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Prevalence of asymptomatic SARS-CoV-2-positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross-sectional study protocol

Massimo Guerriero [ID],[1] Zeno Bisoffi [ID],[2,3] Albino Poli,[2] Claudio Micheletto,[4] Carlo Pomari[5]

For numbered affiliations see end of article.

Correspondence to
Professor Zeno Bisoffi;
zeno.bisoffi@sacrocuore.it

ABSTRACT

Introduction As of 30 April 2020, the novel betacoronavirus SARS-CoV-2 had infected more than 3 172 000 individuals, killing over 224 000 people and spreading to more than 200 countries. Italy was the most affected country in Europe and the third most affected in the world in terms of the number of cases. Therefore, the aims of this study are: (1) to estimate the prevalence of asymptomatic SARS-CoV-2-positive individuals among the general population of Verona; (2) to assess the accuracy (sensitivity, specificity and predictive values) of an ELISA serological test for the screening of SARS-CoV-2.

Methods and analysis The study will be carried out on a random sample of subjects aged at least 10 years from the general population of Verona. Participants will undergo the measurement of vital parameters (oxygen saturation measured by oximeter, respiratory rate and body temperature detected by laser thermometer), the administration of a COVID-19-related symptoms questionnaire, the collection of a blood sample and a nasopharyngeal swab. Our evaluation will include the statistical technique of Latent Class Analysis, which will be the basis for the estimation of prevalence.

Ethics and dissemination The study protocol has been approved by the Ethics Committee of Verona and Rovigo provinces on 15 April 2020 (internal protocol number 2641CESC). The study results will be submitted for publication in international, peer-reviewed journals and the complete dataset will be deposited in a public repository. Most relevant data will be made available to policy-makers as well as disseminated to stakeholders and to the community.

INTRODUCTION

As of 30 April 2020, the novel betacoronavirus SARS-CoV-2 had infected more than 3 172 000 individuals, killing over 224 000 people and spreading to more than 200 countries.[1] Italy was at the time the most affected country in Europe and the third most affected in the

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Strengths and limitations of this study

► Study based on the random sample of a general population.
► Very low estimation standard error (max 1.5%).
► Simultaneous comparison of symptoms, real-time PCR test and ELISA serological test.
► Results will depend on the response rate.

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world in terms of the number of cases. The epidemic was posing an extremely difficult challenge to healthcare establishments, health workers and to the general population. The identification of asymptomatic SARS-CoV-2-positive individuals is crucial in reducing the spread of the virus throughout the world. The frequency of such cases is unknown, despite asymptomatic cases regularly being referred to in the literature.[2–4]

Data from the cruise ship 'Diamond Princess' have shown that the percentage of asymptomatic SARS-CoV-2-positive cases among all passengers and crew members tested prior to disembarkation was about 50%. This rate has since been revised as 17.9.[5]

A similar study focusing on a Japanese population returning from China found the percentage of asymptomatic cases to be 33.3%.[6]

Finally, a small group of residents in a skilled nursing facility has also been screened: 13 of 23 residents were defined as asymptomatic, but 10 of these developed symptoms over the subsequent 7 days.[7]

Currently, SARS-CoV-2 detection is based on accepted and standardised molecular methods that require about 4 hours to carry

out in our laboratory, although more rapid molecular tests are being made available. Samples are accumulating in many laboratories that are at risk of being overwhelmed. This causes further critical delays in managing SARS-CoV-2-positive cases and obtaining a definitive COVID-19 diagnosis.

Serological tests would be extremely useful for identifying asymptomatic carriers of SARS-CoV-2. Despite this, to date, the accuracy of these tests is insufficient to replace the current laboratory diagnosis. Serological tests focus on the detection of immunoglobulin (Ig)M and/ or IgA and IgG. As it was noted during the previous SARS epidemic, a possible problem with serologic tests may be a cross-reaction with other coronaviruses.[8] A recent publication has evaluated the median seroconversion time for antibodies IgA, IgM and IgG. The authors evaluated 173 SARS-CoV-2 -positive subjects and reported a median time of 11, 12 and 14 days, respectively. Additionally, antibodies were found in >40% of patients within 1 week of symptom onset, rapidly increasing to 100.0% (IgA), 94.3% (IgM) and 79.8% (IgG) from day 15 after symptom onset. In contrast, RNA detectability decreased from 66.7% (58/87) in samples collected before day 7 to 45.5% (25/55) during days 15–39. Combining RNA and antibody detection significantly improved the sensitivity of COVID-19 diagnosis (p<0.001), even in the early phase during the first week of symptoms (p=0.007). Moreover, a higher titre of the antibody was independently associated with a worse clinical classification (p=0.006).[9] Therefore, the aims of this study are as follow:

1. To estimate the prevalence of asymptomatic SARS-CoV-2-positive individuals among the general population of Verona.
2. To assess the accuracy (sensitivity, specificity and predictive values) of two, commercially available serological tests for the screening of SARS-CoV-2.

METHODS AND ANALYSIS
Study design
This will be an observational cross-sectional prevalence study and an observational prospective diagnostic study in Verona (Veneto, Italy). Samples will be collected and analysed at Istituto di Ricovero e Cura a Carattere Scientifico(IRCCS) Sacro Cuore Don Calabria Hospital.

Study population
The study will be carried out on a random sample of the general population of Verona. Subjects eligible for the study must reside in Verona, be at least 10 years of age and provide consent for the participation in the study and for the donation of biological samples for study purposes.

Subjects will be randomly selected from the municipality of Verona's registry and invited to participate.

According to official sources, the cumulative number of SARS-CoV-2 infections in Verona as of 25 May 2020 was 1528 cases (0.7% of the total population), of which 144 deaths, for a death rate of 9.4%.[10]

Procedures
Each randomly selected citizen will receive an invitation letter to their place of residence, outlining the reasons for the study and how to take part.

Those who decide to participate will be invited to contact a dedicated telephone number, at which point general information will be given and their contact details collected.

The specially trained staff at IRCCS Sacro Cuore Don Calabria Hospital will contact citizens both to confirm participation by verbal consent and to arrange an appointment (according to a pre-established calendar, automatically managed by a suitable software that limits the formation of queues). During the same phone call, a COVID-19-related symptoms questionnaire (online supplemental annex 1) will also be administered and all information related to the logistics and implementation of the study will be provided (eg, mask and gloves use, methods of sample collection and so on). This is to minimise the duration of physical contact and length of stay by participants in the centre, as well as to maximise protection against possible contagion.

In the case of participants under the age of 18, the phone call and questionnaire will be conducted with a parent (preferably the primary caregiver), whereas the minor will be required for the sample collection only.

Each participant that attends the IRCCS Sacro Cuore Don Calabria Hospital will be asked to deliver signed informed consent forms (customised according to the age of the participant), which will be verified and countersigned by the principal investigator or by delegated staff. Participants who fail to bring their consent form can obtain another copy directly at the centre.

Participants will then undergo the measurement of vital parameters (oxygen saturation measured by oximeter, respiratory rate and body temperature detected by laser thermometer), the collection of a blood sample and a nasopharyngeal swab. All procedures will be performed by specialised personnel of the IRCCS Sacro Cuore Don Calabria Hospital in dedicated clinical settings with suitable anticontagion equipment.

Auxiliary staff will monitor the movement of people outside and inside the hospital.

So as not to potentially contaminate hospital rooms and to avoid the need for continual sanitisation, samples will be taken outdoors in a designated tented facility located inside the hospital grounds, so as to allow examinations to be carried out even in the event of adverse weather. For those arriving by motorised vehicle, swabs may be collected from participants while they remain seated inside it, as a final step, when they are going out after concluding the other procedures including blood sampling.

Up to 280 samples per day are expected to be collected.

The collected samples will be stored in a refrigerator at 4°C until their transfer, within 24 hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital. Blood samples will be immediately stored there

on reception at −80°C, until their processing and analysis that will be carried out in the following weeks, whereas swabs are processed on reception and then also stored at −80°C.

Sample collection procedures will take place with 10 min intervals between the end of one sample collection and the start of the next one to ensure privacy and to avoid any close contact between subjects.

To further guarantee the safety of participants, the route inside the centre will be one-way only eliminating the need to return to spaces already frequented. Visual indications, such as strips on the ground, will allow all individuals to maintain the recommended safe distance from each other.

If a subject is unable to travel to the testing centre, then the home collection of the sample will be arranged during the initial phone call to confirm verbal consent.

The results of the examinations will be communicated to participants and in case of a positive SARS-CoV-2 result, appropriate procedures will be activated.

All essential information, including completed questionnaires and selected laboratory findings, will be recorded in an electronic Case Report Form using the platform OpenClinica.

Measurements

This protocol refers to STAndards for the Reporting of Diagnostic accuracy studies (STARD) guidelines[11] for the reporting of diagnostic test accuracy. Based on an assessment methodology already used at the IRCCS in diagnostic studies,[12 13] the assessment will be carried out using an approved molecular test as the gold standard, assuming that the sensitivity will not be 100% (due to variances in nasopharyngeal swabbing technique). The evaluation will also include the statistical technique of latent class analysis (LCA), which will be also the basis for the estimation of prevalence.

Anti-SARS-CoV-2 ELISA IgA/IgG (Euroimmun, Germany) will be performed according to the manufacturer's instructions, detecting SARS-CoV-2 antibodies of classes IgA (described as early markers of acute respiratory tract infections) and IgG (indicating a persisting or past infection). In a recent study,[14] the value of specific IgA detection, in the early detection of acute SARS-CoV-2 infections, has been confirmed. The assay uses the S1 domain of the spike protein on the surface of SARs-CoV-2 as its antigen, which is considered to be more specific for the serological detection of SARS-CoV-2 antibodies.

The SARS-CoV-2 IgG assay (Abbott Laboratories, USA) is a chemiluminescent microparticle immunoassay for the detection of IgG antibodies to SARS-CoV-2. This will be also performed according to the manufacturer's instructions.

According to the most recently published study on both tests,[15] the sensitivity and specificity of the Euroimmun test were found to be 78.3% and 96.7% for IgG, and 86.7% and 82.7% for IgA; for the Abbott test (IgG), they were 81.8% and 99.3%, respectively. Another study

in preprint[16] found (for IgG) a sensitivity of Euroimmun test varying from 76.9% to 87.1%, and for Abbott from 96.2% to 97.1%, depending on the population group sampled; specificity was 97% for Euroimmun and 99% for Abbott. These figures were obtained on small groups of patients, some of whom with active or recent infection when they might have not yet developed detectable antibodies.

The Primary Reference Standard test is a real-time reverse transcription PCR (RT-PCR), executed at our department, that has been set up according to the procedures followed by the Regional Reference Laboratory (Department of Microbiology, University Hospital of Padua) and cross-validated.

RNA will be extracted from nasopharyngeal swabs in accordance with our routine laboratory practice. Briefly, total RNA will be extracted using a MagnaPure LC.2 instrument (Roche Diagnostic, Monza, Italy), and MagNA Pure LC RNA Isolation Kit-High Performance (Roche), according to the manufacturer's instructions for the cell-containing samples. Eluted RNA will be analysed following the routine in-house real-time RT-PCR protocol for the COVID-19 diagnostic test. The remaining RNA aliquots will be stored at −80°C until they are required for further tests.

True positive subjects will be those with a positive real-time RT-PCR result as this indicates the presence of SARS-CoV-2 RNA. True negative subjects will be subjects with a negative real-time RT-PCR result that have no detectable SARS-CoV-2 RNA. The outcome for indeterminate results is outlined later.

In detecting SARS-CoV-2, RT-PCR revealing specific viral RNA sequences may be considered the gold standard, being a test with virtually 100% specificity and therefore acceptable as a gold standard for the sensitivity of index tests. However, the sensitivity of RT-PCR cannot truly be considered to be 100% in detecting SARS-CoV-2 RNA, as is becoming clear to reference laboratories performing the test, due to the viral load being too low for the sequences to be revealed or a flawed swabbing technique. In cases that only use this gold standard, the classification of discordant results (negative gold standard, positive index test) would be subject to error. Using a composite reference standard (CRS) is one of the alternative methods when a 'perfect' gold standard is not available.[17 18] However, this method has its limitations too, as when a CRS is used, its accuracy cannot be assumed 'a priori'.[19] Alternative methods to address a lack of a gold standard are LCMs.[20] LCA is planned using the available tests for SARS-CoV-2 as well as other, selected, clinical and paraclinical variables.

Each test will be executed independently by experienced laboratory personnel. Laboratory professionals will not be aware of the clinical data of the subjects and will not know in advance the results of any other test.

Subjects found positive

Subjects with a positive test result will be informed of the test result and managed according to the routine procedures for clinical assessment and isolation.

Sample size calculation

To the best of our knowledge, there is no information published in an accredited scientific journal that indicates the prevalence of asymptomatic SARS-CoV-2-positive individuals among the general population. In Italy, epidemiologists have reported a potential prevalence equal to 5 or 10 times higher than the number of detected SARS-CoV-2-positive individuals. Other similar sources indicate that the prevalence of asymptomatic SARS-CoV-2-positive subjects is 9%–10% of the general population.[21 22]

Therefore, assuming an objective population of 235 034 inhabitants (residents in the municipality of Verona on 1 January 2020 who are aged at least 10 years—source Istituto nazionale di statistica - ISTAT), a prevalence of asymptomatic SARS-CoV-2-positive individuals of 10.0% and an alpha value (type 1 error) of 5%, then a random sample of 1527 subjects is required to obtain a SE of no more than 1.5%, that is, a 95% CI of lower amplitude or equal to 3%. Assuming a drop-out rate of 35%, it will be necessary to enrol 2061 subjects.

A systematic probabilistic sampling technique will be used to perform the sample list.

Data analysis plan

Demographic and clinical data will be summarised using descriptive statistics, measures of variability and precision, and plots. Statistical tests will be used based on the type of variables, test assumptions and sample dimension. All parameters will be reported with 95% CIs. Statistical models and estimations will be adjusted for covariates if necessary. Information criteria and likelihood ratio methods will be used to compare candidate models.

Test results will be displayed in contingency tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% CIs.

For the LCA, we will use latent class models (LCMs) to boost the diagnostic accuracy of the gold standard test by adding to these models the results of multiple tests used in the same samples, as well as other key information from the CRF.

The basis of LCA is that each subject belongs to one of a finite number of classes; in our study, we have two classes: with and without COVID-19. Each class is described by a set of parameters that define the statistical distribution of outcomes; here the conditional probability is that the subject has or does not have the condition COVID-19 (specificity and sensitivity) and the probability that the condition COVID-19 is present (prevalence).[20] Observations with missing reference standard results will be excluded from the analysis.

DISCUSSION AND CONCLUSIONS

Currently, there are no published data that reliably estimate the prevalence of asymptomatic SARS-CoV-2-positive individuals in Italy. SARS-CoV-2 cases are now reported worldwide but at differing incidences depending on the region. Developed regions with a temperate climate and a medium to high population density seem to be the most affected. However, the true prevalence of asymptomatic SARS-CoV-2-positive individuals is unknown, as is the prevalence of those who have never contracted the virus. Furthermore, it is not yet established whether now recovered, previously SARS-CoV-2-positive individuals can become reinfected.

This study will be useful in understanding the spread of SARS-CoV-2 in a city with a total population of around 260 000 inhabitants and a population density of approximately 1300 inhabitants per square kilometre (ISTAT source). Most importantly, the prevalence of asymptomatic SARS-CoV-2-positive individuals and the prevalence of those who are negative for SARS-CoV-2 can be estimated. This will allow phase II of Italy's outbreak management strategy, in which day-to-day activities will gradually be reintroduced and population contact will resume, to be planned more effectively.

In addition, the study will allow us to better understand the symptoms of SARS-CoV-2 infection and what potential role they may have in disease prognosis. Finally, the study will allow us to evaluate the diagnostic accuracy and value of the ELISA serological test as a screening tool for the general population.

Ethics and dissemination

This protocol will be registered at http://www.clinicaltrials.gov. The full study protocol will be made accessible at a public repository on publication.

This study adheres to the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research and the principles of the Declaration of Helsinki.

The study could involve vulnerable groups within the community, and so it is imperative that the study is conducted in a sensitive and culturally appropriate manner. On an invitation to the study, subjects will be given the opportunity to review all study materials and ask any questions. Furthermore, subjects who feel overwhelmed or anxious at any point during study participation will be referred to an appropriate support service. Subjects will also be reassured that they are free to withdraw from the study at any time without reason or consequence. Results from the study will be disseminated through a presentation at national and international conferences and publications in peer-reviewed journals.

Patient and public involvement statement

Patient and public involvement (PPI) representatives worked with us to refine the research question, however it was difficult to involve patients in other areas of the study design due to the very technical methods required to do a

data linkage analysis. PPI representatives will write a plain language summary and design a leaflet for dissemination to their peers and distributing them to patient groups.

Ethics approval

This study was reviewed and approved by the CESC (Comitato Etico per le Sperimentazioni Cliniche—approval number 28, 17 April 2020).

Author affiliations
[1]Clinical Research Unit, IRCCS Sacro Cuore Don Calabria Hospital, Negrar di Valpolicella, Verona, Italy
[2]Department of Diagnostics and Public Health, University of Verona, Verona, Italy
[3]Department of Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital, Negrar di Valpolicella, Verona, Italy
[4]Unit of Pneumology, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy
[5]Department of Internal Medicine, Unit of Pneumology, IRCCS Sacro Cuore Don Calabria Hospital, Negrar di Valpolicella, Verona, Italy

ORCID iDs
Massimo Guerriero http://orcid.org/0000-0003-1310-539X
Zeno Bisoffi http://orcid.org/0000-0001-9530-8742

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