) the first-wash agent is found in the amount of 800 µl in each well; in columns 3 (A3 to H3) and 9 (A9-H9) the reagent for the second wash is found in the same quantity as at the first wash; the spaces belonging to columns 4 (A4-H4) and 10 (A10 to H10) each contain 200 µl of ddH2O and 10 µl of magnetic particle, the 100 µl of elution agent is found in the spaces in columns 5 (A5-H5) and 11 (A11-H11), where the viral RNA that will be pipeted to be amplified is collected; and in the spaces

in columns 6 (A6-H6) and 12 (A12-H12) there is nothing. After the temperature of the plates has been balanced with that of the ambient environment, in columns 1 (A1 to H1) and 7 (A7 to H7) pipette 200 µl of the test sample and then add 20 µl lysis 2 [13].
Then the plates are carefully placed in the specially designed space of the Nucleic Acid Extraction system model BNP32, the plastic arms in the kit are mounted and the working session is started.
This procedure takes about 10 minutes. After the viral RNA is extracted, the plates are removed from the extractor, and the device is given on the mode of sterilization with UV rays (30 minutes, according to the clinic protocol).

During this time we pipette 10 µl of the enzyme needed to amplify in each well on the amplification plate (from Nest Biotechnology, identified by the catalogue code 402101); the spaces being corresponding to those on the extraction plate.
Then the genetic material is removed from columns 5 (A5-H5) and 11 (A11-H11) of the extraction plate with the help of the pipette and put in its homologous place on the amplification plate. After we have finished putting the RNA over the amplification enzyme, the plate is sealed with a special transparent film that will not interfere or influence the results of the RT-PCR test [13, 14].

b) Amplification

The One-Step RT-PCR COVID-19 kit. According to the leaflet of the kit (identified by the catalog number: PT. COVID.100) produced by PISHTAZ TEB DIAGNOSTICS (Iran) the principle of operation of their enzyme (preparations prior to amplification) during the amplification process is as follows: the mixture consisting of primer and sample uses the design of the gene with a double target, aimed at the sequence that encodes the region / gene RdRp (which is RNA polymerase and RNA dependent) but also the nucleocapsidic protein N (encoded by the N gene).

The primer and the sample together with the reaction buffer make it possible to monitor the amplification (figure number 3) of the model in a quantitative way due to the increase in the fluorescent signal that is detected by a Real Time PCR instrument.

The PCR detection system includes both the primer and the internal control sample (identified in the working session of the program as the ROX channel); the result of the internal control ensuring the accuracy of the extraction and sampling procedure, thus avoiding false negatives [14].
The graph of the amplification curve shows 3 phases [12] (fig. 2):

- linear – now there is no fluorescence;
- exponential growth – PCR products increase;
- of the plateau – this is where the components of the reaction are exhausted.

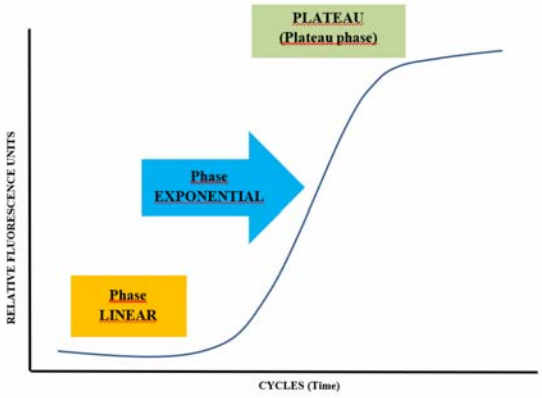

Figure 2. RT-PCR amplification curve (scheme)

In vitro enzymatic amplification of RNA (RT-PCR). After the amplification plate is inserted into the thermocycle (Accurate model 96-x4, no. 5084102300 catalog, produced by the Chinese from DLAB Scientific Co., Ltd.) and the work session is started, testing can begin.

The thermocycle must go through the following steps [14], according to the recommendations:

- reverse transcriptase – a cycle that lasts 20 minutes at a temperature of 50°C;
- initial complementary DNA denaturation – a step that is achieved at 95°C, a cycle for 3 minutes;
- denaturation – the temperature decreases by one unit from the previous stage, the duration of a cycle of this stage being 10 seconds, but repeating 45-50 times;
- recombination, expansion and fluorescence measurement – this stage involves lowering the reaction temperature to 55°C, the cycle having 40 seconds and repeating itself 45-50 times;
- cooling – the reaction temperature is brought up to 25°C in a time of 10 seconds.

Because the PCR technique uses a DNA polymerase, the RNA obtained from extraction cannot be used for any purpose; it will therefore be converted using the reverse transcriptase (DNA polymerase-dependent RNA) enzyme into single-stranded complementary DNA (DNA_C). The RNA is then degraded and the DNA_C will then be amplified by a standard PCR technique.

Thus complementary DNA will be amplified with the help of fluorescently marked primers specific to SARS-Cov-2. If specific genetic regions are present in RNA, then the DNA_C corresponding to these regions is amplified leading to the emission of fluorescence. It should be noted that the amplification cycle from which the fuorescent signal can be detected is called the "cycle threshold" (Ct).
Genes of interest.

The reagent from Pishtaz Teb Diagnostics allows the detection of N (for screening purposes, determines the formation of nucleocapside of

phosphoproteic nature) and RdRp (for confirmation) [11, 12, 15].

c) Interpretation of results

Normally, in order to correctly interpret a result, the package leaflet of the kit used should be consulted.

Thus the manufacturer informs us about the channels homologous to the target genes: the ROX channel to detect internal control, the HEX channel for the N gene and for the RdRp gene the FAM channel.

Positive or Negative. To be able to establish the result we must first check if the ROX channel (internal control) has a value of Ct ≤ 40; that means the detection is valid [14].

- If the FAM channel or HEX channel detects a typical "S"-shaped amplification curve, and the Ct value ≤ 40, it means that the sample is POSITIVE (Figure 3), so the patient has the SARS-CoV-2 virus present in the body.
- If the FAM and HEX channels did not detect a typical amplification curve or Ct□ 40, then the virus is not present in the analyzed sample, so the patient is declared NEGATIVE.

If the internal control channel has not detected any Ct value or has detected a value greater than 40, testing shall be repeated [14].

Gray area. The package leaflet says that if the fluorescent signal of any test sample indicates a significant increase in the FAM and HEX channels, but the Ct value jumps above 40, the sample result falls into the gray area. That just means that the sample needs to be retested. If the sample falls back into the grey area, the final result will be considered positive [14].

RESULTS AND DISCUSSIONS

September

This month the total number of people tested is 8060 and the number of positive results is 56. Figure number 4 is the number of patients tested each day and also the number of positive daily results.

The maximum number of positive patients (12) reported daily is recorded on September 27th, 2021; while the minimum number (0) is recorded in several days of the same month (1,2,3,4,8,10,11,13,14,15,19,20,21,22).

This month, the number of people detected positive was found to be higher for the male sex (35) and lower for the female sex (21).

The month of September showed that the highest incidence of the virus is found in the age group 31-40 years old (15 years old), followed by the 14 patients who were aged between 21-30 years old (fig. 5). At the opposite pole we find the 3 people in the age group 71-80.

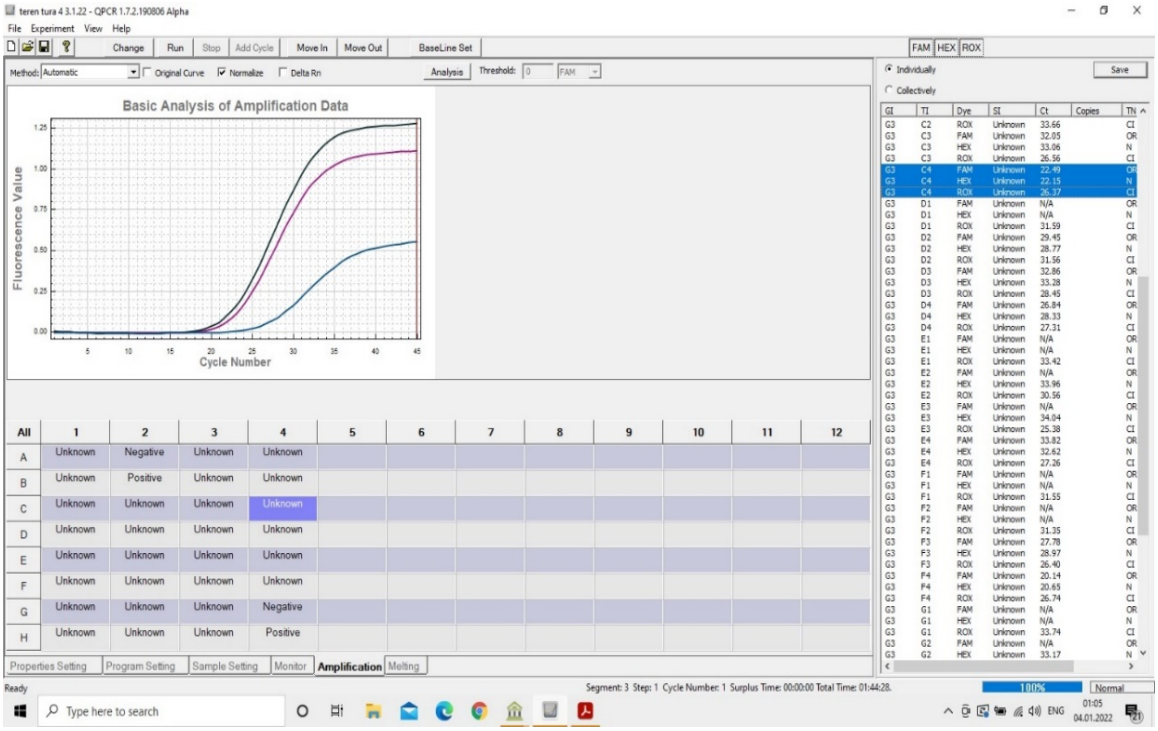

Figure 3. RT-PCR amplification curve of a positive patient

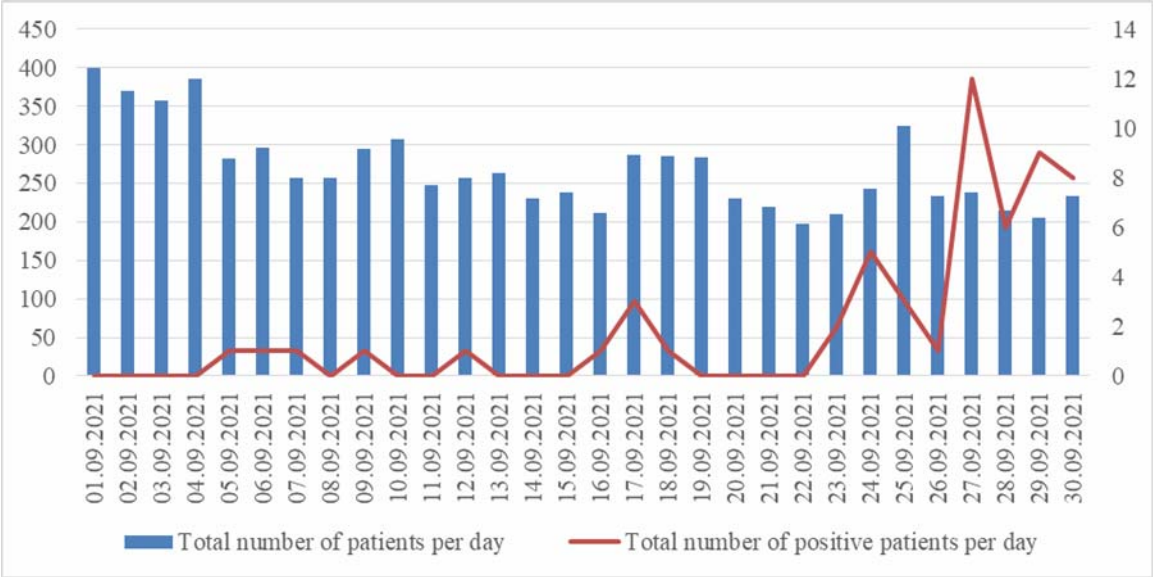

Figure 4. Graphical representation of the total number of patients tested and those who tested positive, per day in September

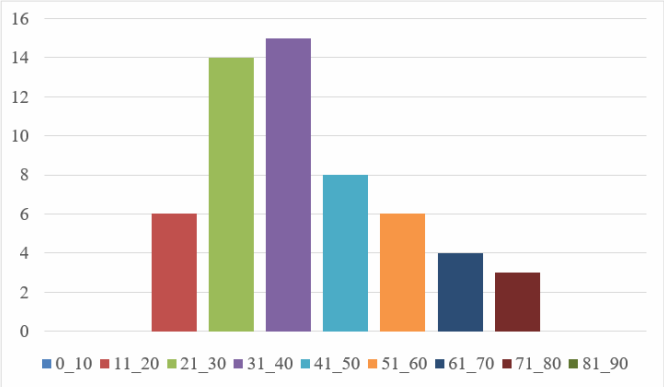

Figure 6. Distribution of the number of positive tests by age group (years) – September

The patient with the youngest age is of sex M, is 13 years old, is from Bacău and did not specify the appearance of symptoms until the time of testing.

The oldest patient is of sex F, is 80 years old, is from Galați and did not report symptoms at the time of testing.It is worth mentioning that out of the total of 56 patients tested positive in the 30 days of this month, only three reported symptoms (from Iași, Galați and Focșani), from the mildest ones such as fever or cough to those specific to COVID.

Table 1 lists the centralization of the number of people tested positive in each city. Thus, we can see that the highest incidence is recorded in the city of Iasi (14), followed by Bacău with 10 cases.

October

In October, the total number of tests is 6535; of these the number of patients detected positive is equal to 828. The following figure (number 6) shows the number of patients tested in the 31 days, and the number of patients detected positive each day. As can be seen from Figure 6, no day is without positive cases.

Table 1. Centralization of the number by cities of positive people from September

Cities	No of positive patients
Bacău	10
Botoşani	7
Braşov	4
Bucureşti	1
Buzău	4
Făgăraş	2
Focşani	3
Galați	6
Iași	14
Sibiu	4
Suceava	1
Tulcea	0

The highest number of positive patients is recorded on day 24 (52 cases), followed by day 18 on which 51 positive people were detected. The smallest number of people validated positive is found on the 1st and 10th days, every day 2 sick people are found.

Unlike the previous month, in October the incidence in terms of the sex of people detected positive is higher in women (435 cases) while the male sex records a number of 375 confirmed cases.

The data gathered shows that an increased incidence is found in the age group 31-40 years old (here finding 107 cases), followed as the previous month by the group 21-30 years old (fig. 7) where we encounter 75 confirmed cases. On the other hand, the lowest incidence reported in the age group is found in the elderly 81-90 years old, where a number of 5 positive tests are recorded. The patient with the youngest age is of sex M, is 1 year and 10 months old, is from Buzău and did not show symptoms at the time of testing.

The oldest patient is of sex M, is 84 years old, is from Galați and did not report symptoms at the time of testing.

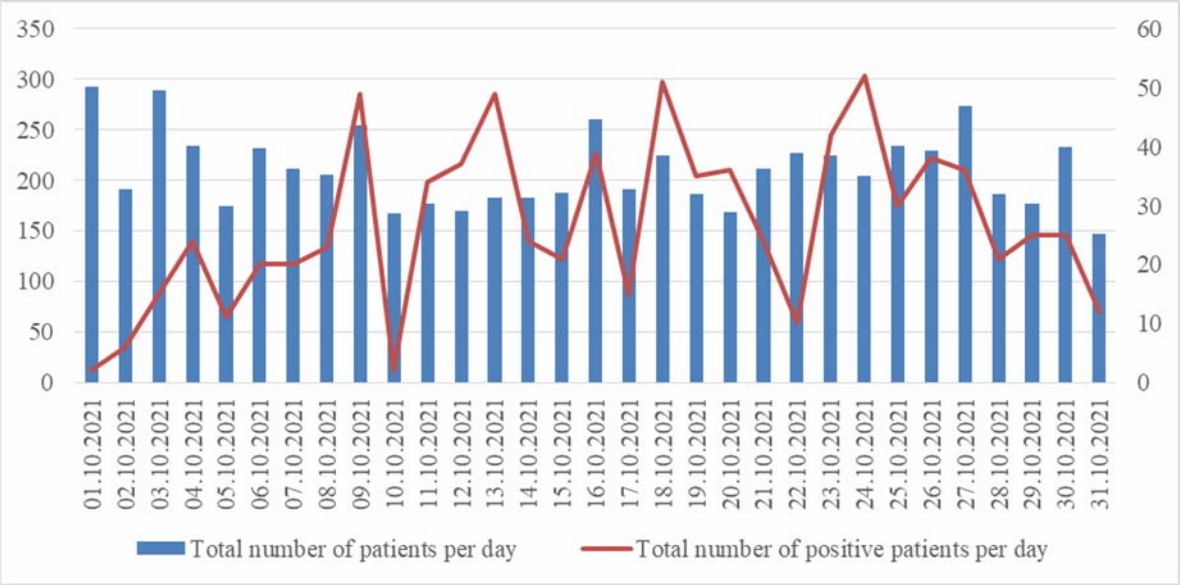

Figure 6. Graphical representation of the total number of patients tested and those who tested positive, per day in October

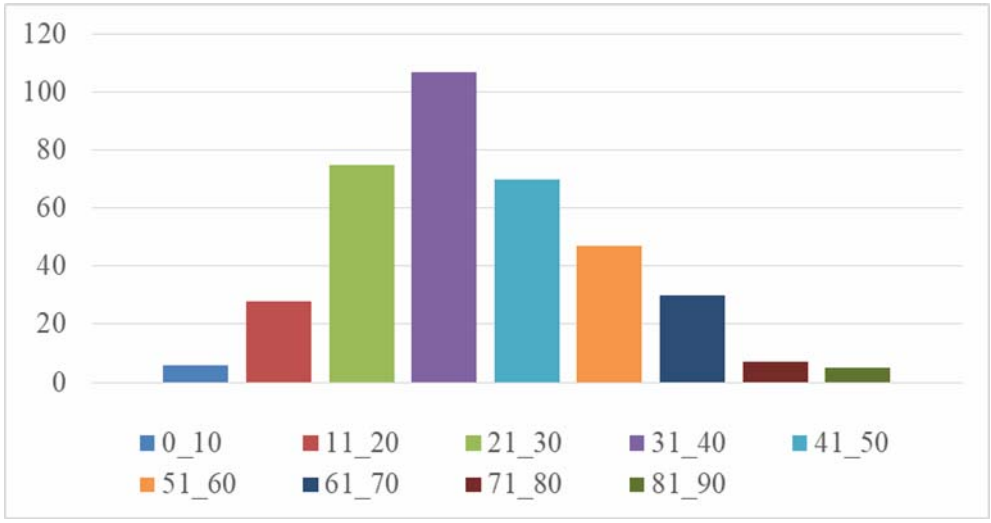

Figure 7. Distribution of the number of positive tests by age group (years) – October.

It is worth mentioning that out of the total of 828 patients detected positive in the 31 days of this month, 82 people reported symptoms (Table 2), from the mildest ones such as fever or cough to those specific to COVID (lack of taste, lack of smell).

Table 2. Centralization of the number of people by cities positive and people who have reported symptoms – October

Cities	No of positive patients	No of positive patients who reported symptoms
Bacău	23	2
Botoşani	25	5
Braşov	44	11
Bucureşti	41	13
Buzău	49	9
Făgăraş	13	6
Focşani	10	4
Galaţi	35	20
Iaşi	94	4
Sibiu	20	5
Suceava	3	0
Tulcea	8	3

In terms of the number of positive patients, the highest is in Iasi with 94 patients, followed by Buzău with 49 positives and Braşov (44 positive). The smallest number of people tested positive is found in Suceava, 3 people.

November

In November, 326 positive cases were registered out of the total of 4706 people tested.

Figure 8 in blue shows the number of tests carried out each day and in red the number of people tested positive each day.

The lowest number of people found positive is recorded on 20 days (3 positive), 29 (2 positive people) and 30, a day when 3 positive people were validated. Of the 30 days of November, in the 22nd (27 people confirmed positive) and the 25th (26 positive people) the peak of the confirmed cases was recorded compared to the other days of the same month.

Of the total 326 positives, 164 are male and 157 female; the number of females and males being almost equal.

The data gathered during November shows that, as in September and October, the highest number of positive cases falls into the age group of 31-40 years, that is, 80 people.

The next age group in which a significant number of people found to be positive are recorded (64) is 41-50 years, as shown in Figure 9.

The lowest incidence is found in the age group 0-10 years where we find 5 cases; category followed by that of positive people between 81 and 90 years of age (7 people).

The patient with the youngest age is of sex F, is 1 year old, is from Bacău; and the parents stated that their baby had mild symptoms at the time of testing (cough, fever, diarrhea).

The oldest patient is of sex M, is 89 years old, is from Buzău and did not report symptoms at the time of testing.

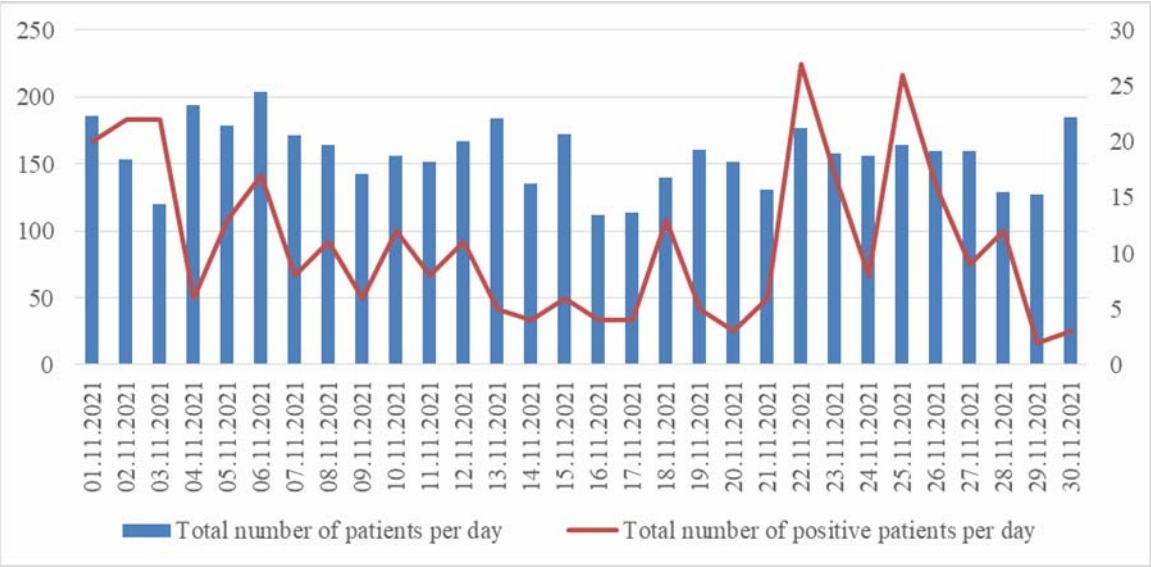

Figure 8. Graphical representation of the total number of patients tested and those who tested positive, per day in November

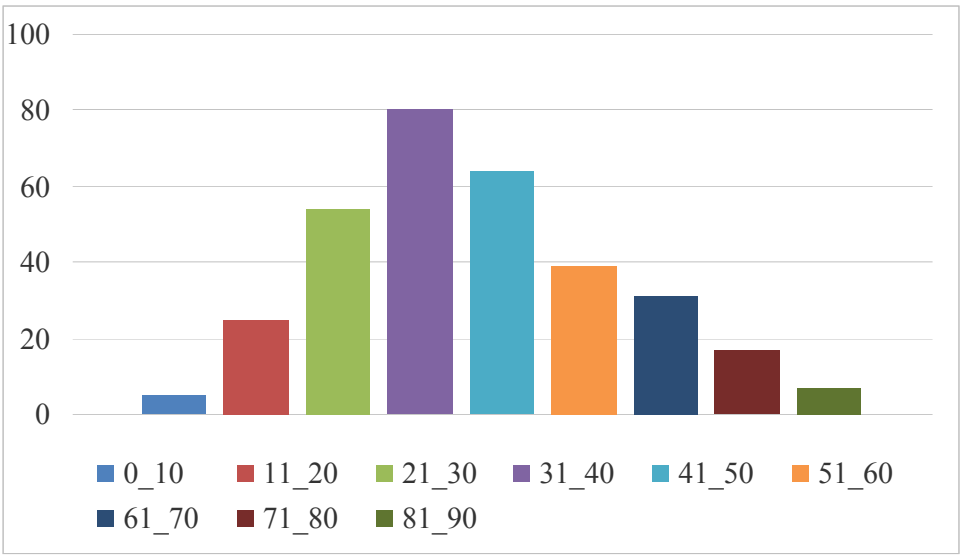

Figure 9. Distribution of the number of positive tests by age group (years) – November

Analyzing the information gathered in table 3, we can say that out of the total of 326 people found positive, the highest number is found in the city of Bacău (80), also in this city being the most people who said they had symptoms at the time of testing. The cities where the fewest cases were registered are Botoşani (6 people) and Suceava with 5 people. Regarding the lowest number of people who had symptoms, we see that we can talk here about the cities of Suceava and Tulcea where 0 such cases are registered, so the people being positive but asymptomatic.

Table 3. Centralization of the number of people by cities positive and people who have reported symptoms – November

Cities	No of positive patients	No of positive patients who reported symptoms
Bacău	80	45
Botoşani	6	2
Braşov	40	14
Bucureşti	25	9
Buzău	30	1
Făgăraş	12	8
Focşani	12	5
Galaţi	35	5
Iaşi	30	1
Sibiu	32	3
Suceava	5	0
Tulcea	14	0

CONCLUSIONS

Following the information gathered, we can conclude that the data changes a lot from one month to the next, so the whole situation of the pandemic is very dynamic. This makes it very difficult to anticipate the number of positives, the possible mutations that may occur when transmitting from one person to another, the future variants of the virus, the number of future epidemiological waves and even the end of the pandemic.

In conclusion relevant are the following:

Although the highest number of tests was recorded in September (8060, of which only 56 positive), the highest number of people tested positive is 828 and is recorded during October.

Out of the entire 19,301 people tested in the three months, 1210 were found positive, and of these only 178 reported symptoms.

September is the month in which most days were recorded (14) in which no positive was detected.

The highest incidence reported at the age of the subjects is found in the group of 31-40 years old, followed by the group 21-30 years old of age in both September and October. In November the category 21-30 years old is replaced by that of people aged 41 to 50.

Out of the three months and the 19,301 patients tested, the youngest patient has at the time of testing 1 year, is of sex F and resides in Bacău, and the parents said that the baby had mild symptoms such as cough, fever and diarrhea. The oldest person is an 89-year-old man from the town of Buzău, the gentleman did not report the presence of symptoms. Both people (1 year old and 89 years old respectively) tested positive in November. We conclude here that neither children (babies even) nor the elderly are safe from being infected with the new SARS-CoV-2 virus.

Reporting the number of positive people to the cities where their exudates were taken shows that out of the three months (September, October and November) in October, the highest number was detected in the city of Iaşi, 94 people; followed by the city of Bacău in November with 80 people.

In Bacău, in November, most people were also registered who reported symptoms (45), from the mildest to the most serious, specific.

Calculating the percentage of patients with symptoms out of the total number of positive patients in each month we get the following numbers: in September (56 positive) the percentage is 5.36%, in October (828 positive) this percentage increases to 9.90%; and in the last month (November with 326 positive people) the percentage increases and registers 28.52 percent.

The RT-PCR test remains the golden standard in terms of detecting the new coronavirus, regardless of whether the patient has symptoms or whether he agrees with the given result or not.

ABSTRACT

The pandemic was declared worldwide in early 2020, since then things have taken an unexpected turn for all of humanity. The pandemic still exists and the epidemiological waves are coming one after the other. I firmly believe that such a study was necessary, with incidence studies being very important.The purpose of the study was to see which age groups are most prone to SARS-CoV-2 infection, which sex shows an increased tropism compared to the new virus. I also wanted to see which of the 12 cities studied have the most cases of positive people tested at our clinic. The method with which we were able to test the people who took part in the study was RT-PCR (Reverse transcription – polymerase chain reaction).The results obtained in the three months in which we have gathered information prove that the pandemic exists and is present every day, new people tested positive appear almost every day (September) and even every day (in October and November). The results also show that children can also be infected with the COVID-19 virus. I also conclude that although few people have symptoms, many do not; the percentages of people with symptoms reported to positives each month increased from 5.36% in September to 9.90% in October to rise to 28.52% in November.

ACKNOWLEDGMENTS

My thanks go to a special lady Diana Bianca Lucan without whom this study would not have existed, and last but not least to Andrei Constandache without whose help this study would not have been finished yet.

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