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Article

SARS-Cov-2 in Asymptomatic and Mild Infections in a Hungarian Outpatient Cohort in the First Year of the Pandemic

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Abstract: We aimed to estimate the proportion of the population infected with SARS-CoV-2 in the first year of the pandemic. The study population consisted of outpatient adults with mild or no COVID-19 symptoms, and was divided into subpopulations with different levels of exposures. Of the subpopulation without known previous COVID-19 contacts 4143, of the subpopulation with known COVID-19 contacts 594 persons were investigated. IgG- and IgA-seroprevalence and RT-PCR positivity were determined in context with COVID-19 symptoms. We hope to have contributed to the understanding of the significance of the asymptomatic and mild infections in the long persistence of the pandemic.

Keywords: SARS-CoV-2; COVID-19; outpatient cohort; seroprevalence; mild infections; asymptomatic cases; COVID-19 contacts; PCR-positivity and symptoms; first year of the pandemic; Hungary

1. Introduction

The World Health Organization (WHO) declared the outbreak of the Coronavirus Disease-19 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) a Public Health Emergency of International Concern under International Health Regulations in January of 2020 (WHO, 2020.01.30)[1], and a pandemic on March 11, 2020 [2].

The appearance of SARS-CoV-2 in Hungary, based on the first detection of the virus in the swab samples of two foreign university students living in the capital, Budapest, was reported in March 2020 [3]. In a few days after the detection of the viral RNA in these two students, the viral RNA was demonstrated in small clusters of university students in Budapest, some with COVID-19 symptoms. The pandemic has started in Hungary.

Relatively to the short time (less than 2 years), since the official recognition of the SARS-CoV-2 in January 2020 [1], causing deadly diseases in the human population, a great number of results have been published about the characteristics, the diagnosis, the possible mechanisms of the disease, about the immune responses to the SARS-CoV-2 in people suffering from COVID-19 and about the virus itself [4–7]. Much less is known about the SARS-CoV-2 infections causing mild COVID-19 symptoms with no need of hospitalization or special medical treatment of the patients, or subclinical infections with no symptoms at all. However, there are more and more data suggesting that individuals experiencing mild disease or symptomless infections spread the virus to other people with the possibility of causing new cases of severe COVID-19 with deadly outcome or even new waves of the pandemic [8–10] .

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Our aim was to determine the prevalence and some characteristics of asymptomatic and mild forms of COVID-19 in an outpatient subpopulation of Budapest, from April 2020 to March 2021. The outpatients included individuals of whom none needed hospitalization during the study period. The patients were evaluated for cold symptoms and their samples were tested for the antibody responses by IgG- and IgA- specific ELISA and for the presence of viral genetic material by real time reverse-transcription polymerase chain reaction (RT-PCR).

2. Materials and Methods

2.1. Collection of blood and nasopharyngeal swab samples

The blood samples and the nasopharyngeal swab samples were collected at the Complex Medical Center (CMC) South Clinic, Budapest. CMC is a private outpatient clinic located in a residential area of the first district of Budapest, serving outpatient individuals with various health problems. The CMC is also functioning as a laboratory for SARS-CoV-2 testing in Hungary. Typically, no university students are the outpatient visitors of the clinic and no university is located in the neighborhood. The participants of the study include patients who visited the CMC clinic during the time period of April 2020 and March 2021 with mild symptoms, supposedly of COVID-19, or because of some COVID-19 unrelated medical problems, but were interested in their possible SARS-CoV-2 infection, or were the employees of the clinic, or persons who needed to know their SARS-CoV-2 related status for business or travel purposes.

Collection of data was based on WHO recommendation for every sample tested in the laboratory [11]: laboratory identification number, sample collection date (dd/mm/yyyy), symptoms, type of sample, type of test, the date of test (dd/mm/yyyy), the results, i.e., SARS-CoV-2-antibody and RT-PCR results (dd/mm/yyyy). All the persons attended for testing signed informed consent (available in paper form) and some agreed to follow-up testing. In case of antibody testing, blood sample, in case of viral RNA detection, nasopharyngeal swab samples were obtained. The data collection was managed according to the current version of the declaration of Helsinki and approved by the local Ethics Committee. All data were stored in an electronic database.

ELISA and RT-PCR were carried out at the laboratories of the Department of Microbiology and Infectious Diseases of the University of Veterinary Medicine, Budapest, and of the Veterinary Diagnostic Directorate of National Food Chain Safety Office, Budapest. Mild COVID-19 is defined as symptomatic disease without evidence of viral pneumonia or hypoxia [12].

2.2. Questionnaire

Participants completed a pseudonymized questionnaire at the study site at the beginning of the study. The questionnaire contained the name, address, demographic data as well as symptoms, if reported, such as fever (≥ 38 ○C), chills, fatigue, myalgia, sore throat, cough, rhinitis, shortness of breath, chest pain, headache, anosmia, dysgeusia, and gastrointestinal symptoms. The severity of symptoms was not recorded. Yes or no questions were asked regarding co-existing diseases, such as heart-disease, hypertension, diabetes, high cholesterol-level, asthma, allergy and tumors. Self-reported information on SARS-CoV-2 RT-PCR test in the past 10 days, as well as on previous close contacts with COVID-19 cases were included. The SARS-CoV-2 vaccination program started in December 2020 in Hungary, but none of the participants included in the study in January-March 2021 were vaccinated.

2.3. SARS-CoV-2 RT-PCR

Detection of SARS-CoV-2 in nasopharyngeal and pharyngeal wash samples was performed by RT-PCR amplification of SARS-CoV-2 N-gene fragments. Two hundred microliters (200 μL) of the pharyngeal washes (swabs washed in DNase, RNase free wa-

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ter) were first processed for RNA extraction in the Thermo Scientific™ KingFisher™ Flex Purification System (Thermo Fisher Scientific, Waltham, MA USA), using the IndiMag® Pathogen Kit (QIAGEN® GmbH, Hilden, Germany). Subsequently, the detection of N-gene of SARS-CoV-2 was performed by using the 2019-nCoV-2 RUO kit (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) and One-Step RT-PCR Kit (QIAGEN® GmbH) on a Rotor-Gene Q real-time PCR cycler (QIAGEN® GmbH). The amplification protocol consisted of a reverse transcription step at 50°C for 30 minutes, a denaturation step at 95°C for 15 minutes and subsequent 45 cycles at 95°C/56°C/72°C for 30/30/60 seconds, respectively. A positive result was defined as amplification of N-gene in a sample with each cycle threshold value (ct) less than 37. Virus shedding time was defined as the interval between the date of the first PCR positive test and date of the last PCR positive test.

2.4. SARS-CoV-2 culture, inactivation and purification

The experiments with active SARS-CoV-2 were performed in the BSL-3 facilities of the Veterinary Diagnostic Directorate of the National Food Chain Safety Office, Budapest. Vero E6 cells were grown to a confluence of 80–90% and infected with the SARS-CoV-2/Hungary/CMC-1/2020 (GISAID No…….) strain at a MOI 1 in serum free RPMI-1640 medium () completed with non-essential amino acids (ThermoFisher) and Penicillin-Streptomycin (ThermoFisher, 10,000 U/mL). The infected cells were incubated for 4 days at 37°C with 5% CO2 when cytopathic effect was visible. Virus containing supernatant was ultrafiltered using 0,22 μm pore size filter. The filtered supernatant was inactivated with 1:2000 diluted formaldehyde solution at 25°C for 18 h. The inactivated supernatant was purified by ultracentrifugation at 29,000 rpm (Thermo Scientific™ S58-A Fixed Angle Rotor) for 1.5 h and 4°C. The pellet was resuspended in PBS. Inactivation was validated by inoculation of Vero E6 cell monolayers. The virus preparation was analyzed by RT-PCR assay. The total protein content was tested by a BRC assay kit (Sigma-Merck).

2.5. Anti-SARS-CoV-2 IgG and IgA antibody testing

IgGs against SARS-CoV-2 were assessed on serum samples obtained from the participants during the period of April – August 2020, by a commercially available ELISA kit (Dia.Pro Diagnostic Bioprobes S.r.L.Sesto San Giovanni, Milan, Italy), as suggested by the manufacturer. This assay is based on a microplate coated with a recombinant antigen of both nucleocapsid and spike proteins, the reported sensitivity and specificity were 98% and ≥90%, respectively. To achieve the maximal sensitivity of the antibody detection, an ELISA based on inactivated whole-virion (IWV) of SARS-CoV-2 was developed in our laboratory, and serum samples obtained from the participants during the period of September 2020 to March 2021 were tested by this ELISA. Coating conditions were optimized by antigen dilution and testing with convalescent sera collected from 50 SARS-CoV-2 RT-PCR-positive patients and 50 pre-pandemic control serum samples(Serum Collection of the National Public Health Center, Budapest). Validation of the IWV-based ELISA was based on a comparative analysis with the Dia.Pro Diagnostic Bioprobes S.r.L. kit.

Briefly, 96-well MaxiSorp ELISA plates (Nunc, Thermo Fisher Scientific, NY, USA) were coated with inactivated and purified SARS-CoV-2 whole virus antigen diluted 1:10 in PBS overnight, then were blocked with 3% bovine serum albumin (BSA) for 2 hours at room temperature. The plates were then washed three times with washing-diluting PBS containing 0.05% Tween 20 (Diavet Ltd., Budapest, Hungary). The serum samples diluted 100-fold with washing-diluting buffer were added to each well in a volume of 100 μl. After 1 h incubation at 37^0C, the wells were washed three times. One hundred μl of a peroxidase-conjugated goat anti-human IgG (H+L) or a goat anti-human IgA alpha chain (Abcom, Cambridge, UK) diluted 10,000-fold with PBS-Tween-20 buffer was added to wells, respectively. After 1 h incubation and washing procedure 100 μl of tetra-

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methylbenzidine substrate (TMB) (Diavet Ltd.) was added into the wells for 5 minutes. The color reaction was stopped with 4N H_2SO_4 solution. The absorbance was measured at 450 nm using a FLUOstar Optima (Thermo Fisher Scientific, Waltham, MA USA) microplate reader. Positive and negative controls were included in the respective wells in the test. Cut-off values were determined as the mean plus 2 SD of a set of 10 negative reference sera. Randomly selected 100 sera obtained from the study participants in April-August 2020 and tested by the Dia.Pro Diagnostic Bioprobes S.r.L. ELISA kit were retested with our IWV ELISA; a full (100%) matching was observed as for IgG positivity or negativity of the sera. Further, 100 pre-pandemic control serum samples (Serum Collection of the National Public Health Center, Budapest) were tested by the IWV ELISA; 8% of the sera showed positive reaction with the IWV antigen, suggesting cross-reactivity with antibodies to other human coronaviruses, as was reported by multiple studies [5,13]

Seroprevalence was defined as the prevalence SARS-CoV-2 specific IgG antibodies at or above a designated OD value in the IgG ELISA. The main analysis is based on IgG antibodies, because these isotypes are elevated for a longer period post-infection than IgM and IgA antibodies [14]. However, since the IgA response in the early stage of the disease seems to be more pronounced than IgM [15], IgA detection for the serology assessment was included.

2.6. Statistical analysis

For evaluation of the data, we used the seroprevalence estimate and 95% CIs. Categorical variables were presented as percentages and were compared by the Z-probe test. A two-tailed p-value of less than 0.05 was statistically significant.

3. Results

The immunological and clinical consequences of different levels of exposures were evaluated by dividing the participants into two groups: 1. participants without known previous COVID-19 contacts, or 2. participants with known previous COVID-19 contacts. Only data from the first sample collection were analyzed in both groups to limit selection bias. The characteristics of SARS-CoV-2 infection were also investigated by testing blood and swab samples obtained at consecutive occasions from 33 selected participants.

3.1. Clinical symptoms, seroprevalence and RT-PCR positivity of participants without known COVID-19 contacts

Table 1 shows the results of the first wave and the following time period with a low-level infection rate from April to August 2020; 12.6% of the persons reported symptoms and 3.81% of the tested persons had detectable levels of IgG antibodies. Table 1 also shows the results of participants obtained in the period of September 2020 to March 2021, the time frame comprising partially the second and third waves of the pandemic in Budapest. Symptoms, serum IgG antibodies, IgA antibodies and viral RNA were detected in 11.51%, 16.34%, 7.35% and 8.42%, respectively. During the overall study period of April 2020 to March 2021 COVID-19 symptoms were observed in 11.51% of the tested participants and SARS-CoV-2-specific serum IgG antibodies were detected in 12.94% samples.

Table 1. Symptoms, seroprevalence and RT-PCR positivity of participants without known COVID-19 contacts

Characteristics	Total No. of tested persons	Time period	No. of persons with positive results	% of positive persons (95% CI)
Symptoms	1023 of 1023	04.2020–08.2020	129	12.6 (10.64-14.80)
IgG antibodies	1023 of 1023	04.2020–08.2020	39	3.8 (2.72-5.17)
Symptoms	3120 of 3120	09.2020–03.2021	348	11.1 (10.07–12.31)

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IgG antibodies	2748 of 3120	09.2020–03.2021	449	16.3 (14.98–17.78)
IgA antibodies	2409 of 3120	09.2020–03.2021	177	7.3 (6.34–8.46)
Viral RNA	2423 of 3120	09.2020–03.2021	204	8.4 (7.34-9.6)
Symptoms	4143 of 4143	04.2020–03.2021	477	11.5 (10.56–12.52)
IgG antibodies	3771 of 4143	04.2020–03.2021	488	12.9 (11.89–14.05)

Figure 1 shows that the monthly distribution of participants with symptoms broadly followed the epidemic curve of Budapest, especially in the second and third waves of the pandemic. The results demonstrated that in April 2020, 2.4%, from May to August 14.4%–21.0%, and from September 2020 to March 2021 7.6%–16.9% of the participants experienced some of the COVID-19 symptoms (Figure 1A).

The monthly distribution of the IgG positive sera reflects the 2–3 weeks seroconversion period, low rates in April–August 2020 (1.8%–3.3%), except for the high 13.9% IgG positivity in June 2020. The lowest rate of IgG positivity was observed in September (0.5%), then it slowly increased in October–November, followed by a sharp increase of 14%–48,9% in December 2020 to March 2021 (Figure 1B). The monthly distribution of IgA-positive serum samples showed relatively high rates (16.3%–9.1%) in September to October, then low rates in November to December (3.2%–3.4%), that followed by an increase in January to March 2021 (4.9–17.7%) (Figure 1C). The monthly distribution of PCR positivity showed low rates in September 2020 (1.7%) and January 2021 (1.8%), and the highest rate in November (11.9%) and March 2021 (11.4%), while relatively similar rates, between 6.7% and 8.3%, in the rest of the months (Figure 1D).

Figure 1A

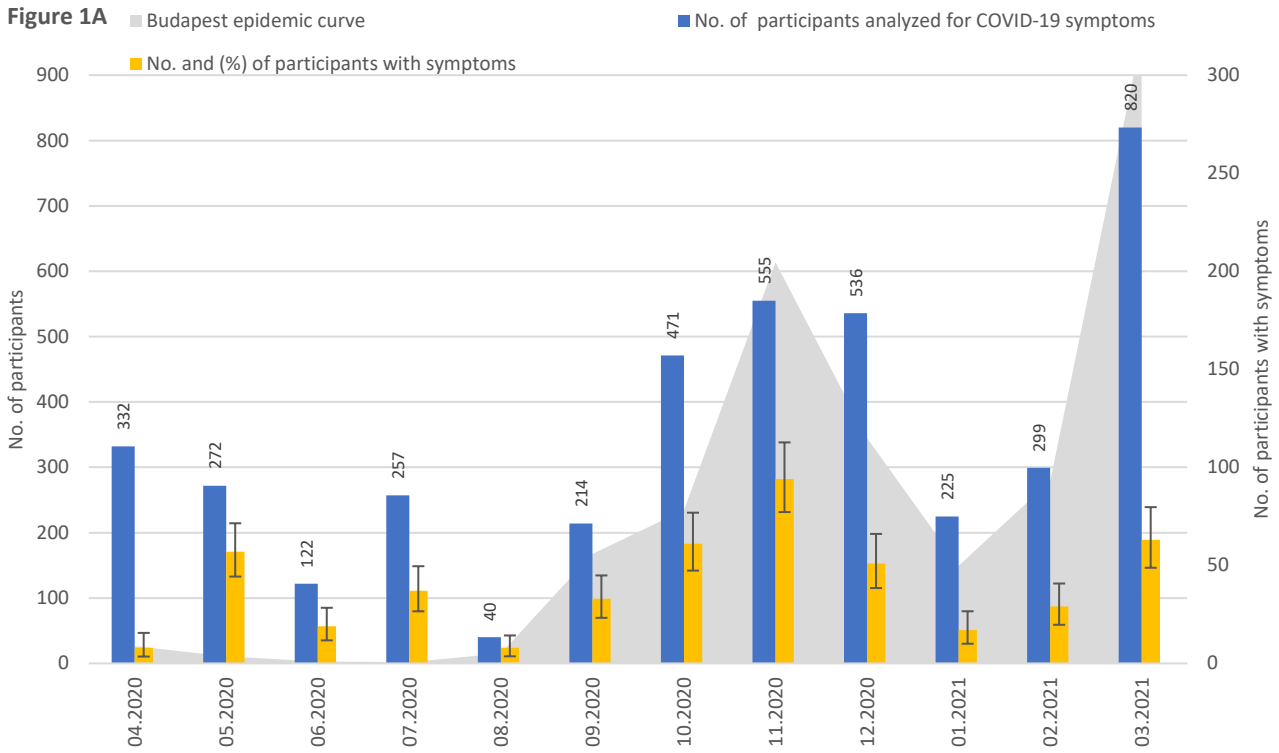

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Figure 1B

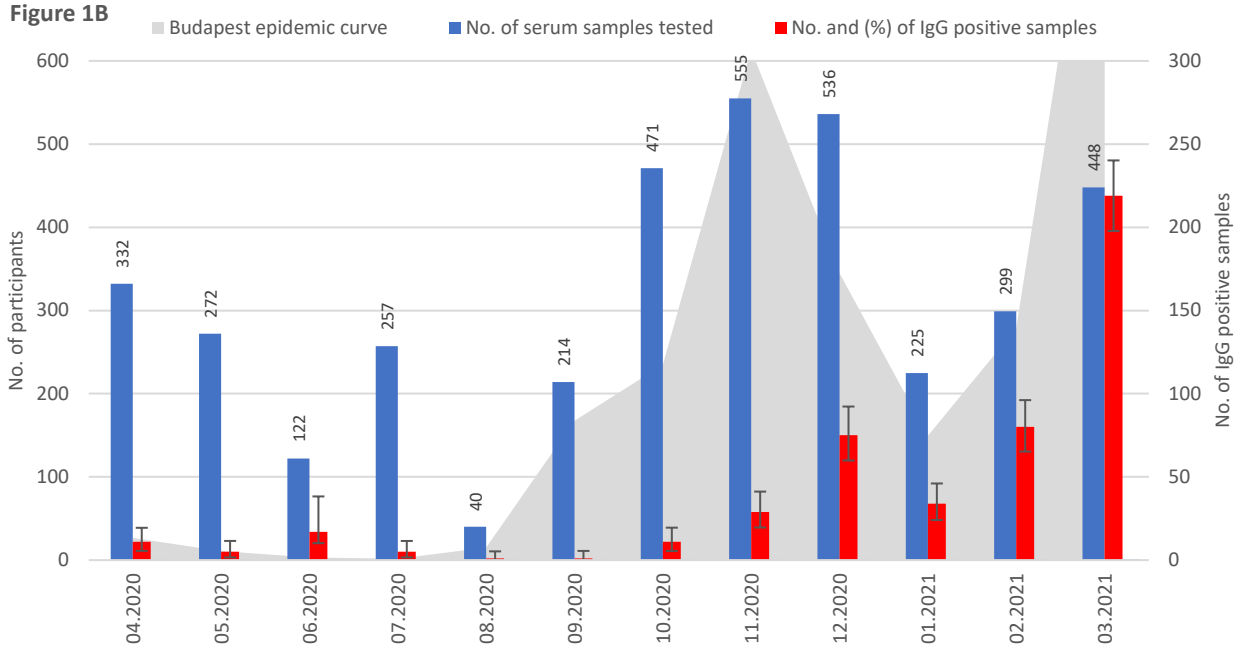
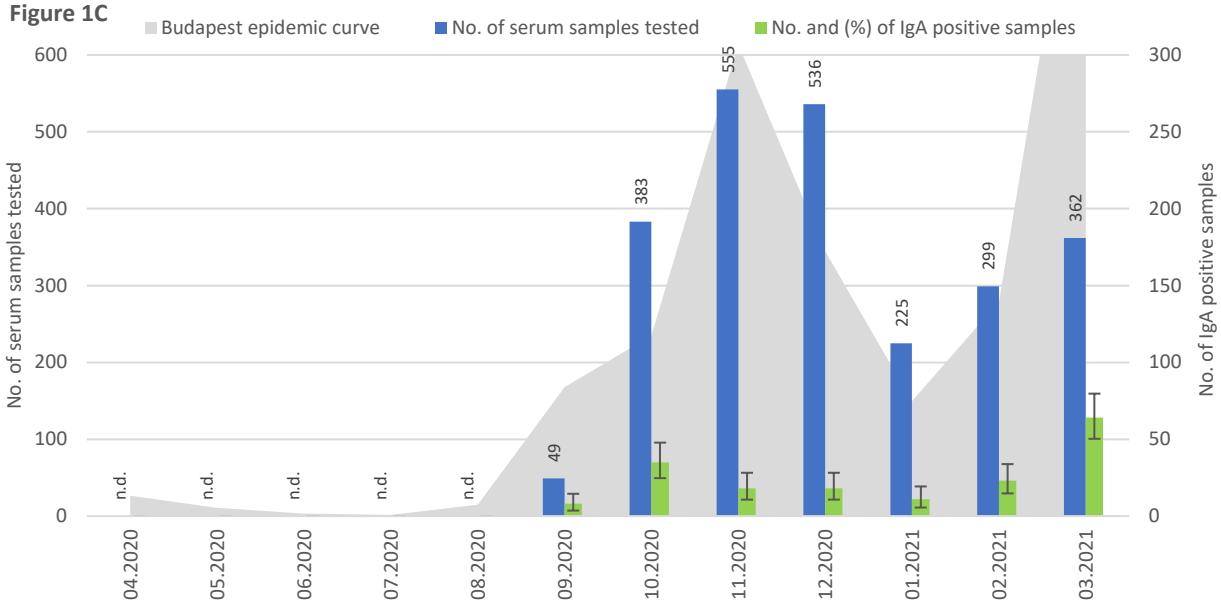


Figure 1C



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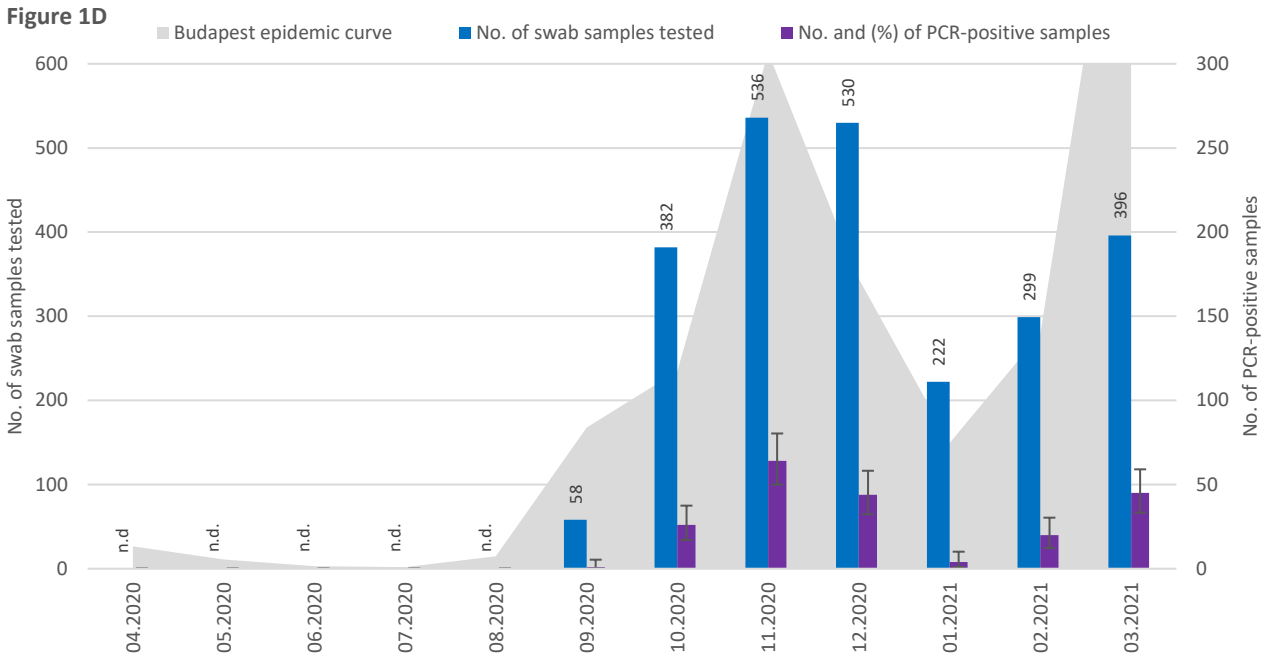

Figure 1. Monthly distribution and results of samples obtained from participants with no known COVID-19 contacts. The participants were investigated for IgG and IgA antibodies by ELISA, for symptoms by analyzing the Questionnaire, and for the presence of SARS-CoV-2 RNA in naso-pharyngeal swab samples by RT-PCR. (A) Monthly distribution of participants with COVID-19 symptoms (B) Monthly distribution of serum samples tested for IgG results. (C) Monthly distribution of serum samples tested for IgA results (D) Monthly distribution of swab samples and PCR-results

The gender distribution of the IgG seroprevalence showed that of the 2353 female participants 296 (12.58%) and of the 1938 male participants 263 (13.57%) were IgG positive, indicating no difference in seroprevalence between genders in the group of participants without COVID-19 contacts.

3.2. Symptoms, seroprevalence and RT-PCR positivity of participants with known COVID-19 contacts

Table 2 shows the summarized data of 182 participants who were tested for IgG antibodies and symptoms in April, and 3 participants in May 2020. Of the 185 participants 80 (43.2%) reported symptoms and 9 persons (4.9%) were IgG positive. From March, Hungary applied physical distancing measures, such as workplace and school closures, wearing face masks, cancellation of public events and stay-at-home requirements, which reduced the transmission of SARS-CoV-2, thus no persons with previous contacts asked for COVID-19 testing at CMC from May to August 2020.

Table 2. Symptoms, seroprevalence and RT-PCR positivity of participants with known COVID-19 contacts

Characteristics	Total No. of tested persons	Time period	No. of persons with positive results	% of positive persons (95% CI)
Symptoms	185 of 185	04.2020–05.2020	80	43.2 (35.99–50.71)
IgG antibodies	185 of 185	04.2020–05.2020	9	4.9 (2.25–9.03)
Symptoms	409 of 409	09.2020–03.2021	233	57.0 (52.01–61.82)
IgG antibodies	386 of 409	09.2020–03.2021	52	13.5 (10.23–17.29
IgA antibodies	320 of 409	09.2020–03.2021	31	9.7 (6.68–13.47)

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Viral RNA	335 of 409	09.2020–03.2021	47	14.0 (10.46–18.16)
Symptoms	594 of 594	04.2020-03.2021	313	52.6 (48.59-56.77)
IgG antibodies	571 of 594	04.2020-03.2021	61	10.6 (8.27-13.51)

However, 409 individuals previously in contact with COVID-19 cases requested COVID-19 testing in the period of September 2020 to March 2021. These participants reported COVID-19 symptoms with a rate of 57.0%. Of the 409 individuals 52 (13.5%) were shown to have SARS-CoV-2-specific IgG antibodies at or above the designated OD level to define a seropositive result. IgA antibodies were detected in 31 (9.7%) of the tested participants. Nasopharyngeal swab samples were tested by RT-PCR; 47 (14.0%) of these samples proved to be positive. During the overall study period of 04.2020 to 03.2021 COVID-19 symptoms were reported by 52.6% of the individuals and 10.6% had detectable level of IgG antibodies.

As shown in Figure 2, the number and percentage of participants with symptoms was 44.0% in April 2020 and varied within a range of 45.2%–68.0% from September 2020 to March 2021 (Figure 2A). The monthly distribution of the IgG positive sera showed 4.9% positive samples in April 2020, the rate of seroprevalence continuously increased from September 2020 to March 2021 from 0% to 55.2% (Figure 2B). The monthly distribution of IgA positive serum samples showed low rates (or low number of tested persons) in September 2020–January 2021, and higher rates of positivity in February and March 2021 (26.0% and 19.2%) (Figure 2C). Monthly distribution of the PCR-positive samples varied between 16.7%–5.6% from September 2020 to January 2021, the highest rates of 28.0% and 25.6% PCR positivity were detected in February and March 2021 (Figure 2D).

Figure 2A

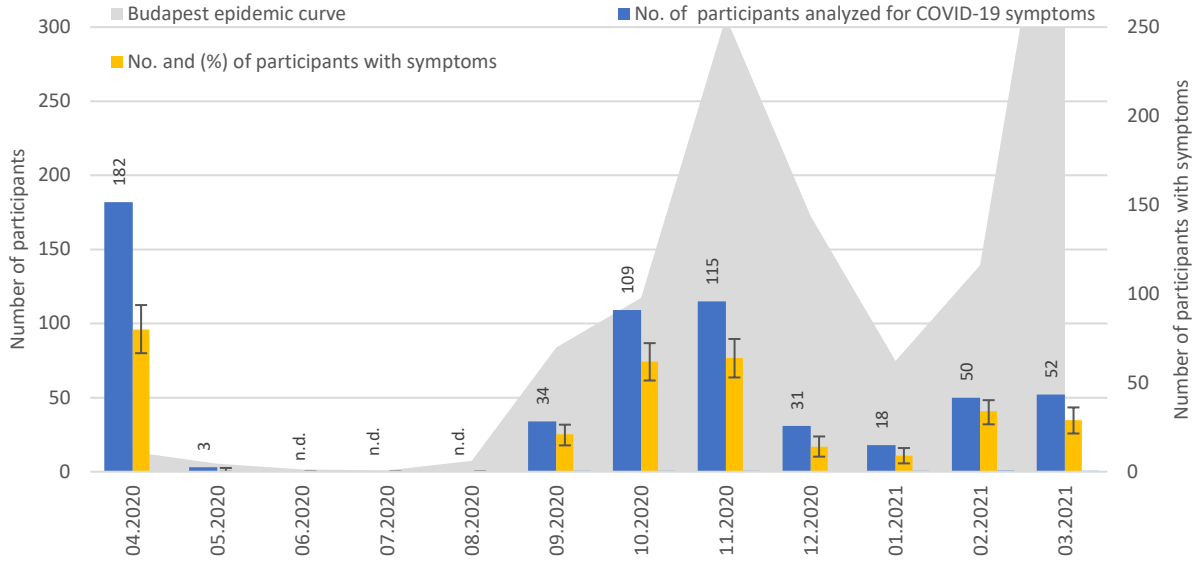

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Figure 2B

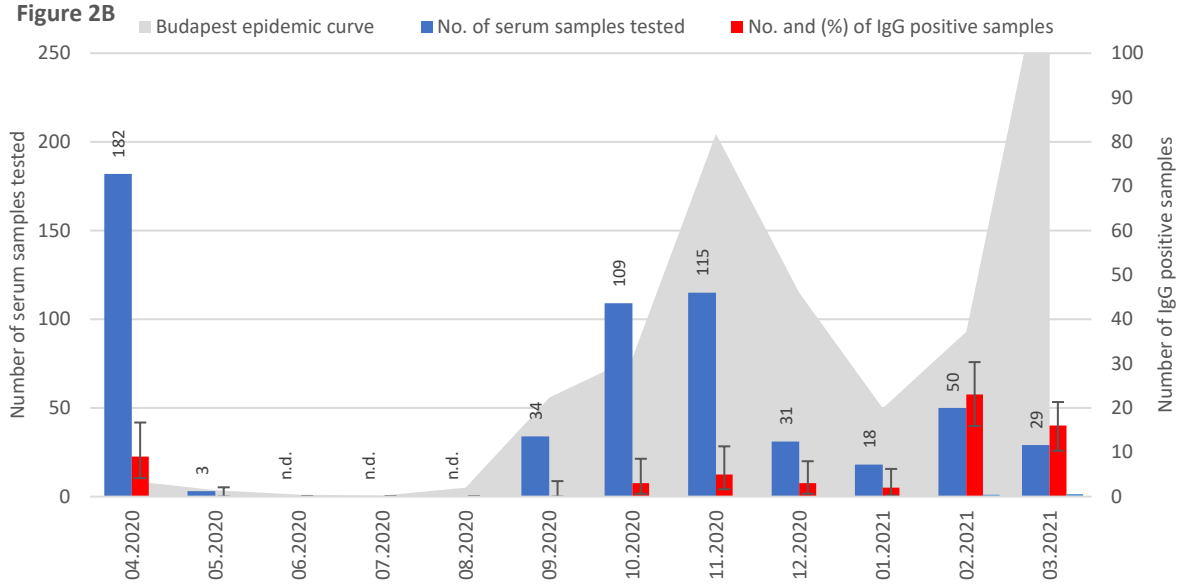
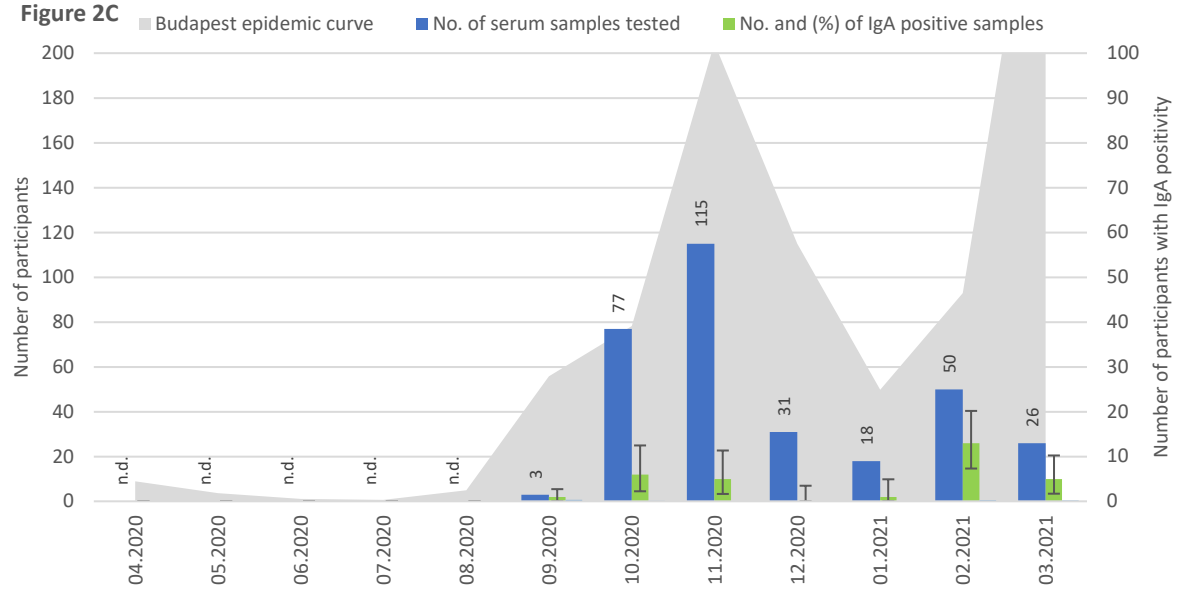


Figure 2C



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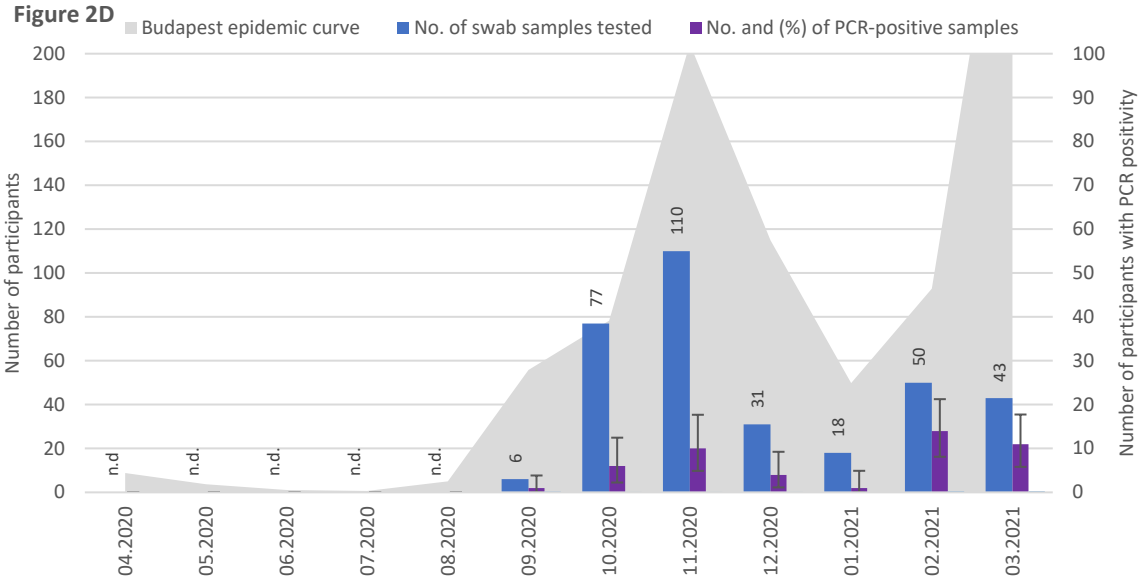

Figure 2. Monthly distribution and results of samples obtained from participants with known COVID-19 contacts. The participants were investigated for IgG and IgA antibodies by ELISA, for symptoms by analyzing the Questionnaire, and for the presence of SARS-CoV-2 RNA in naso-pharyngeal swab samples by RT-PCR. **(A)** Monthly distribution of participants with COVID-19 symptoms **(B)** Monthly distribution of serum samples tested for IgG results. **(C)** Monthly distribution of serum samples tested for IgA results **(D)** Monthly distribution of swab samples and PCR-results.

Similarly to participants without known COVID-19 contacts, there were no gender differences between male and female participants for the rate of IgG, IgA and PCR positivity (not shown).

The age distribution of IgG seroprevalence of participants without COVID-19 contacts and of the participants with known COVID-19 contacts (Figure 3 A and B) showed that the highest number of participants appeared for testing belonged to the age group of 40-49 years old, followed by the age group of 30–39-year-olds, then the 50–59-year-olds, 20–29-year-olds and 60–96-year-olds, in both groups. The results did not show major differences in the IgG seroprevalence in the age groups of participants without COVID-19 contacts (10.7%–13.7%) or with contacts (7.6%–15.5%). Surprisingly, however, in the groups of participants without contacts the number of persons with symptoms was significantly higher in the group of 20–29-year-old persons than in all the older age groups ($p< 0.05$). As expected, the percentage of participants with symptoms was higher in the group with contacts than in the group without contacts: 61.5% (CI 51.5–70.9) vs. 17.5% (14.7–20.7) (20–29-year-old); 53.2% (CI 45.1–61.3) vs. 13.5% (CI 11.3–15.8), (30–39-year-old); 50.0% (42.4–57.6) vs. 11.8% (CI 10.1–13.7) (40–49-year-old); 54.8% (CI 43.5–65.7) vs. 7.5% (CI 5.7–9.7) (50–59-year-old); 60.5% (43.4–76.0) vs. 6.3% (4.3–8.9) (60–96-year-old). The p-value was <0.001 in all age groups. (Figure 3A and B).

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Figure 3A

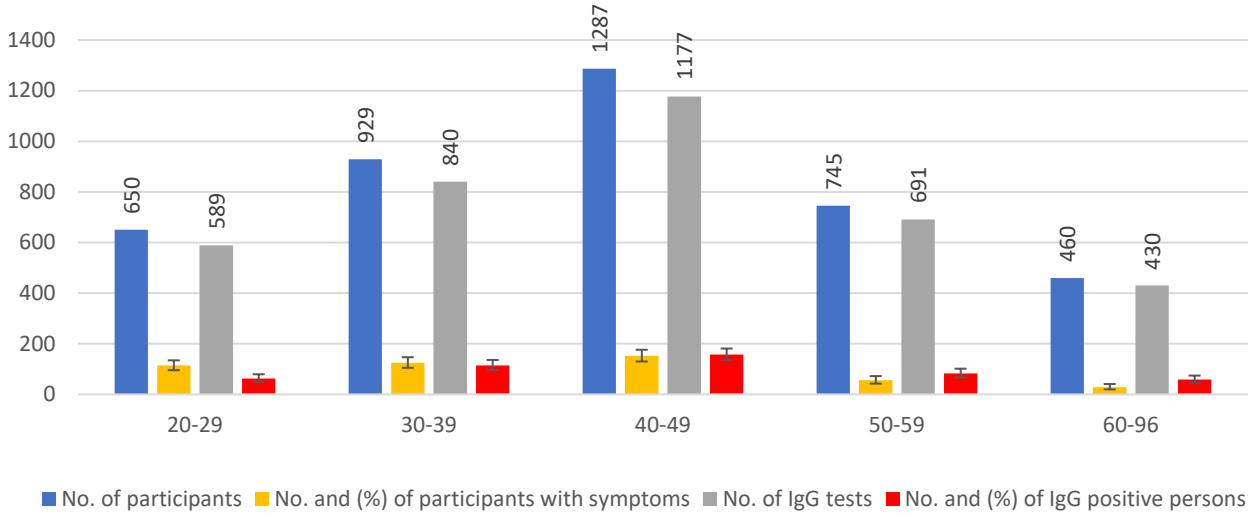

Figure 3B

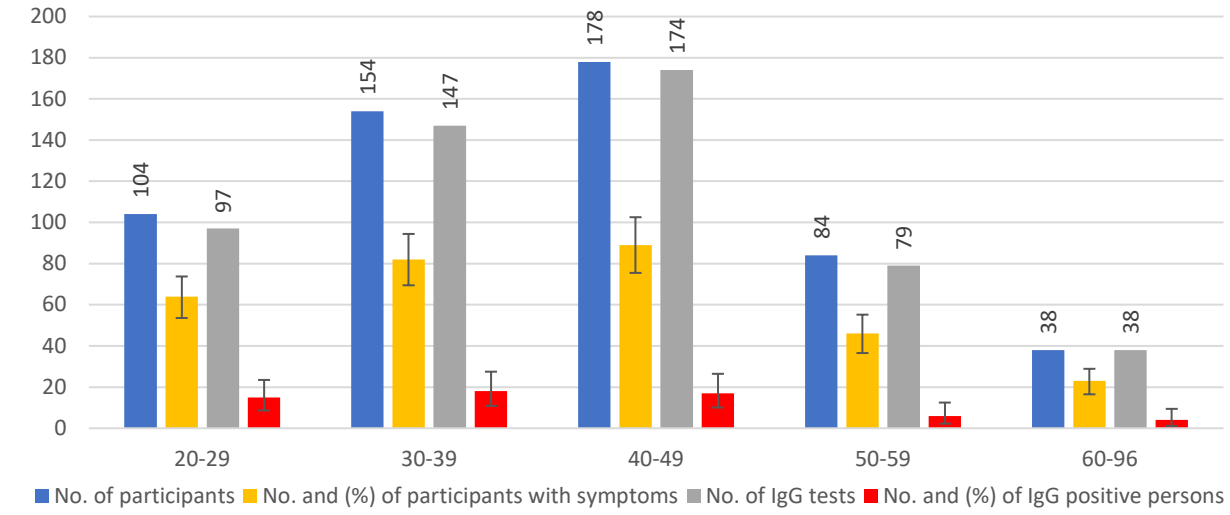

Figure 3. Age distribution of IgG-positive and symptom-positive persons in the group of participants without known COVID-19 contacts (**A**) and in the group with known COVID-19 contacts (**B**)

3.3. The relation of IgG and IgA antibodies in the participants without or with previous COVID-19 contacts

In the group of participants without COVID-19 contacts the relation of IgG to IgA antibodies in serum samples obtained in September 2020 to March 2021 showed a lower rate of IgA than that of the IgG antibody responses (7.35% IgA vs. 16.34% IgG) (Table 1.). Similarly, in the group of participants with known COVID-19 contacts the percentages of IgA (9.7%) were lower than that of the IgG (13.5%) responses (Table 2). The difference between the IgA levels in the participants without or with contacts was not significant (p=0.138). Further, IgA responses were higher at the beginning of the second wave of the

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pandemic in the no contact group of participants, and higher at the beginning of the third wave in the contact group (Figure 1.C. and Figure 2.C.), indicating that at the beginning of the waves more persons were in an acute phase of the infection at the time of blood sampling than at later phases of the waves. However, the low number of IgA positive persons in both groups did not allow a statistical calculation for this observation.

3.4. Coexisting diseases and COVID-19 symptoms in context with PCR positivity in participants without or with previous COVID-19 contacts

Concerning coexisting diseases such as heart disease, diabetes, cancer, asthma and allergy, no significant differences were observed between PCR positive and PCR negative persons in either group of participants (not shown). However, as Table 3 summarizes, the PCR-positive persons demonstrated a significantly higher percentage (1.5%–19.6%) of all the symptoms except shortness of breath, than the PCR-negative persons (0.6%–1.5%), in case of participants without COVID-19 contacts. The order of the frequency of the symptoms is listed in Table 3.

Table 3. Clinical characteristics of 2423 PCR-tested participants without COVID-19 contacts

symptoms*	PCR positive participants n=204	%	PCR negative participants n=2219	%	p-value
cough	40	19.6	68	3.1	<0.001
fatigue	33	16.2	98	4.4	<0.001
headache	33	16.2	125	5.6	<0.001
sore throat	27	13.2	80	3.6	<0.001
rhinitis	24	11.8	84	3.8	<0.001
chills	21	10.3	32	1.4	<0.001
myalgia	23	11.3	38	1.7	<0.001
anosmia	15	7.4	19	0.9	<0.001
dysgeusia	15	7.4	19	0.9	<0.001
fever	14	6.9	19	0.9	<0.001
chest pain	7	3.4	22	1.0	0.002
gastrointestinal symptoms	6	2.9	13	0.6	<0.001
shortness of breath	3	1.5	13	0.6	0.135

*the number and % of the participants with the indicated symptoms are shown

Table 4 shows that in the group of participants with known COVID-19 contacts, except for the gastrointestinal manifestations, headache and shortness of breath, the COVID-19 symptoms were present in a significantly higher percentage (10.6%–48.9%) in the PCR positive participants than in the PCR negative ones (3.1%–35.1%). The myalgia was present in the highest percentage of PCR-positive participants, (55.3%), while headache was the most frequent symptom in the PCR-negative participants (35.1%).

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Table 4. Clinical characteristics of PCR-tested participants with COVID-19 contacts

symptoms*	PCR positive participants n=47	%	PCR negative participants n=288	%	p-value
sore throat	22	46.8	59	20.5	<0.001
cough	20	42.6	41	14.2	<0.001
fatigue	23	48.9	57	19.8	<0.001
rhinitis	20	42.6	57	19.8	0.001
headache	20	42.6	101	35.1	0.322
myalgia	26	55.3	22	7.6	<0.001
anosmia	11	23.4	9	3.1	<0.001
dysgeusia	11	23.4	9	3.1	<0.001
chills	12	25.5	20	6.9	<0.001
fever	10	21.3	9	3.1	<0.001
chest pain	5	10.6	8	2.8	0.010
shortness of breath	5	10.6	15	5.2	0.145
gastrointestinal symptoms	0	0.0	19	6.6	0.070

*the number and % of the participants with the indicated symptoms are shown

3.5. Direct comparison of PCR-positivity and symptoms in participants without or with previous COVID-19 contacts

Table 5 presents that 53 of 204 (26%) PCR-positive participants in the group of persons without contacts exhibited mild COVID-19 symptoms, while 151 (74.0 %) PCR-positive persons were symptomless. In the group of participants with previous COVID-19 contacts symptoms were detected in 36 of the 47 PCR-positive participants (76.6%), and 23.4% of the persons in this group remained symptomless SARS-CoV-2 carriers at the time of testing. These results indicate significantly higher percentage of participants with symptoms in persons with contacts then in the group with no contacts.

Table 5. Direct comparison of PCR positivity with COVID-19 symptoms in groups of participants without or with COVID-19 contacts from 09.2020 to 03.2021

Groups	No. of PCR positive persons	No. and (%) PCR-positive persons with symptoms	No. and % of PCR-positive persons with no symptoms
Participants without COVID-19 contact	204	53 (26.0)*+	151 (74.0)+
Participants with COVID-19 contact	47	36 (76.6)*×	11 (23.4)×

*=p<0.001, +=p<0.001 , ×=p<0.001

The PCR-positive persons were divided according to the presence or absence of symptoms as recorded in the Questionnaire.

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3.6. Repeated sampling and testing of 33 selected participants: IgG-positivity, PCR-positivity and symptoms.

Participants who provided samples 2-3 times after the first visit were selected. The results are summarized in Tables 6 and the details are shown in Table S1.

Table 6. Results of samples obtained from participants sampled and tested 3-4 times.

Participants	PCR-positive	IgG-positive	IgG-negative
10 with symptoms	4 (1 sample is positive of 3-4 samples)	2	2
	6 (more than 1 samples are positive of 3-4 samples)	2	4
23 without symptoms	8 (1 sample is positive of 3-4 samples)	3	5
	12 (more than 1 samples are positive of 3-4 samples)	1	11
	3 (intermitting positive and negative samples)	1	2

Samples were obtained repeatedly from participants and tested by PCR and ELISA. A significantly lower (p=0.004) rate of IgG positivity in participants without symptoms (5/23) than in participants with symptoms (4/10)

Tables 6 and S1 present that of the participants who were PCR-positive at least once or more than 1 time of the 4 sampling and testing, 10 (30.3%) reported symptoms (participants identification numbers 1, 2, 9, 10, 13, 16, 19, 27, 29 and 33), while 23 (69.7%) participants were symptomless. Of the participants without symptoms intermitting viral RNA shedding was detected in 3 participants (participants identification numbers 7, 18, 23), i.e. PCR-positivity was followed by a PCR-negative result, that is followed by a PCR-positive result by tests carried out by using swab samples obtained from the same person at different times during the investigation period. The duration of shedding was detected as at least 30 days (participant identification number 13). There were 9 IgG responder persons (27.3%), while 24 persons remained IgG-nonresponder (72.7%), including 6 persons with symptoms (participants identification numbers 9, 10, 13, 16, 19, 28).

4. Discussion

Epidemiological, statistical and mathematical analyses summarized the first one and a half years of the pandemic in Hungary. The first wave of the pandemic, concentrating in Budapest and Pest-county, started in March 2020. A flat curve of the COVID-19 cases was seen, ending in July. The middle of July is considered to be the beginning of the second wave, characterized first by a slowly, then a rapidly increasing number of cases until 19 December 2020, then slowing down until the middle of February 2021. It is considered that the third wave started on 17 February 2021 [16–18]. The Budapest epidemic curve showed an earlier peak of the second wave in the middle of November 2020 [19].

In April–July 2020 we tested 983 outpatients without COVID-19 contacts by ELISA (Figure 1B). An interesting finding was that even in the first month after the official beginning of the pandemic in Hungary, i.e. in April 2020, a surprisingly high rate of IgG seroconversion (3.3%) was seen. Similarly, of the 182 sera collected in April from participants with known contacts 4.9% were IgG positive (Figure 2B). These results indicate that the spread of the virus started earlier in Hungary than the presence of the virus was officially recognized in university students with typical COVID-19 symptoms in March 2020 [3]. Interestingly, SARS-CoV-2 RNA was identified in an oropharyngeal swab specimen collected from a child with suspected measles in early December 2019, 3

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months before the first identified COVID-19 case in Italy [20]. In addition, the presence of the SARS-CoV-2 RNA was demonstrated in the untreated wastewater of Milan, Italy, as early as mid-December 2019, suggesting the beginning of the outbreak in Europe as late autumn 2019 [21]. In our study, in case of the participants without contacts, the IgG curve in the first wave was flat, with the highest rate of IgG positive participants (13.9%) in June. Considering the 2 weeks incubation time of the infection and the 2–3 weeks IgG seroconversion time, the 13.9% IgG positivity in June might reflect the highest rate of active COVID-19 cases in May in Budapest, reported earlier [18]. Participants with COVID-19 contacts appeared at the CMC clinic for testing from September 2020, the highest percentages of IgG-positivity were determined in February to March 2021. IgA antibodies were determined in lower rate than the IgG antibodies, but were present in a higher percentage in the participants with known COVID-19 contacts than in participants without contacts. Also, higher rates of IgA antibodies were observed at the beginning of the second and third waves than at later stages of the waves (Figure 1C and 2C). These results are in correlation with published results reporting 88% IgG and 10% IgA positivity rate in PCR-positive ambulatory patients [15]. A case study identified a connection between the early appearance of IgA antibodies and disease severity [15], confirming the importance of IgA detection in COVID-19 laboratory diagnosis.

In a representative, cross-sectional population survey of Hungarian individuals the number of active infections and prevalence of seroconversion was investigated using swab and serum samples collected in May 2020 [22]. The investigated individuals were selected from the population registry from several regions of the country. In this survey a low active SARS-CoV-2 infection rate (0.029 %) and a low overall seropositivity rate (0.68%) was identified considering the whole country, while higher prevalence of seropositivity (0.9%) was found in Budapest [22]. Since our study population included outpatients with mild COVID-19 symptoms and symptomless individuals visiting the CMC clinic, the 3.3%-1.8% (Figure1B) and 4.9%-0% (Figure 2B) IgG seroprevalence we observed in April-May 2020, may be comparable with the 0.9% seropositivity in Budapest detected by Merkely et al. [22] in individuals selected from the population registry.

Our data showed no significant differences in the IgG seroprevalence between the age groups, neither in the no contact, nor in the contact groups of participants. Elder age has been accepted as risk factor for severe COVID-19 infections. The age-dependent susceptibility to infection may explain the more common severe illnesses in the elderly, or the increase in severity may be the result of the elder age and the existence of comorbidities, which is likely with aging [23]. Our results support the second idea, i.e., young and elderly persons can be infected at a similar rate. No significant differences in susceptibility for younger adults versus older adults were found in earlier studies [23–25]. However, surprisingly, in our study, higher number of individuals were found with symptoms in the age group of 20–29-year-olds than in the elder groups of the no contact participants (Figure 3A). Our interpretation of the age groups does not differentiate between persons tested at the beginning or at the end of the 1-year period we investigated. It was reported that early in the pandemic in the USA COVID-19 incidence was highest among older adults, but in June–August 2020, COVID-19 incidence was highest in persons aged 20 to 29 years, constituting the largest proportion of cases, and indicating a decline of median age of COVID-19 cases [25. A similar age shift in 2020 was reported in Europe [26].

We observed that in the participants without or with COVID-19 contacts who developed a mild disease, the most frequent symptoms were cough, fatigue, headache, sore throat, rhinitis, and similarly to an earlier study [9], fever was only the 10th in the order of frequency of the 13 symptoms we documented. In a study of 656 severe cases of COVID-19 high fever (88.7%) cough (57.6%) and shortness of breath (45.6%) were the most prevalent manifestations [4]. We report a significantly higher frequency of most of

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the 13 symptoms in the PCR-positive groups than in the PCR-negative groups of the investigated participants (Tables 3 and 4).

When analyzing the 33 participants sampled and tested at consecutive occasions (Tables 6 and S1), our serology test determined only 4 seropositive persons of the 10 outpatients with mild disease. Five persons (15.1%) of the 23 symptomless, but PCR positive persons had detectable level of IgG antibodies in 30 days after the first results of PCR positivity. Much higher rate of seropositivity was reported for patients hospitalized for COVID-19; nearly 100% of these individuals exhibited IgG seropositivity [8,27,28]. One of the reasons of the low seropositivity rate in our groups of PCR positive persons might be that the virus replication in persons with mild infection was limited, thus producing less antigenic stimuli for the immune system. These results indicate that there are differences between PCR positive cases as for the magnitude of viral exposure and replication, suggesting the importance of the ct values of the RT-PCR runs of the swab samples. The ct values would indicate the severity of the COVID-19 cases, thus would be informative for the estimation of seroconversion [29]. Our results show a significantly ($p=0.004$) lower rate of IgG positivity in asymptomatic PCR positive outpatients (5/23 persons) than in PCR positive ones experiencing mild symptoms of COVID-19 (4/10 persons). We observed a duration of the virus shedding up to 30 days in one participant with mild COVID-19 symptoms. However, since swab samples were not obtained at later days in our study, the virus shedding might exist longer than we observed. Earlier studies documented a variable duration of viral shedding of 19 days [8] or 5-16 days [30]. It should be noted, however, that PCR-positivity does not necessarily mean active virus infection, but it can be the result of a previous infection with the presence of not infectious viral RNA fragments in the swab sample.

Further, in 3 cases (identification numbers: 7, 18 and 23) of the 33 selected outpatients intermitting PCR positivity was observed (Tables 6 an S1). Similar results were reported earlier by other investigators [31, 32]. False-positive RT-PCR results are highly improbable because repeated RT-PCR assays on the same swab samples revealed consistent results. False-negative RT-PCR results may occur because of inadequate swab type and time since symptom or infection [31,32]. In our study nasopharyngeal swab samples were used, which provide more reliable RT-PCR results than oropharyngeal samples. We observed that after a negative RT-PCR results repeated testing could lead to positive PCR results, and that PCR testing at a single time point can underestimate the number of infected persons with no symptoms.

Monthly distribution of PCR-positive samples and the symptoms showed similar pattern in the groups of participants without or with COVID-19 contacts (Figure 1A and D; Figure 2A and D), indicating a relationship between the detectability of viral RNA and the development of symptoms in the patients. Direct comparison of PCR-positivity and the presence of symptoms showed that depending on the study population, 23.4% (group of persons with known COVID-19 contacts), 74.0% (group of persons without known COVID-19 contacts) and 70.5% (group of consecutively sampled and tested persons) were symptomless at the time of positive PCR results (Table 5 and Table S1). However, since the symptoms were assessed at a single time point, or with a relatively short follow-up period in some cases of the consecutively tested persons, it is not clear how many of these symptomless participants had pre-symptomatic or post-symptomatic infections or were truly persistently asymptomatic. An appropriate follow up to capture pre-symptomatic or post-symptomatic cases should include the maximum duration of the incubation period of 14 days, excluding pre-symptomatic cases, and the median duration of nasopharyngeal swab shedding of 22 days [33] or 30 days (our observation) to exclude post-symptomatic cases [34]. Nevertheless, it is of great public health significance to identify and manage asymptomatic individuals and their close contacts to con-

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trol possible outbreaks. In an earlier study the proportion of patients with asymptomatic infections was 20.8% [8].

Limitations of our study include that the serum and swab samples were not obtained always according to a well-designed plan, but were collected according to the availability and request of the participants. Individual differences in time in serum and swab sampling since symptom onset may influence the RT-PCR and ELISA results in mild COVID-19 cases, which might be true for asymptomatic persons as well. Some imperfections arose from the limited availability of certain laboratory diagnostic methods at the very early phase of the pandemic, i.e., IgA-specific ELISA and RT-PCR were not available at the beginning of the pandemic, and at the beginning of our study but were included in the study later. Also, the number of participants in certain settings was low, thus calculation of percentages might not be accurate, and the results should be confirmed.

5. Conclusions

Our observational study on symptomless or mildly infected persons has begun at the very early phase of the pandemic, providing valuable data for this period. It shows a surprisingly high, 3.3%- 4.9% seroprevalence in April 2020, indicating a fast spread or early appearance of SARS-CoV-2 in Budapest. Our study also shows a 12.9% IgG seroprevalence in the group of participants without COVID-19 contacts in the one-year-period of April 2020 to March 2021 in Budapest, comparing with the 7% registered COVID-19 cases in Budapest [19,35] during the same time. Our results suggest no significant age- and gender-related differences between participants for IgG positivity but indicate that COVID-19 symptoms were the most frequent in persons aged between 20 and 29 years old. We identified 23.4%–74.0% PCR-positive persons, depending on the study population, who were symptomless SARS-CoV-2 carriers at the time of the investigation, and a duration of viral shedding up to 30 days in one person. We also recognized that 72.7% of the PCR-positive persons remained seronegative within 30 days or for a longer period of time after the first PCR-positive results, indicating the need for vaccination after an asymptomatic or mild SARS-CoV-2 infection.

More work needs to be done to estimate the rate of the asymptomatic and mild infections with SARS-CoV-2 in various clinical settings and in the general population. It is of great importance to understand the significance of these infections in the long persistence and repeated outbreaks of the pandemic, as well as its possible role in achieving a herd immunity of the population. The goal is challenging for many reasons, including the emerging mutated variants of the virus.

Supplementary Materials: Table S1: Results of samples obtained at the first visit of the participants at the CMC Clinic and days (in brackets) after the first visit.

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Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. You might choose to exclude this statement if the study did not report any data.

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