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Seroprevalence of SARS-CoV-2 antibodies in the county town of Slovakia – a pilot study from the Trencin city

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Summary. – Slovakia is a country with only 5.45 million inhabitants. However, the past two years of the COVID-19 pandemic have shown huge inter-regional differences. These were represented by different numbers of diagnosed SARS-CoV-2 cases and the vaccination rates in the regions, as well as by the willingness of the inhabitants to comply with anti-pandemic measures or to undergo testing. The occurrence of such regional disparities provided a rational basis for monitoring the epidemic situation within smaller areas, e.g. at city level. Trencin is a medium-sized Slovak county town with about 55 000 inhabitants. The city administration gave its residents the opportunity to assess their current level of antibodies against the SARS-CoV-2 virus, and received an additional benefit in the form of data on the real epidemic situation in the city, which helped in further management of anti-pandemic measures. The primary aim of the study, conducted in January and February 2022, was to determine the levels of antibodies against the SARS-CoV-2 virus in the inhabitants of Trencin. The results showed that 75% of the study participants, representing the adult population of the city, had detectable IgG antibodies against the SARS-CoV-2 spike protein. Noteworthy, at the time of the study, 13% of the Trencin city population who were unaware of overcoming COVID-19 had specific antibodies against the virus. Furthermore, the antibody levels in recovered unvaccinated subjects increased not only with the severity of their COVID-19 symptoms, but also after multiple recoveries from the disease. On the other hand, the severity of side effects after vaccination did not influence the antibody levels. The results of the study are in line with the current view that hybrid immunity (vaccination plus SARS-CoV-2 infection in any order) offers greater protection than immunity elicited by vaccination or COVID-19 separately.

Keywords: SARS-CoV-2 coronavirus; COVID-19; ELISA; seroprevalence; antibodies; vaccination

Introduction

Slovakia has recorded the first case of COVID-19 on March 6th and the first death on April 1st, 2020 (https://www.worldometers.info/coronavirus/country/slovakia/). Thanks to rapid epidemiological measures such as the introduction of mandatory mask wearing on public places, social distancing, ban on assembly in public places and events, recommendation of home office, introduction of quarantine for people coming from abroad and several others, the country has passed first pandemic wave relatively successfully. A dramatic change has occurred in early autumn as the second wave of the pandemic hit the country. In addition to epidemiological measures applied in the first wave, the government ordered nationwide antigen testing, but even these measures did not prevent rapid spread of the virus. The second wave began in October 2020 and lasted until the end of April 2021. Within

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Abbreviations: BMI = body mass index; COVID-19 = Coronavirus Disease 2019; C-19 = COVID-19; IgG = immunoglobulin G; RT-PCR = reverse transcription PCR; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; SAS = Slovak Academy of Sciences; S/C = sample-to-calibrator ratio (unit)

this period, precisely on December 27th, the country launched its vaccination program against COVID-19. The third pandemic wave started in late September and early October 2021 and ended in mid-January 2022. The subsequent fourth wave hit the country from the second half of January 2022, as a consequence of the Omicron variant occurrence and its decline was recorded in late April 2022 (https://korona.gov.sk/). While in the initial phase of the pandemic, Slovakia was one of the most successful countries in the world in terms of mortality per 100 000 inhabitants, in the following waves this situation changed dramatically (total 19 839 deaths, or 3648 deaths per million people at the end of April 2022). Undoubtedly, one reason for this unfavorable situation was the relatively low vaccination rate of the country population against the SARS-CoV-2 virus. At the end of April 2022, the share of fully vaccinated people was only 50.3%, which is one of the lowest shares among the EU countries. In addition, there are significant regional differences in vaccination coverage within the country, being highest in urban areas and lower in rural areas.

The severity of the COVID-19 disease and the associated hospital admissions are related to the level of antibodies in the susceptible population (Garcia-Beltran *et al.*, 2021; Ou *et al.*, 2021). This level mainly depends on exposure to either SARS-CoV-2 virus, vaccination, or a combination of both. Many European countries are monitoring the antibody levels in nationwide seroprevalence studies (Gornyk *et al.*, 2021; Ward *et al.*, 2021), thus trying to predict the current resilience of the population to pandemic waves due to the emergence of new SARS-CoV-2 variants. In Slovakia, larger seroprevalence studies are absent or have been performed only on a limited number of participants or specific areas (Kajanova *et al.*, 2021). It has been also shown that there is a need for close cooperation between local authorities and state or public institutions in effectively combating the consequences of the COVID-19 pandemic. A good example in Slovakia is the county town Trencin, which already carried out comprehensive antigen/PCR testing of the town's population for the presence of the SARS-CoV-2 virus during the second wave of the pandemic in order to control the spread of the virus at the local level. The consistency of the city's anti-pandemic activities lies also in the determining antibody levels in the city of Trencin. Results of this study may provide useful information about the epidemiological situation in the city and motivates hesitant citizens to undergo vaccination against the disease. These activities are in line with the efforts of other European cities to combat the pandemic (Zinszer *et al.*, 2021; Richard *et al.*, 2022).

The current study was undertaken to assess the levels of antibodies against the SARS-CoV-2 virus in willing inhabitants of the city of Trencin according to their vaccination and/or COVID overcoming status. Furthermore, the aim of the study was also to evaluate the effect of self-reported side effects after vaccination and severity of symptoms during COVID-19 on the antibody level, as well as to examine the antibody levels in relation to the number of vaccine doses administered. The second aim was to validate the methodological approach of dry blood spot sampling on a diverse range of probands in cooperation with the local authorities.

Materials and Methods

Study participants. All study participants had to meet the following inclusion criteria: permanent residence in the city of Trencin; age 18 and over; consent to participate in the study (signature of informed consent). Participation in the study was exclusively on a voluntary basis. The city authorities informed about the study via local media. Willing inhabitants filled out an online form containing informed consent and anamnestic questionnaire. Subsequently, the volunteers picked up the sampling kits at one of the city's designated contact points. They collected their capillary blood at home according to a detailed schematic instruction manual or tutorial video. Collection cards with dry blood samples were returned within 2–4 days following sampling to contact points created by the city and subsequently delivered by city employees to the laboratory at the Biomedical Research Center of the SAS and analyzed as described below. The sampling was performed in two phases. The first phase involving non-vaccinated persons was accomplished within the last two weeks of January 2022 (20.1.–3.2.). The second phase was performed within the last three weeks of February 2022 (11.2.–2.3.) and included all other voluntary participants irrespective of whether they were vaccinated, positively tested for SARS-CoV-2 or considered themselves naïve persons with respect to SARS-CoV-2 infection.

Collection of data and ethical approvals. The design of the study was approved by an independent Ethics Committee of the Trencin self-governing region on January 13, 2022 (TSK/2022/00). All participants received information about the purpose, design, and interpretation of results, and provided informed consent as well as anamnestic information including date of birth, gender, height and weight, vaccine type, dates of vaccination, post-vaccination side effects and/or date of onset and severity of COVID-19 disease. Severity of the disease was classified as asymptomatic, mild, moderate, and severe by probands themselves according to the description of symptoms as defined in the COVID-19 Treatment Guidelines Panel, NIH. Severity of the post-vaccination side effects was classified as none, minimal, mild, moderate and severe by probands themselves according to the description of the symptoms. All personal data provided by the probands were handled in

compliance with the Personal Data Protection Act and other generally binding legal regulations.

Sample collection and preparation for ELISA. Dry blood spot samples were obtained by participants performed self-collection of capillary blood by lancet pricking of fingertip using the in-house collection set assembled at the Biomedical Research Center of the SAS. Blood drops were blotted onto the collection card and left to dry for 3–4 h on air. Defined area of the card was punched out and submerged into the sample buffer of the ELISA and incubated for 1 h at 37°C. The extracted blood sample was then used for the serological analysis using ELISA as described below. The use of dry capillary blood spot samples was validated by parallel testing of venous blood samples of the selected individuals previously (data not shown). This highly effective, safe and low-cost method was applied in this seroprevalence study. The study was conducted on the voluntary basis and in a close cooperation with the city authorities and with the assistance of regional public health authority.

Serological analysis. Seroprevalence of antibodies to SARS-CoV-2 virus was evaluated using an anti-SARS-CoV-2 IgG ELISA (EUROIMMUN Medizinische Labordiagnostika AG, Germany), detecting IgG antibodies specifically binding to the SARS-CoV-2-encoded spike protein subunit 1 (S1) containing the immunologically relevant receptor binding domain (RBD), which comprises the vast majority of antibodies capable of neutralizing the virus. This type of antibodies is produced both after contact with the virus and after vaccination. The specificity of the anti-SARS-CoV-2 IgG ELISA of 99.6% and sensitivity in samples collected after day 10 post-symptoms of 94.4% was determined by the manufacturer. The assay was performed according to the manufacturer's protocol and recommendations, a sample-to-calibrator ratio (unit S/C) of < 0.8 was considered negative, ≥ 0.8 to < 1.1 borderline, and ≥ 1.1 positive. The numerical values for a positive test range were from 1.1 to 11. Participants were informed of their antibody level with a comment that the test only provides partial information about immunity status and cannot predict individual risk of subsequent infection. Each participant had the opportunity to consult the test results with health care professional by telephone.

Statistics. Data were categorized according to selected characteristics and subjected to the statistical analysis. Statistical analyses of the obtained data were performed using the IBM SPSS Statistics version 19 (SPSS Inc., USA). Categorical variables were expressed as number (%), continuous variables as mean (± standard deviation, SD), or median (interquartile range, IQR) depending on the normality of their distribution. Pairwise comparisons were performed by the Student's *t*-test or Mann-Whitney U test, as appropriate. Group differences were analyzed by one-way analysis of variance (ANOVA) or ANOVA on ranks, depending on the normality of the data distributions with the appropriate post hoc tests for pairwise multiple comparison procedures. The prevalence of different variables was compared using the χ^2-test. A p-value of < 0.05 was considered statistically significant.

Results

Basic characteristics of the study cohort

From a total of 3300 prepared sampling kits, 3137 were actually picked up and 3013 blood samples were returned for ELISA testing what represents 96.05% return rate.

Of the 3013 participants, who returned the blood sample, 1291 were unvaccinated (42.85%) and 1722 were vaccinated (57.15%). The median age was 49 years, with the following distribution in individual age categories: 888 people (29.47%) in the age group of 18–39 years, 1235 people (40.99%) in the category of 40–59 years and 890 (29.54%) in the category of 60 and over. A total of 1215 men (40.33%) and 1798 women (59.67%) participated in the study. There was no considerable difference between females and males in age, vaccination status, COVID-19 experience and seropositivity. On the other hand, women showed significantly ($p < 0.001$) lower BMI than men. The detailed cohort characteristics are summarized in Table 1a.

Out of a total of 3013 samples, 2244 (74.48%) were positive for SARS-CoV-2 spike protein IgG antibodies. The presence of antibodies was detected in 538 (41.67%) of 1291 unvaccinated participants (median level of antibodies in the unvaccinated seropositive individuals: S/C 3.68) and in 1706 (99.07%) of 1722 vaccinated volunteers (median level of antibodies in the vaccinated seropositive individuals: S/C 8.28), X^2 (1, N = 3013) = 10.7271, p = 0.001. The characteristics of the subjects based on their seropositivity are summarized in Table 1b. Although the seronegative vaccinated subjects were slightly older than their seropositive vaccinated counterparts, the difference did not reach statistical significance.

Antibody levels of vaccinated and non-vaccinated participants

In the group of vaccinated volunteers, there was a significant difference between individuals who did not overcome COVID-19 (median S/C 8.15) and those who (before or after vaccination) recovered from COVID-19 (median S/C 8.46), $p < 0.001$. This difference is present in all groups, regardless of vaccine type/manufacturer (Fig. 1). We did observe slight difference in antibody levels between participants who recovered from COVID-19 before vaccination (median S/C 8.36) and those who overcame the disease after vaccination (median S/C 8.55) ($p < 0.05$). Individuals, who did not overcome COVID-19 and were vaccinated with Comirnaty, Spikevax or Vaxzevria

vaccines, had comparable antibody levels, whereas those vaccinated either with Janssen or SputnikV vaccines had significantly lower antibody levels than those in the three aforementioned groups ($p < 0.001$). There was no significant difference in the antibody levels among all vaccine types in individuals, who recovered from COVID-19. We observed major variations in antibody levels within the Janssen and SputnikV vaccines, however, these results may be skewed by the low number of subjects, absence of the 3rd dose or receiving of the 3rd dose from a different manufacturer (Fig. 1).

Among the unvaccinated participants who have been tested positive for SARS-CoV-2, we observed already proven differences in antibody levels depending on the severity of the disease (moderate and severe vs. asymptomatic and mild, $p < 0.001$). Median levels range from S/C 2.48 in the asymptomatic group to S/C 6.77 in the group with severe symptoms. In this study, we were also able to compare antibody levels between individuals who overcame COVID-19 only once (median S/C 2.99) and multiple times (median S/C 7.16). It is evident that after repeated recovering from the disease, the level of antibodies rises significantly ($p < 0.001$). The results for the individual groups of participants are shown in Fig.1.

Side effects of vaccines in relationship to antibody levels

Vaccinated participants were asked to describe side effects after receiving the vaccine. They could choose one of the six options offered. We wondered if there was any connection between the intensity of the symptoms after the vaccine administration and the level of subsequently produced antibodies. Median antibody levels ranged from S/C 7.82 ("don't remember" group) to 8.36 ("severe" group). There was no statistical difference in measured antibodies among the groups created according to self-reported severity of vaccine side effects. However, the groups of people who do not remember or felt severe side effects were statistically very small (Table 2).

From the results of the questionnaire shown in Table 2, it is clear that among the three most frequently administered vaccines in Slovakia, people experienced

Table 1a. Basic characteristics of the study cohort as reported by the participants in the questionnaire

Cohort characteristics	Participants (n)	%	Females (n)	%	Males (n)	%
Total	3013	100	1798	59.67	1215	40.33
Vaccinated	1722	57.15	1022	59.35	700	40.65
Vaccinated only	1100	36.51	654	59.45	446	40.55
Vaccinated + COVID-19	622	20.64	368	59.16	254	40.84
Non-vaccinated	1291	42.85	776	60.11	515	39.89
COVID-19 only	543	18.02	334	61.51	209	38.49
No vaccine, no COVID-19	748	24.83	442	59.09	306	40.91
Age (years), median (IQR)	49 (25)	-	49 (24)	-	48 (25)	-
Age group 18–39	888	29.47	510	57.43	378	42.57
Age group 40–59	1235	40.99	752	60.89	483	39.11
Age group >60	890	29.54	536	60.22	354	39.78
BMI (kg/m^2), median (IQR)	25.7 (6.4)	-	24.4 (6.5)	-	27.1 (5.1)	-

Table 1b. Basic characteristics of the study cohort based on the seropositivity (the data are expressed as median (IQR))

	Seropositive			Seronegative		
	Total	Vaccinated	Non-vaccinated	Total	Vaccinated	Non-vaccinated
n	2244 (74.48%)	1706 (99.07%)	538 (41.67%)	769 (25.52%)	16 (0.93%)	753 (58.33%)
Gender F/M	1341/903	1013/693	328/210	457/312	9/7	448/305
Age (years)	49 (25)	49 (24)	48 (22)	48 (26)	53 (18)	47 (26)
BMI (kg/m^2)	25.7 (6.3)	25.7 (6.3)	25.9 (6.2)	25.4 (6.6)	25.8 (5.3)	25.4 (6.6)
S/C	8.0 (3.1)	8.3 (1.2)	3.7 (3.7)	0.4 (0.2)	0.7 (0.3)	0.4 (0.2)

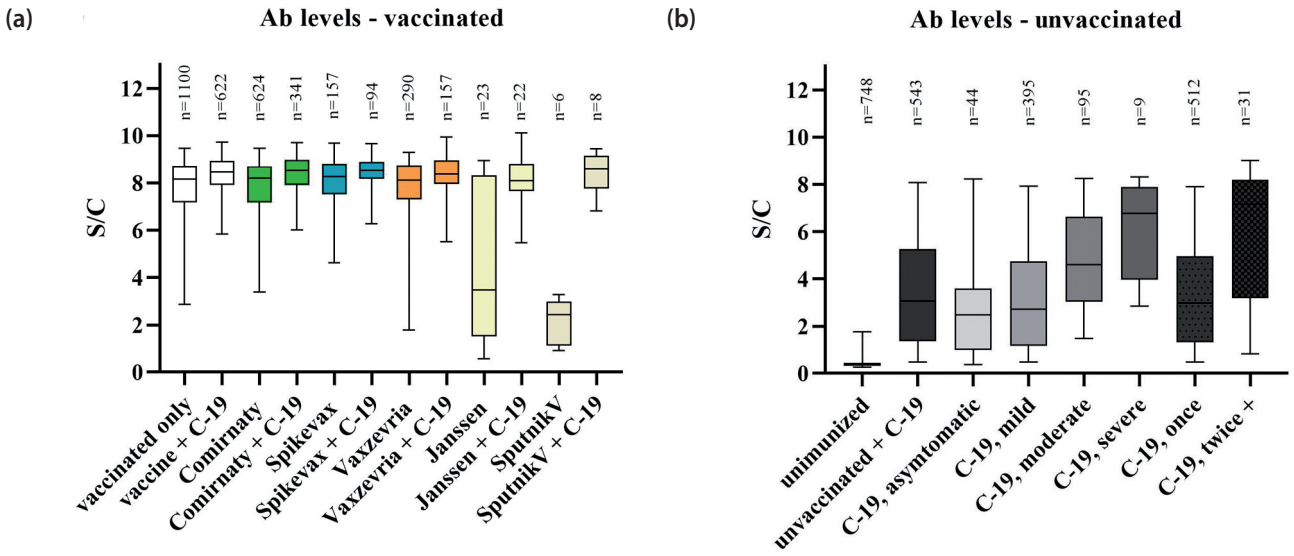

Fig. 1

Relative SARS-CoV-2 S-protein IgG antibody levels in participants who reported vaccination and/or COVID-19 (C-19)
(a) Participants were stratified according to the vaccine type (the first dose of vaccine given to an individual) and self-reported severity of COVID-19 symptoms. **(b)** The "unimmunized" group includes participants who reported neither vaccination nor a positive test for COVID-19. The data are expressed as a ratio of signal to calibrator (S/C). Median is represented by a horizontal line within boxes. The area of boxes represents 25.–75. percentile and whiskers 5.–95. percentile of the measured values in the group. The number of samples in each group is shown at the top of the graphs as „n".

negligible (up to 76.7% for "none" + "minimal") side effects after administration of the Comirnaty vaccine. On the contrary, according to the participants' selfassessment, major adverse reactions ("mild" + "moderate" + "severe") occurred after Spikevax and Vaxzevria vaccines without statistical difference between those two – X^2 (1, N = 1206) = 46.237, p <0.001 for Comirnaty vs. Spikevax, X^2 (1, N = 1404) = 47.327, p <0.001 for Comirnaty vs. Vaxzevria.

Differences in antibody levels according to number of vaccine doses administered

In connection with the application of booster doses of the COVID-19 vaccine in recent months, we compared groups of vaccinated individuals with/without the booster dose, who considered themselves as COVID-19 naïve. For this purpose, we divided the 1100 COVID-19 naïve vaccinated participants in three groups: 1 dose – subjects, who received one dose of Janssen vaccine or only one dose of any 2-dose vaccine and decided not to continue the vaccination process; 2 doses – subjects, who received two doses (completed the baseline vaccination schema) of any 2-dose vaccine or received one dose of Janssen and additional booster of any other vaccine; 3 doses – subjects, who received two doses of any 2-dose vaccine and an additional booster. For all vaccine types analyzed, we observed significantly higher antibody levels in individuals, who did not report overcoming COVID-19 in the group received the 3rd dose compared to

Table 2. Vaccination side effects (self-reported)

Side effects (self-reported)	All vaccines (n)	%	Comirnaty (n)	%	Spikevax (n)	%	Vaxzevria (n)	%	Other (n)	%
Don't remember	7	0.41	6	0.62	1	0.40	0	0.00	0	0.00
None	326	18.93	201	20.87	24	9.60	80	17.90	21	33.87
Minimal	856	49.71	538	55.87	114	45.60	185	41.39	19	30.65
Mild	449	26.07	185	19.21	93	37.20	153	34.23	18	29.03
Moderate	75	4.36	28	2.91	16	6.40	27	6.04	4	6.45
Severe	9	0.52	5	0.52	2	0.80	2	0.45	0	0.00

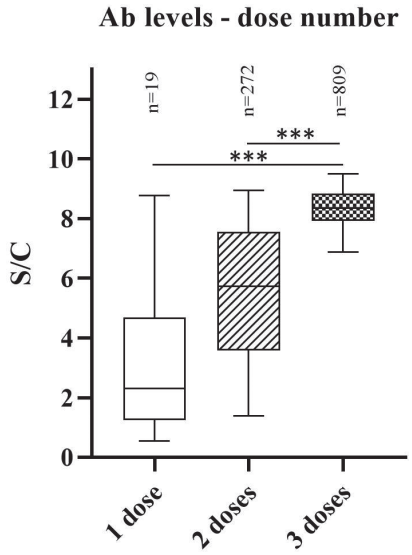

Fig. 2

Relative SARS-CoV-2 S-protein IgG antibody levels in vaccinated participants (never tested positively for COVID-19) according to the number of vaccine doses

The data are expressed as a ratio of signal to calibrator (S/C). Median is represented by a horizontal line within boxes. The area of boxes represents 25.–75. percentile and whiskers 5.–95. percentile of the measured values in the group. The number of samples in each group is shown at the top of the graphs as „n". ***p <0.001 vs. 3 doses.

those received only 2 doses (5.6 (4.1) vs. 8.4 (0.9), p <0.001) (Fig. 2). However, it should be noted that the level of antibodies decreased over time and the average time elapsed after the second dose was 213 days, compared to 68 days after the third dose at the time of testing. Only mRNA vaccines were administered as a booster dose in Slovakia, and this combination of vaccines in the Vaxzevria group could have contributed to a considerable increase of antibody levels in this group (2 doses: 2.5 (3.8) vs. 3 doses: 8.2 (1.1), p <0.001).

At the time of the study, 62.7% of vaccinated had decided to administer the 3rd dose. This percentage is comparable to the overall percentage of people who received a booster dose in Slovakia – 59.29% (data from March 2022 korona.gov.sk).

Change in antibody levels over time

It is already known, that the levels of antibodies obtained after recovery from the disease as well as in response to vaccination against the SARS-CoV-2 virus gradually decrease over time. This also reduces the protection against infection. Fig. 3 shows antibody levels at multiple time intervals since the last dose of vaccine (vaccinated who were never tested positive for SARS-CoV-2) or since the last positive SARS-CoV-2 test (in those who were not

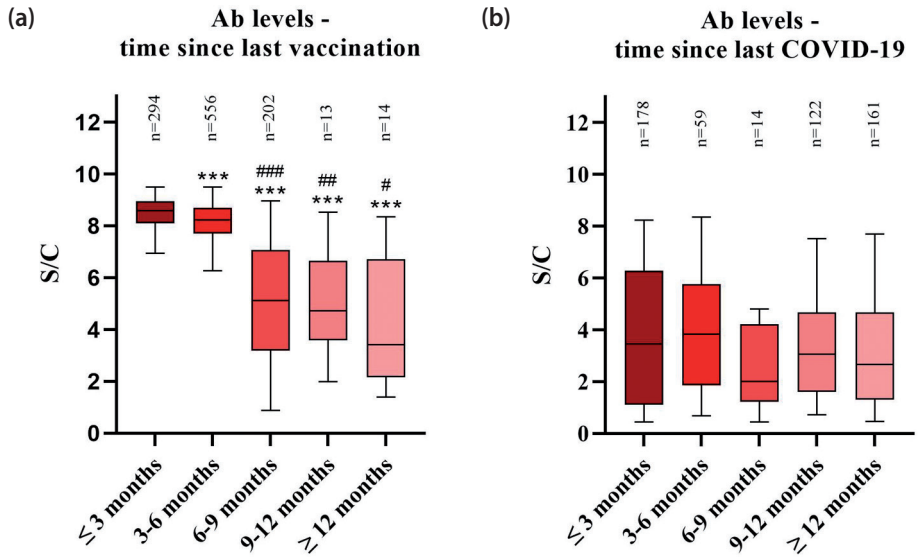

Fig. 3

Relative SARS-CoV-2 S-protein IgG antibody levels in time

Participants stratified according to the time elapsed **(a)** from the day of their last vaccine dose given (vaccinated, who did not overcome COVID-19) or **(b)** to the time elapsed from their last positive RT-PCR or Ag test (unvaccinated only). Only data from participants who filled in all the dates in the correct form in the questionnaire are included. The data are expressed as a ratio of signal to calibrator (S/C). Median is represented by a horizontal line within boxes. The area of boxes represents 25.–75. percentile and whiskers 5.–95. percentile of the measured values in the group. The number of samples in each group is shown at the top of the graphs as „n". ***p <0.001 vs. ≤ 3 months; ### p <0.001, ## p <0.01, # p <0.05 vs. 3–6 months.

vaccinated). The highest antibody levels were observed in subjects vaccinated with their last dose within 6 months prior the study (Fig. 3). The median level of antibodies in COVID-19 naïve subjects vaccinated within 6 months prior the study reaches more than twice the value in the group of unvaccinated subjects, who recovered from COVID-19 during the last 6 months (8.3 (0.9) vs. 3.5 (4.9), p <0.001). The dynamics of the decline over time is not clearly visible after infection, there are no significant differences between the groups in respect to the time interval since the last positive test (p = 0.067), although overall antibody levels are lower compared to vaccinated group (Fig. 3). This phenomenon is probably caused by the existence of different variants of the SARS-CoV-2 virus in the observed time frame. It is possible that different variants of the virus have different immunogenic potential and induce the production of antibodies with different degrees of stability (Servellita *et al.*, 2022).

Probability of SARS-CoV-2 infection in different study groups

As there are obvious differences in antibody levels in vaccinated and non-vaccinated as well as in individuals with or without the 3rd dose of the vaccine, it may also be interesting to look at the degree of protection against infection in these groups. Overall, more than 38% of study participants, regardless on their vaccination status, were tested positive for SARS-CoV-2 since March 2020. Among the unvaccinated participants, 42.1% were tested positive for SARS-CoV-2. Overall, 36.1% of subjects in the vaccinated group were tested positive for SARS-CoV-2 sometimes within the last 2 years. However, many of them (44%) became infected before they completed the baseline vaccination schema, so their protection against the infection was insufficient at that time. Out of 884 people who were vaccinated with the 3rd dose and remained COVID-19 naïve until that time, only 9% were infected with SARS-CoV-2 for the first time after the 3rd dose. Interestingly, 12.7% of subjects, who reported neither vaccination, nor the COVID-19, had positive antibody levels against the virus (7% from the whole not-vaccinated group) (Fig. 4).

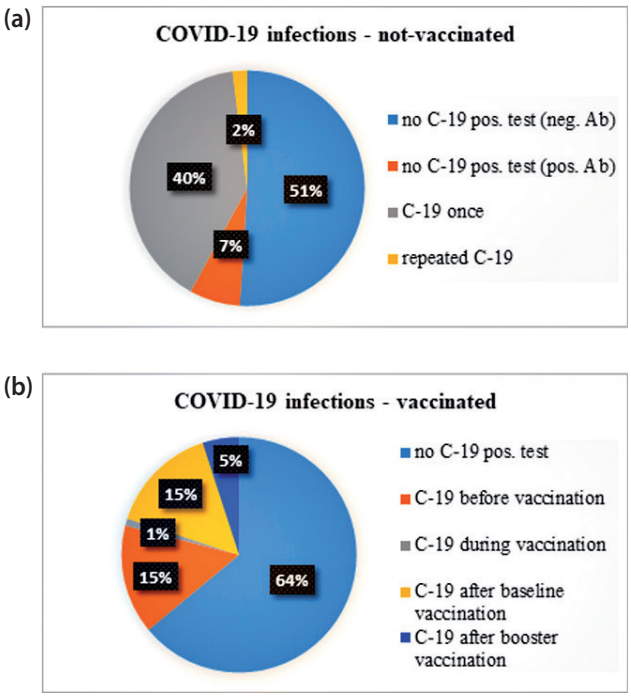

Fig. 4

Percentage of SARS-CoV-2 infections in the population
Percentage of SARS-CoV-2 infections in the **(a)** non-vaccinated and **(b)** vaccinated population. (no C-19 pos. test = no positivity of any diagnostic test for SARS-CoV-2 reported; neg. Ab = no antibodies against SARS-CoV-2 detected; pos. Ab = antibodies against SARS-CoV-2 detected; C-19 once = only one infection with SARS-CoV-2 reported; repeated C-19 = repeated infection with SARS-CoV-2 reported; C-19 before vaccination = infection with SARS-CoV-2 before start of vaccination reported; C-19 during vaccination = infection with SARS-CoV-2 between 1st and 2nd dose of a 2-dose vaccine; C-19 after baseline vaccination = infection with SARS-CoV-2 after 2nd dose of a 2-dose vaccine or after a single dose of Janssen; C-19 after booster vaccination = infection with SARS-CoV-2 after a 3rd dose following a 2-dose vaccine or after a 2nd dose following Janssen vaccination).

Discussion

The SARS-CoV-2 pandemic has lasted for more than 2 years so far. Throughout its almost entire duration, intensive testing (PCR/antigen) was carried out worldwide to determine the current presence of the virus in the body. This testing is of great importance, especially in regulating the spread of the virus, since it allows the isolation of positive people, their correct diagnosis and subsequent treatment. However, such testing cannot capture all cases of the disease, and thus provides only partial information on the prevalence in the population. The accuracy of this information depends to a large extent on the testing capacities of individual countries and regions. Testing for the presence of specific antibodies to SARS-CoV-2 in the blood provides much more reliable information on the epidemiological situation. Unlike PCR and antigen testing, it also provides a glimpse into the past. The results of sero-epidemiological studies indicate the proportion of the population that has encountered SARS-CoV-2 viral disease in the past, or (for some types of antibodies monitored) is vaccinated against it. Such knowledge at the level of institutions, regions or entire countries can be very useful for the management of anti-pandemic measures. Therefore, it is not surprising that the results of such

sero-epidemiological studies are presented in dozens of scientific publications (Anda *et al.*, 2022; Fernández-Rojas *et al.*, 2022; Goldfarb *et al.*, 2022; Lewin *et al.*, 2022; Mahallawi *et al.*, 2022; Mercado-Reyes *et al.*, 2022; Olariu *et al.*, 2022; Prakash *et al.*, 2022; Prguda-Mujic *et al.*, 2022).

Most seroprevalence studies evaluate results only at the level of positivity and do not handle quantitative data. According to our experience (Kajanova *et al.*, 2021), an anti-SARS-CoV-2 IgG ELISA (EUROIMMUN Medizinische Labordiagnostika AG, Germany) can be used for semi-quantitative analysis. With this approach, it is possible for individual categories of participants not only to determine whether they have present antibodies against SARS-CoV-2, but also the average level of this immune protection with respect to the observed characteristics of the groups.

Humoral immunity is only one component of the immune system and the degree of protection against SARS-CoV-2 infection as well as the severity of the disease depend on the overall readiness of the immune system. Besides specific antibodies, specific T lymphocytes also play an important role. The response to the SARS-CoV-2 virus is given by the cooperation of the entire immunity. From the point of view of the effectiveness of humoral immunity, the proportion of virus-neutralizing antibodies and, of course, the total amount of specific antibodies in the blood is important. Due to the significant interindividual differences in the immune response and the fact that antibodies are only part of this response, it is essentially impossible to set a specific antibody level threshold that provides individual protection against infection. In addition, new variants of the SARS-CoV-2 virus are emerging, in which the efficacy of the antibodies may be significantly altered (Zhang *et al.*, 2022). However, there is a presumption, that higher levels of specific antibodies increase the likelihood of protection against infection as well as the likelihood of a milder course of the disease (Tang *et al.*, 2021).

The evaluation of such studies is very challenging due to the large number of variables that apply to each individual. In addition to the basic data such as vaccination status and disease overcoming, consideration should be given to: number of vaccine doses, vaccine type, time elapsed since the last vaccination, multiple COVID-19 overcoming, time elapsed since the last overcoming, variant of the SARS-CoV-2 virus, disease severity, etc. These and many other circumstances can have a significant impact on the measured levels of specific antibodies.

We found, that subjects with hybrid immunity, i.e. those who were vaccinated against SARS-CoV-2 after recovery from COVID-19 or vice versa, had significantly higher antibody levels than their vaccinated COVID-19 naïve counterparts. This finding is in line with other findings (as reviewed in t.j. Pilz *et al.*, (2022)) on hybrid immunity appearing to be the most protective one. Similarly to the study of Park *et al.* (2022), we found a positive relationship between the severity of COVID-19 symptoms and the antibody levels in recovered unvaccinated subjects.

Regarding the average levels of specific antibodies to the SARS-CoV-2 virus in the vaccinated and non-vaccinated groups, the results of the current study in Trencin city largely confirmed our previous results from a study conducted in employees of the Slovak Academy of Sciences (Kajanova *et al.,* 2021). On the other hand, changing circumstances give us the opportunity to compare. In our cohort, there was a group of individuals, who have overcome COVID-19 twice or more. Comparing this group with a group of participants who overcame the disease once, we found that their antibody levels were more than twice as high on average (multiple vs. once – median S/C 7.16 vs. S/C 2.99). However, in addition to the booster effect of reinfection, the time that has elapsed since the last infection also influences the antibody level. At the time of the current Trencin study, a significant part of the population had already been vaccinated with the 3rd dose of vaccine (about a third of the Slovak population). Therefore, the levels of antibodies in the vaccinated group in the current study are slightly higher than in our previous study with only 2 doses of vaccine administered. In 3 doses group we observed a significant increase in antibody levels compared to the group in which participants are vaccinated with only two doses of vaccine (2 doses: 2.5 (3.8) vs. 3 doses: 8.2 (1.1), $p < 0.001$). Since only two manufacturers introduced the 3rd dose of the vaccine, it was common that the third administered dose was from a different manufacturer than the previous two doses. This is likely to cause the differences in antibody levels between vaccines from various manufacturers to be blurred. The highest antibody levels were found in the group with 3rd dose of a vaccine, however besides the effect of the booster dose, waning immunity with time elapsed since last vaccination plays an important role too, as protection against infection decreases with increasing time since last vaccination (Goldberg *et al.*, 2021, 2022). This is in line with our other finding of highest antibody levels in subjects vaccinated within the last 6 months prior the study, irrespective of the number of doses received.

Over time, the number of individuals who have overcome COVID-19 has increased in the unvaccinated, as well as in the vaccinated population. Nevertheless, a significant difference of COVID-recovered individuals between the vaccinated and non-vaccinated groups is noticeable, suggesting non-vaccinated COVID-19 naïve individuals being at higher risk getting infected with SARS-CoV-2 than the vaccinated.

Often, limiting factor of such studies is collecting blood from a large number of study participants in a short pe-

riod of time. A possible and very effective solution seems to be the use of the method of dry blood collection from the fingertips. This method was successfully used by the Biomedical research Center of the Slovak Academy of Sciences in testing of employees of the Slovak Academy of Sciences (Kajanova *et al.*, 2021). The return rate of more than 95% and high-quality samples in Trencin study confirmed high suitability and reliability of the chosen dry-blood-spot sampling method for massive population screening. The professional organization of registration, distribution and subsequent collection of the sampling kits by the city of Trencin was very important for the success of this phase of study. The high quality of the samples themselves was probably related to less stress from at home blood collection and to high-quality tutorial video for the collection of capillary blood from the finger, which was prepared by the city of Trencin.

In conclusion, vaccinated subjects, who recovered from COVID-19 (either before or after vaccination) had significantly higher antibody levels than vaccinated subjects without SARS-CoV-2 infection, suggesting hybrid immunity to be more protective than the vaccination induced only. Furthermore, the antibody levels in recovered unvaccinated subjects increased with severity of their COVID-19 symptoms, as well as after multiple recoveries from the disease. In our study, the severity of side effects after vaccination did not influence the antibody levels. Decrease in antibody levels after 6 months from the last vaccination could be observed.

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