



Seroprevalence of infection-induced SARS-CoV-2 antibodies among health care users of Northern Italy: results from two serosurveys (October–November 2019 and September–October 2021)

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ABSTRACT

Objectives: The objective was to estimate the seroprevalence of SARS-CoV-2 in autumn 2019 (before case zero was identified in Italy) and 2021 among residual sera samples from health care users in the Piedmont region of northwestern Italy.

Methods: Two serosurveys were conducted. Using a semiquantitative method, samples were tested for the presence of immunoglobulin G (IgG) antibodies against the S1 domain of the spike protein. Samples with positive test results from the 2019 survey were independently retested using a multiplex panel to detect IgG antibodies against the receptor binding domain, S1 and S2 domains, and nucleocapsid. Samples with positive test results from the 2021 survey underwent repeat testing with enzyme-linked immunosorbent assay to detect anti-nucleocapsid IgG antibodies. Prevalence rates according to gender and age groups, together with their respective 95% confidence intervals (CIs), were calculated.

Results: Overall, the proportion of samples with positive test results was 2/353 in 2019 and 22/363 in 2021, with an estimated seroprevalence of 0.27% (95% CI 0–1.86) and 6.21% (95% CI 3.9–9.31) in 2019 and 2021 respectively.

Conclusion: Results of this study support the hypothesis that the virus was circulating in Italy as early as autumn 2019. The role of these early cases in broader transmission dynamics remains to be determined.

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Introduction

COVID-19, caused by SARS-CoV-2, was declared a public health emergency of international concern on January 30, 2020. As of June 12, 2022, there have been 533,160,628 confirmed cases worldwide, including over 6 million deaths (WHO, 2022).

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Italy was the first European Union country hit by the pandemic. The Italian Council of Ministers declared a state of emergency throughout the country on January 31, 2020. Since then, Italy has faced four epidemic waves; in response, the government has implemented strict containment measures during the peak phases (Vicentini et al., 2020a). Italy has reported nearly 18 million cases of COVID-19 and over 160,000 deaths (WHO, 2022). Meanwhile, the vaccination campaign against COVID-19 began in Italy at the end of December 2020, using mRNA and viral-vector-based vaccines (Sacco et al., 2022).

Although the first officially identified COVID-19 cases in Italy were reported in late January 2020 in the region of Lombardy (in

Table 1

Sample size, according to age group and gender, employed for each seroprevalence survey conducted on residual sera obtained from health care users in Piedmont, Italy (first survey, October–November 2019; second survey, September–October 2021).

Number of samples	Male	Female	Total
Age group, years			
6–20	82	83	165
21–40	70	70	140
41–50	5	5	10
51–64	8	8	16
65–74	8	8	16
>75	8	8	16
Total	181	182	363

northwestern Italy), previous Italian studies have highlighted the presence of SARS-CoV-2 in samples collected and stored for other reasons before February 2020 (Apolone et al., 2021; Gragnani et al., 2021; La Rosa et al., 2021). Apolone et al. (2021), analyzing blood samples collected from September 2019 to January 2020, found positive test results for immunoglobulin M (IgM) antibodies in 97 patients (10.1%); immunoglobulin G (IgG) antibodies were found in 16 (1.7%). Furthermore, an environmental study identified the presence of SARS-CoV-2 RNA in wastewater from the neighboring regions of Lombardy and Piedmont in December 2019 (La Rosa et al., 2021). Other studies have also identified the presence of anti-receptor binding domain (RBD) antibodies before the date of the first official cases in Italy (Deslandes et al., 2020; Gragnani et al., 2021). However, false-positive results have been documented, and the utility of testing archival samples for SARS-CoV-2 has been questioned (Latiano et al., 2021; Wang et al., 2020).

This study aimed to estimate the prevalence of antibodies against SARS-CoV-2 in October–November 2019 (before case zero was identified in Italy) and in September–October 2021 (after the first three pandemic waves and the beginning of the vaccination campaign) among residual sera samples from health care users in the Piedmont region of northwestern Italy.

Methods

Study design and study population

Two seroprevalence surveys were conducted on residual sera obtained from health care users. For the first survey, samples were collected in October–November 2019; for the second survey, samples were collected in September–October 2021. For both surveys, residual sera were obtained from outpatients aged 6–90 years who were undergoing routine hematological tests at the Città della Salute e della Scienza, a tertiary-care and teaching hospital of Turin and an important referral center for the entire Piedmont region of northwestern Italy. Patients who had cancer or immunodeficiency or were undergoing immunosuppressive therapy were excluded from the study.

The first serosurvey was conducted on samples collected for a previous seroprevalence study with the objective of investigating the prevalence of antibodies against endemic infectious diseases, stratified by age groups. The sample size of the study was calculated on the basis of antibody prevalence estimates for measles, varicella, hepatitis A, tetanus, and diphtheria in each age group, with a confidence interval (CI) of 95% and a power of 80%, resulting in a sample size of 363 sera distributed among age groups as listed in Table 1. Anti-SARS-CoV-2 antibody prevalence estimates were not considered in sample size calculations, as the virus had not yet been officially identified in Italy in early autumn 2019. For

both serosurveys reported in this study, the same sample size and age and gender distributions were applied.

This study was approved by the Institutional Review Board of the University of Turin (protocol number 0063529). Informed consent was not requested, as all specimens were deidentified, and only basic demographic information was obtained, in accordance with standard ISO/FDIS 20916.

Serological analyses

Samples were delivered to the “Laboratory of Serology and Microbiology applied to Hygiene” of the Department of Public Health and Pediatrics of the University of Turin. After centrifugation, sera were collected and stored at -20°C until testing, which was performed in January 2022 on samples from both serosurveys.

Samples obtained from both the 2019 and 2021 serosurveys were tested by EUROIMMUN Anti-SARS-CoV-2 enzyme-linked immunosorbent assay IgG (EUROIMMUN Medizinische Labordiagnostika AG) to detect SARS-CoV-2 IgG antibodies against the S1 domain of the spike protein, including the immunologically relevant receptor binding domain. Peroxidase activity was quantified after color development and optical density (OD) determination at 450 nm. Sera were diluted 100-fold in the analysis. IgG results were assessed using a semiquantitative method by calculating the ratio of the OD value of the sample to the OD value of the calibrator. The result was interpreted as negative if the ratio is <0.8 , borderline if $0.8\text{--}1.1$, and positive if ≥ 1.1 . Samples with results categorized as borderline were considered seronegative and did not undergo further testing. According to instructions of the manufacturer, this cutoff produced a sensitivity of 94.4% and a specificity of 99.6%.

After initial testing, sera were stored at -20°C . Samples with positive results from the 2019 survey were sent to the Laboratory of the Hygiene Unit, Scientific Institute for Research, Hospitalization and Healthcare, Ospedale Policlinico San Martino Genova (Genoa, Italy) to be independently retested. Samples were tested using BioPlex 2200 SARS-CoV-2 IgG multiplex panel (Bio-Rad Laboratories, Inc.) to detect IgG antibodies against the RBD, S1 and S2 spike domains, and nucleocapsid (N) of SARS-CoV-2. The manufacturer ensures an overall sensitivity of 99.8% and a specificity of 99.7%. The combined sensitivity and specificity of the assays used for 2019 were 94.21% and 99.6%, respectively.

All SARS-CoV-2 vaccines approved in Italy function by eliciting an immunological response to the spike protein (Sacco et al., 2022). To differentiate between vaccinated individuals and individuals exposed to natural infection, samples with positive test results from the 2021 survey underwent repeat testing with Anti-SARS-CoV-2 QuantiVac ELISA IgG (EUROIMMUN Medizinische Labordiagnostika AG) to detect anti-N antibodies (using the same semiquantitative method and cutoff as described for the detection of anti-S1 IgG). The manufacturer ensures a sensitivity of 94.6% and a specificity of 99.8%. The combined sensitivity and specificity of the assays used for 2021 samples were 89.3% and approximately 100%, respectively. This analysis was not conducted on 2019 sera, as vaccination against SARS-CoV-2 in Italy was introduced in December 2020 (Sacco et al., 2022).

A flowchart summarizing serological analyses is presented in Figure 1. As depicted in the Figure, individuals whose test results were negative for anti-S1 IgG in both surveys were considered not exposed to SARS-CoV-2 (and to have neither infection-induced nor vaccination-induced immunity). Regarding 2019 samples, only individuals whose test results were positive for anti-S1, anti-S2, and anti-N IgG were considered seropositive (infection-induced seroprevalence). Regarding 2021 samples, individuals who were seropositive for anti-S1 IgG and seronegative for anti-N IgG were considered vaccinated and unexposed to SARS-CoV-2 (vaccination-induced seroprevalence), whereas individuals who

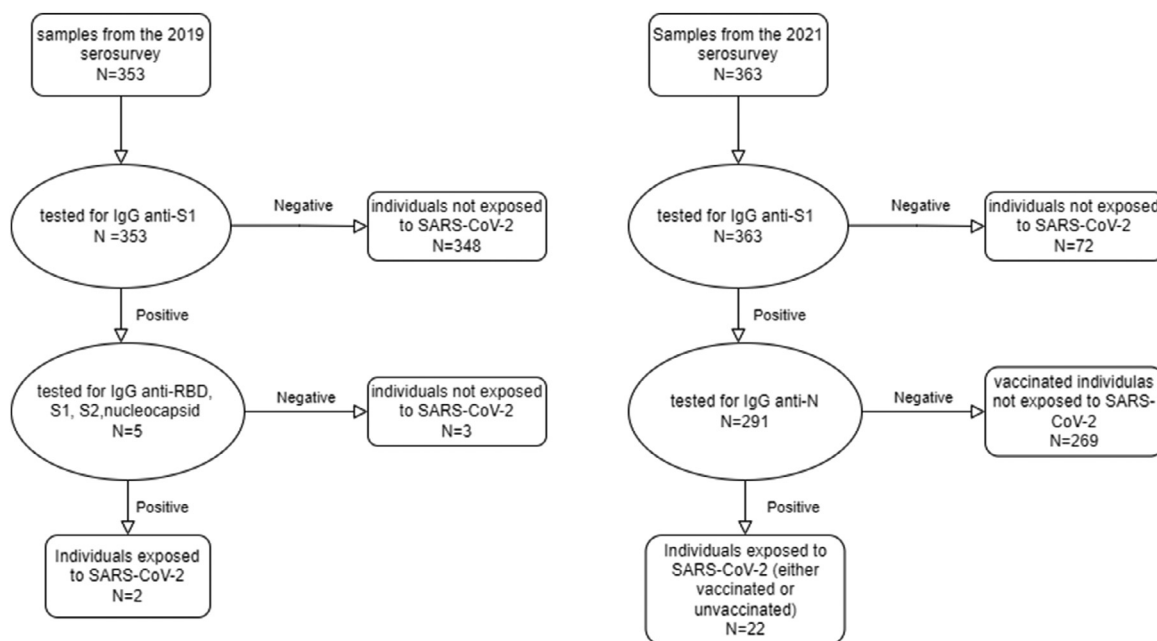


Figure 1. Flowchart summarizing serological analyses conducted on the 2019 and 2021 residual sera samples.

Samples categorized as borderline based on seroreactivity (optical density ratio) in the first round of testing were considered seronegative and did not undergo further testing.

IgG, immunoglobulin G; RBD, receptor binding domain; S1, spike protein domain 1; S2, spike protein domain 2.

were seropositive for both anti-S1 and anti-N IgG were considered exposed to SARS-CoV-2 (either vaccinated or unvaccinated; infection/vaccination-induced seroprevalence).

Statistical analysis

Prevalence rates according to gender and age groups, together with their respective 95% CIs, were calculated using the adjustment to the Rogan-Gladen formulas proposed by Lang and Reiczigel (2014). Results of the 2021 survey were compared, using Yates-corrected chi-square test, to (1) the proportion of vaccinated individuals and (2) the proportion of cumulatively infected individuals (based on swab samples with positive SARS-CoV-2 polymerase chain reaction test results) across the population of the Piedmont region in the same months (Istituto Superiore di Sanità [ISS], 2021; Sacco et al., 2022). Statistical significance was set at $P < 0.05$; analysis was two-tailed. Analyses were performed using SPSS Statistics 28.0 (IBM Corp., Armonk, New York).

Results

A total of 353 of the 363 residual sera originally collected in 2019 were retrieved and tested. One sample, obtained from a 14-year-old male, had borderline test results (OD ratio 0.84). Five samples had positive test results for anti-S1 IgG (OD ratio range 1.23–4.31); these were obtained from a 35-year-old female and four males aged 8, 8, 10, and 39 years. Upon retesting with the multiplex panel, two of these five sera had positive results for anti-S1, anti-S2, and anti-N IgG but negative results for anti-RBD IgG. These two sera were those with the highest OD ratios in the previous round of testing (3.56 and 4.31) and were obtained from the 39-year-old male and 35-year-old female. The remaining three sera had negative test results for anti-S1, anti-S2, anti-N, and anti-RBD IgG.

Of 363 residual sera from 2021, five had borderline test results for anti-S1 IgG (OD ratio range 0.82–1.05), and 291 had positive test results (OD ratio range 1.13–9.99). Of the 291 sera retested for anti-

N IgG, five had borderline test results (OD ratio range 0.82–1.02), and 22 had positive results (OD ratio range 1.23–7.99). The 22 sera samples with positive test results for both anti-S1 and anti-N IgG were obtained from nine male and 13 female individuals.

Tables 2 and 3 list seroprevalence estimates stratified by age groups, based on residual sera samples from 2019 and 2021. Overall, the proportion of samples with positive test results was 0.57% in 2019. In 2021, the overall proportion of anti-S1 and anti-N seropositivity was 6.06%; among the samples that were seropositive for anti-S1, the proportion of samples that were seropositive for anti-N was 7.56%. The estimated seroprevalence, calculated using the adjustment to the Rogan-Gladen formulas proposed by Lang and Reiczigel (2014), was 0.27% (95% CI 0–1.86) and 6.21% (95% CI 3.9–9.31) in 2019 and 2021, respectively.

In the Piedmont region, 385,860 cumulative cases were registered by October 27, 2021 (ISS, 2021) in a total population of 4,252,279 (Istituto Nazionale di Statistica, 2021), resulting in a proportion of cumulatively infected individuals of 9.07%. This result differed significantly from the proportion of samples with positive test results in residual sera from 2021 ($P = 0.046$) but is within the 95% CI of the seroprevalence estimate for 2021, which was calculated with consideration of test sensitivity and specificity (Lang and Reiczigel, 2014).

Based on results of the 2021 survey, the proportion of vaccinated individuals was 80.17%. The total number of vaccinated individuals with at least one dose in the Piedmont region at the end of October 2021 was 3,324,410 (Sacco et al., 2022); the resulting proportion of vaccinated individuals was 78.18%, which was not significantly different from our estimate ($P = 0.36$).

Discussion

Uncertainty persists on the date of origin of SARS-CoV-2 and the date of its introduction in Europe (Roberts et al., 2021). The precipitous rise in cases in the early stages of the pandemic in Italy has led to speculations that COVID-19 had already been circulating, undetected, before the identification of the first official

Table 2

Frequency of anti-SARS-CoV-2 IgG antibodies and estimates of infection-induced seroprevalence, stratified by age groups, based on residual sera samples from Turin, Italy in 2019 (N = 353).

Age group, years	Proportion of anti-S1 IgG positive samples, 2019	Proportion of anti-S1, -S2, and -N IgG positive samples, 2019	Estimated infection-induced seroprevalence (95% CI) ^a
6-20	3/158	0/158	0 (0-2.51)
21-40	2/137	2/137	1.17 (0-5.16)
41-50	0/10	0/10	0 (0-31.91)
51-64	0/16	0/16	0 (0-22.45)
65-74	0/16	0/16	0 (0-22.45)
>75	0/16	0/16	0 (0-22.45)
Overall	5/353	2/353	0.27 (0-1.86)

IgG, immunoglobulin G; N, nucleocapsid; S1, spike protein domain 1; S2, spike protein domain 2.

^a Considering sensitivity to be 99.8% and specificity to be 99.7%.

Table 3

Frequency of anti-SARS-CoV-2 IgG antibodies and estimates of infection-induced seroprevalence, stratified by age groups, based on residual sera samples from Turin, Italy in 2021 (N = 363).

Age group, years	Proportion of anti-S1 IgG positive samples, 2021	Proportion of anti-S1 and anti-N IgG positive samples, 2021	Estimated seroprevalence (95% CI) ^a
6-20	128/165	7/165	4.28 (1.69-8.89)
21-40	114/140	7/140	5.08 (2.07-10.46)
41-50	9/10	1/10	10.38 (0-44.91)
51-64	11/16	2/16	13.03 (2.06-39.26)
65-74	14/16	1/16	6.41 (0-31.86)
>75	15/16	4/16	26.27 (9.99-52.74)
Overall	291/363	22/363	6.21 (3.9-9.31)

CI, confidence interval; IgG, immunoglobulin G; N, nucleocapsid; S1, spike protein domain 1; S2, spike protein domain 2.

^a Considering sensitivity to be 99.8% and specificity to be 99.7%.

cases (Apolone et al., 2021; La Rosa et al., 2021). Identifying the first introduction of SARS-CoV-2 in a geographic region is of epidemiological relevance, as it is essential for an accurate description of the spread of COVID-19. In addition, current and broader implications stem from the challenge of accurately diagnosing and reporting SARS-CoV-2 infections at the population level, especially considering the emergence of variants and subvariants (which have been shown to affect assay performance characteristics) and the continued lack of clarity regarding the true magnitude of asymptomatic infections (Oude Munnink et al., 2021).

To gain further insight on the temporality of early introductions in Italy, and to test the hypothesis of early SARS-CoV-2 circulation, this study investigated the presence of infection-induced antibodies against SARS-CoV-2 among archival sera samples obtained from health care users in the Piedmont region in autumn 2019. Seroprevalence estimates, stratified according to age and gender, were compared with results of a second serosurvey conducted in autumn 2021 and with official data on cumulative infections. Samples obtained in 2021 served as controls, which were used to validate the study methodology.

Regarding the serosurvey conducted on samples in October–November 2019, two of 353 samples were found to be positive for IgG antibodies against the RBD, S1, S2, and N of SARS-CoV-2, with an estimated seroprevalence of 0.27% (95% CI 0-1.86). This finding suggests that SARS-CoV-2 circulation in the Piedmont predated the first officially reported cases in the Piedmont region (February 2020), in Italy (January 2020), and even in Wuhan, China (December 2019) (Istituto Superiore di Sanità (ISS), 2021). Several Italian and international studies have identified possible COVID-19 cases before these dates (Apolone et al., 2021; Deslandes et al., 2020; Gagnani et al., 2021; Trombetta et al., 2022), and a recent study has suggested that, based on significant changes in hospital traffic and search-engine data in Wuhan, COVID-19 could have originated in late summer 2019 (Nsoesie et al., 2020). Notably, Apolone et al. (2021) identified immunoreactivity to RBD in two samples collected in the Piedmont at the end of September 2019, and 10 of the 111 possible COVID-19 cases identified throughout the en-

tire study period (September 2019–March 2020) were in residents of the Piedmont (Apolone et al., 2021).

However, the significance of positive serological results obtained from asymptomatic individuals or individuals with unknown exposure history—particularly in geographic areas with little to no viral circulation—remains to be determined. Latiano et al. (2021) tested 1150 archival sera collected in southern Italy in 2018–2019 to identify anti-N IgM/IgG. The authors found four and three samples that had positive results for IgM and IgG, respectively, using an enzyme-linked immunosorbent assay; they used the urea dissociation test proposed by Wang et al. (2020) to identify false positives. All but one IgG-positive sample retained positivity, leading the authors to question the validity of conducting serological testing for SARS-CoV-2 on archival samples. Select samples from the previously mentioned study by Apolone et al. (2021) were cross-validated in an external World Health Organization-affiliated laboratory using different serological assays, which failed to confirm results (Apolone et al., 2021; Montomoli et al., 2021). The criteria applied for the validation testing were triple-IgG antigen positivity and neutralization test confirmation.

In our study, five samples from 2019 had positive test results for anti-S1 IgG; upon retesting, two of the five had positive results for anti-S1, anti-S2, and anti-N IgG. Interestingly, the two sera with positive results upon retesting were those with the highest OD ratios in the first round of testing; this could indicate that the cutoff value should be re-evaluated, at least for the purpose of analyzing archival samples. Further, the three samples that did not maintain positive results upon multiplex-panel testing were obtained from children <10 years of age, whereas both samples from adults were confirmed to have positive results. This finding may suggest the detection of cross-reactivity with unexplored factors. Previous studies have investigated the relevance of interfering epitopes such as rheumatoid factor (Latiano et al., 2021; Wang et al., 2020). Other studies have suggested a potential antigenic cross-reactivity between SARS-CoV-2 and other pathogens, such as Dengue and Zika viruses (Lustig et al., 2021) and endemic human coronaviruses (Dowell et al., 2022). Children, in particular, have been associated

with a profile of enhanced humoral immune response to SARS-CoV-2, with substantial cross-reactivity against other human coronaviruses (Dowell et al., 2022).

Based on results of the second serosurvey, conducted on samples collected in September–October 2021, a seroprevalence of 6.21% (95% CI 3.9–9.31) was estimated. A 10-fold increase in the proportion of samples with positive test results for infection-induced antibodies was found between the two serosurveys, and a 20-fold increase in seroprevalence was estimated. It is beyond the remit of the present study to investigate the role of these potential early cases in determining the subsequent spread of COVID-19 in our region; however, these findings may fit with the hypothesis that a less-transmissible progenitor of the virus was silently circulating before the identification of the first official cases (Trombetta et al., 2022). Growing evidence suggests that multiple sporadic introductions of SARS-CoV-2 occurred initially in Italy but did not lead to sustained transmission until the introduction of the D614G mutant in northwestern Italy in February 2020 (Lai et al., 2022).

The two positive samples from 2019 were obtained from working-aged individuals, consistent with work-related importations in the early stages of the pandemic (La Rosa et al., 2021; Montomoli et al., 2021). Regarding the 2021 serosurvey, at least one positive sample was found in every age stratum, with the highest seroprevalence estimated for the age groups 51–64 and >75; these results are in line with the age distribution of reported infections in Italy through the end of October 2021 (ISS, 2021).

Our estimates of both the proportion of vaccinated individuals and the proportion of individuals exposed to SARS-CoV-2 did not significantly differ from official data (ISS, 2021; Sacco et al., 2022). Although these findings may support the validity of our sampling strategy, the latter observation was unexpected; we anticipated obtaining a higher estimate for virus exposure from our seroprevalence data compared with official records. The identification of COVID-19 cases through molecular testing and the reporting of cases to surveillance systems underestimate the full scale of the spread of SARS-CoV-2; this underestimation is due to the high proportion of asymptomatic or moderately symptomatic individuals, to individuals who do not seek medical care, to organizational and logistical issues, and to limited resources for testing. Underascertainment was particularly significant in the early stages of the pandemic (Apolone et al., 2021; Vicentini et al., 2020b). Stefanelli et al. (2021) conducted a seroprevalence study in a high-incidence area in northeastern Italy in May 2020 and found that the ratio of officially reported cases to seropositive results was 1:3. However, seroprevalence estimates are affected by waning immunity—particularly in anti-N titers—(Lavezzo et al., 2022) and do not account for reinfections. Further, it has been suggested that breakthrough infections after vaccination may determine lower anti-N titers (Clarke et al., 2022), and that some individuals—especially those who are asymptomatic—do not develop complete humoral responses after exposure to SARS-CoV-2 (Takeshita et al., 2021).

Our study had several limitations. First, our sample was relatively small and obtained from a single center; therefore, selection bias may affect the generalizability of our results. Second, sera samples were obtained from health care users, which may have led to the overrepresentation of individuals with greater health care needs or more frequent health care access. Third, due to the use of deidentified samples, no information on health status, travel history, or previous polymerase-chain-reaction-confirmed SARS-CoV-2 infection was available for the individuals from whom sera samples were obtained. Fourth, for 2019 results in particular, although the tests we employed are assumed to have high sensitivity and specificity, we cannot exclude the possibility that the test results for the two seropositive samples were false positives or the possibility that the results indicate a cross-reaction with other seasonal coronaviruses (Dowell et al., 2022; Latiano et al., 2021; Lv et al.,

2020). However, samples with positive test results underwent repeat testing, which was conducted in an independent laboratory in the case of 2019 samples. Likewise, we did not account for the possibility of cross-reactivity (for anti-S1 IgG in particular) in samples from 2021.

Despite these limitations, this study provided seroprevalence estimates for SARS-CoV-2 antibodies at two time points, contributing information for understanding the epidemiology of SARS-CoV-2 in Italy. Further, this study may have identified exceptionally early introductions of SARS-CoV-2 in our region, supporting the hypothesis that the virus was circulating in Italy as early as autumn 2019. The role of these early cases in broader transmission dynamics remains to be determined (Roberts et al., 2021).

Author's Contribution

Conception and design: CV, CMZ. Data collection: VB, ARC, NM, DM. Laboratory analysis: SD, GF, MG, GM, GM, VR, GI. Statistical analysis: VB, CV. Manuscript - first draft: CV. Manuscript - revision and editing: GI, CMZ.

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Ethical approval

This study was performed in alignment with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Turin (October 6, 2021; No. 0063529). Informed consent was not requested, as all specimens were deidentified and only basic demographic information was obtained, in accordance with standard ISO/FDIS 20916.Z.

Data availability

The datasets generated and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Declarations of competing interest

The authors have no competing interests to declare.

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