BMJ Open

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.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-040036
Article Type:	Protocol
Date Submitted by the Author:	06-May-2020
Complete List of Authors:	Guerriero, Massimo; University of Verona, Bisoffi, Zeno; University of Verona, Department of Diagnostics and Public Health; IRCCS Sacro Cuore Don Calabria Hospital, Department of Infectious - Tropical Diseases and Microbiology Poli, Albino; University of Verona, Department of Diagnostics and Public Health Micheletto, Claudio ; Azienda Ospedaliera Universitaria Integrata Verona, Pomari, Carlo; IRCCS Sacro Cuore Don Calabria Hospital, Department of Internal Medicine, Unit of Pneumology
Keywords:	Public health < INFECTIOUS DISEASES, Diagnostic microbiology < INFECTIOUS DISEASES, EPIDEMIOLOGY

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Journal
BMJ Open

Title
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.

Authors

Massimo Guerriero, PhD, Clinical Research Unit, IRCCS Sacro Cuore Don Calabria Hospital, Verona and University of Verona, massimoal.guerriero@gmail.com

Zeno Bisoffi, MD, PhD, Department Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital and Department of Diagnostics and Public Health, Section of Infectious Diseases, University of Verona, zeno.bisoffi@sacrocuore.it

Albino Poli, Department of Diagnostics and Public Health, Section of Hygiene and Preventive, Environmental and Occupational Medicine, University of Verona, albino.poli@univr.it

Claudio Micheletto, MD, Pneumology Unit, Cardio-Thoracic Department, University Hospital Verona, claudio.micheletto@univr.it

Carlo Pomari, MD, Unit of Pneumology, IRCCS Sacro Cuore Don Calabria Hospital, carlo.pomari@sacrocuore.it

Correspondence to:
Prof. Zeno Bisoffi
Department of Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital and Department of Diagnostics and Public Health, Section of Infectious Diseases, University of Verona,
Via Don Sempreboni, 5
37024 Negrar, Italy
Phone: +39 45 6013326
Fax: +39 45 6013694
e-mail: zeno.bisoffi@sacrocuore.it

ABSTRACT

Introduction

As of 30th April 2020, the novel betacoronavirus SARS-CoV-2 has infected more than 3,172,000 individuals, killing over 224,000 people and spreading to more than 200 countries. Italy has been the most affected country in Europe and the third most affected in the world in terms of number of cases.

Therefore, the aims of this study are: i) to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona; ii) 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 old 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, that will be the basis for the estimation of prevalence.

Results

Results from the study will allow us to: i) estimate with a very low margin of error the prevalence of asymptomatic SARS-CoV-2 positive individuals and the prevalence of those who are negative for SARS-CoV-2; ii) better define phase 2 of Italy's outbreak management strategy; iii) better understand the symptoms of SARS-CoV-2 infection and what potential role they may have in disease prognosis; iv) evaluate the diagnostic accuracy and value of the ELISA serological test as a screening tool for the general population.

Ethics and dissemination The study protocol has been approved by the Ethics Committee of Verona and Rovigo provinces on April 15, 2020 (internal protocol number 2641CESC).

Key words
SARS-CoV-2, COVID-19, ELISA serological test, Real-time PCR, Survey Random Sample, Latent Class Analysis

Article Summary section
Strengths and limitations
Study based on 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 response rate

Patient and Public Involvement statement:
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 to patient groups.

INTRODUCTION

As of 30th April 2020, the novel betacoronavirus SARS-CoV-2 has infected more than 3,172,,000 individuals, killing over 224,000 people and spreading to more than 200 countries[1]. Italy has been the most affected country in Europe and the third most affected in the world in terms of number of cases.. The epidemic is posing an extremely difficult challenge to health care 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[2,3,4].

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]. Add that asymptomatic SARS-CoV-2 positive individuals are able to transmit the virus.

Currently, SARS-CoV-2 detection is based on accepted and standardised molecular methods, that require some hours to carry out. 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 IgM and/or IgA and IgG. IgM can be identified in the blood after 3-6 days, while IgG can be detected after 8 days[8]. A recent publication has evaluated the median seroconversion time for antibody 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 antibody was independently associated with a worse clinical classification ($p=0.006$)[9]. Therefore, the aims of this study are:

i. to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona;

ii. to assess the accuracy (sensitivity, specificity and predictive values) of ELISA serological test 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 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 old, and provide consent for the participation to the study and for the donation of biological samples for study purposes.

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

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.

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 (see annex 1) will also be administered and all information related to the logistics and implementation of the study will be provided (e.g. mask and gloves use, methods of sample collection, etc.). 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 care giver), while 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 (PI) 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 anti-contagion equipment.

Auxiliary staff will monitor the movement of people externally and internally 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.
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 twenty-four hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital. Samples will be stored there until their processing and analysis.
Sample collection procedures will take place with 10-minute 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, 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 cases 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 (e-CRF) using the platform OpenClinica.

Measurements

This protocol refers to STARD guidelines[10] for the reporting of diagnostic test accuracy. Based on an assessment methodology already used at the IRCCS in diagnostic studies[11 12], 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, that will be the basis for the estimation of prevalence.

Enzyme-linked immunosorbent assay (ELISA) 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[13] 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 Primary Reference Standard test is a real-time reverse transcription polymerase chain reaction (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 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 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, 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[14,15]. However, this method has its limitations too, as when a CRS is used, its accuracy cannot be assumed "a priori"[16]. Alternative methods to address a lack of a gold standard are latent class models[17]. Latent class analysis (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 lab personnel. Lab 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[18,19].

Therefore, assuming an objective population of 235,034 inhabitants (residents in the municipality of Verona on 1 January 2020 that are aged at least 10 years - source ISTAT), a prevalence of asymptomatic SARS-CoV-2 positive individuals of 10.0% and an alpha value (first species error) of 5%, then a random sample of 1527 subjects is required to obtain a standard error of no more than 1.5%, i.e. a 95% confidence interval 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 sample 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, tests assumptions and sample dimension. All parameters will be reported with 95% confidence intervals. 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 contingence tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% confidence intervals.
For the latent class analysis, we use latent class models (LCM) 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.

In LCM, it is assumed that the subject's true condition is unknown, and it is modelled by two latent classes. Outcomes of this model are interpreted as the probability that the subject has or does not have the condition (specificity and sensitivity) and the probability that the condition 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 in the literature that reliably estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals in Italy or indeed in any part of the world. 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 1,300 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 2 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 upon 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 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 presentation at national and international conferences and publications in peer-reviewed journals.

Acknowledgements

The authors thank Nicoletta De Santis for the editing, Elinor Julie Rae Anderson for language editing and Elvia Malo that contributed to the preparation of documents for ethical approval.

Author Contributions

MG CP conceived the study, MG CP ZB contributed to study design; MG CP contributed to the preparation of the manuscript. MG CP ZB contributed to the preparation of documents for ethical approval including study materials (questionnaire, participant information statement, etc). All authors have contributed to the review of manuscript and are directly involved in the study scientific committee.

Funding. This work was supported by the Italian Ministry of Health "Fondi Ricerca Corrente - Linea 3" to IRCCS Sacro Cuore Don Calabria Hospital.

Patient consent. Required.

Competing interests. None declared

Ethics approval. This study was reviewed and approved by the CESC (Comitato Etico per le Sperimentazioni Cliniche – approval n° 28, 17/04/2020).

Provenance and peer review. Not commissioned; externally peer reviewed.

Word count: 4,185.

Data statement section: The authors confirm that they will have full access to all data and as such, take responsibility for the integrity of said data and the accuracy of all data analysis. Data will be publish in a data repository

References

1. World Health Organization's COVID-19 situation report 64- 24/03/2020 https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200324-sitrep-64-covid-19.pdf?sfvrsn=703b2c40_2 accessed 25th March 2020

2. Chan JF, Yuan S, Kok KH, *et al*. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 2020; 395:514

3. Liu YC, Liao CH, Chang CF, *et al*. A Locally Transmitted Case of SARS-CoV-2 Infection in Taiwan. *N Engl J Med* 2020; 382:1070

4. Wei M, Yuan J, Liu Y, *et al.* Novel Coronavirus Infection in Hospitalized Infants Under 1 Year of Age in China. *JAMA* 2020 doi:10.1001/jama.2020.2131

5. Field Briefing: Diamond Princess COVID-19 Cases, 20 Feb 2020 Update, https://www.niid.go.jp/niid/en/2019-ncov-e/9417-covid-dp-fe-02.html

6. Rocklöv J,Sjödin H,Wilder-Smith A. COVID-19 outbreak on the Diamond Princess cruise ship: estimating the epidemic potential and effectiveness of public health counter measures, *Journal of Travel Medicine* 2020; https://doi.org/10.1093/jtm/taaa030

7. Nishiura H, Kobayashi T, Miyama T, Suzuki A, Jung S, Hayashi K *et al*. Estimation of the asymptomatic ratio of novel coronavirus infections (COVID-19). medRxiv 2020; www.eurosurveillance.org https://doi.org/http://dx.doi.org/10.1101/2020.02.03.20020248

8. Kimball A, Hatfield KM, Arons M *et al.* Asymptomatic and Presymptomatic SARS-CoV-2 Infections in Residents of a Long-Term Care Skilled Nursing Facility — King County, Washington , *MMWR Morb Mortal Wkly Rep* 2020

9. Lee BH, Seok SH *et al*. Production of specific antibodies against SARS- coronavirus nucleocapsid protein without cross reactivity with human coronaviruses 229E and OC43. *J Vet Sci.* 2010;11(2):165-7

10. Zhao J, Yuan Q, Wang H *et al.* Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis*. 2020 ; doi:10.1093/cid/ciaa344

11. La Vecchia C. Doxa: potrebbero essere oltre 5 milioni gli italiani infettati da COVID-19 a marzo 2020. 2020; *La Statale News Università degli Studi di Milano*; lastatalenews.unimi.it/doxa-potrebbero-essere-oltre-5-milioni-italiani-infettati-covid-19-marzo-2020

12. Giuseppe De Natale, The COVID-19 infection in Italy: a statistical study of an abnormally severe 2 disease. pre print, *medRxiv* https://www.medrxiv.org/content/10.1101/2020.03.28.20046243v1.full.pdf

13. Cohen JF *et al.* STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. BMJ Open 2016 ; 6(11): e012799. doi: 10.1136/bmjopen-2016-012799

14. Buonfrate D et al. Accuracy of Five Serologic Tests for the Follow up of Strongyloides stercoralis Infection. *PLOS Neglected Tropical Diseases* 2015;9(2):e0003491. doi: 10.1371/journal.pntd.0003491

15. Beltrame A , et al. Accuracy of parasitological and immunological tests for the screening of human schistosomiasis in immigrants and refugees from African countries: an approach with latent class analysis. *PLOS Neglected Tropical Diseases* 2017;11(6):e0005593. doi: 10.1371/journal.pntd.0005593

16. Okba N MA *et al.* SARS-CoV-2 specific antibody responses in COVID-19 patients. *Emerging Infectious Diseases* 2020; doi: 10.3201/eid2607.200841

17. Rutjes AW, Reitsma JB, Coomarasamy A, et al. Evaluation of diagnostic tests when there is no gold standard. A review of methods. *Health technology assessment* 2007;11(50):iii, ix-51

18. Reitsma JB, Rutjes AW, Khan KS, et al. A review of solutions for diagnostic accuracy studies with an imperfect or missing reference standard. *Journal of clinical epidemiology* 2009;62(8):797-806

19. Dendukuri N, Schiller I, de Groot J, Libman M, Moons K, Reitsma J, van Smeden M. Concerns About Composite Reference Standards in Diagnostic Research *BMJ* 2018;360:j5779. doi: 10.1136/bmj.j5779

20. Goetghebeur E, Liinev J, Boelaert M, Van der Stuyft P. Diagnostic test analyses in search of their gold standard: latent class analyses with random effects. *Stat Methods Med Res* 2000;9(3):231-48. doi: 10.1177/096228020000900304

ANNEX 1

QUESTIONNAIRE
Estimate of the prevalence of asymptomatic subjects affected by COVID-19 in residents of the municipality of Verona

1. Subject Id _____

2. Issuing informed consent
1Yes 0No

3. Enrollment date ____ / ____ / _____

4. Date of birth ____ / ____ / _____

5. Sex
1 Male 0 Female

7. Have you already been diagnosed with Covid-19 positivity?
0=No 1=Yes, with swab 2=Yes, with rapid test 3=Yes, with venous sampling
7.1 If yes, on what date or how many days ago? _____

8. How often are you vaccinated for seasonal flu?
1=Regularly 2=Occasionally 3=Never

9. Presence of comorbidities
1Yes 0No

9.1 If yes, which diseases are these?
1=Pulmonary 2=Cardiological 3=Hypertension 4=Oncological 5=Renal 6=Immunological
7=Metabolic 8=Rheumatological 9=Hepatic 10=Depression and / or anxiety 11=Other

In the past two weeks
10. Have you had loss of taste and / or smell?
1Yes 0No

11. Did you experience burning or feeling of sand in your eyes (conjunctivitis)?
1Yes 0No

12. Have you had a fever (> 37.5)?
1Yes 0No

13. Have you had dry cough and / or productive cough (phlegm)?
1Yes 0No

14. Have you suffered from general muscle pain?

1Yes 0No

15. Have you had an unjustified feeling of tiredness / general asthenia?
1Yes 0No

16. Have you had a headache?
1Yes 0No

17. Have you had a sore throat?
1Yes 0No

18. Did you have chills?
1Yes 0No

19. Have you had diarrhea?
1Yes 0No

20. Have you suffered from shortness of breath / or dyspnea?
1Yes 0No

21. Have you had nausea / vomiting?
1Yes 0No

From 10 March 2020

22. With whom do you live at home (or other residential facility)?
1=alone 2=with another person 3=with more than one other person

23. For any reason, did you leave the house (or other residential facility)?
1Yes 0No

24. Have you had direct contacts (for at least 15 continuous minutes) with people other than your potential cohabitants?
1=Yes 2=No 3=I don't have cohabitants

25. Did any of your cohabitants, if any, leave the house / other facility?
1=Yes 2=No 3=I don't have cohabitants

26. Are you aware that you have been in contact with a positive person at COVID-19?
1=Yes 2=No 3=I am not aware of it

27. Did you use protective equipment during the emergency?
1=Yes, masks and gloves 2=Yes, only masks 3=No

28. Do you currently use protective equipment?
1=Yes, masks and gloves 2=Yes, only masks 3=No

BMJ Open

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.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-040036.R1
Article Type:	Protocol
Date Submitted by the Author:	09-Aug-2020
Complete List of Authors:	Guerriero, Massimo; University of Verona, Bisoffi, Zeno; University of Verona, Department of Diagnostics and Public Health; IRCCS Sacro Cuore Don Calabria Hospital, Department of Infectious - Tropical Diseases and Microbiology Poli, Albino; University of Verona, Department of Diagnostics and Public Health Micheletto, Claudio ; Azienda Ospedaliera Universitaria Integrata Verona, Pomari, Carlo; IRCCS Sacro Cuore Don Calabria Hospital, Department of Internal Medicine, Unit of Pneumology
Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Immunology (including allergy), Epidemiology, Diagnostics
Keywords:	Public health < INFECTIOUS DISEASES, Diagnostic microbiology < INFECTIOUS DISEASES, EPIDEMIOLOGY, Molecular diagnostics < INFECTIOUS DISEASES

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Journal

BMJ Open

Title

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.

Authors

Massimo Guerriero, PhD, Clinical Research Unit, IRCCS Sacro Cuore Don Calabria Hospital, Verona and University of Verona, massimoal.guerriero@gmail.com

Zeno Bisoffi, MD, PhD, Department Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital and Department of Diagnostics and Public Health, Section of Infectious Diseases, University of Verona, zeno.bisoffi@sacrocuore.it

Albino Poli, Department of Diagnostics and Public Health, Section of Hygiene and Preventive, Environmental and Occupational Medicine, University of Verona, albino.poli@univr.it

Claudio Micheletto, MD, Pneumology Unit, Cardio-Thoracic Department, University Hospital Verona, claudio.micheletto@univr.it

Carlo Pomari, MD, Unit of Pneumology, IRCCS Sacro Cuore Don Calabria Hospital, carlo.pomari@sacrocuore.it

Correspondence to:

Prof. Zeno Bisoffi

Department of Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital and Department of Diagnostics and Public Health, Section of Infectious Diseases, University of Verona,

Via Don Sempreboni, 5

37024 Negrar, Italy

Phone: +39 45 6013326

Fax: +39 45 6013694

e-mail: zeno.bisoffi@sacrocuore.it

ABSTRACT

Introduction

As of 30th April 2020, the novel betacoronavirus SARS-CoV-2 (**Severe Acute Respiratory Syndrome Coronavirus 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 number of cases.

Therefore, the aims of this study are: i) to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona; ii) 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 old 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, that will be the basis for the estimation of prevalence.

Results

Results from the study will allow us to: i) estimate with a very low margin of error the prevalence of asymptomatic SARS-CoV-2 positive individuals and the prevalence of those who are negative for SARS-CoV-2; ii) better define phase 2 of Italy's outbreak management strategy; iii) better understand the symptoms of SARS-CoV-2 infection and what potential role they may have in disease prognosis; iv) evaluate the diagnostic accuracy and value of the ELISA serological test as a screening tool for the general population.

Ethics and dissemination The study protocol has been approved by the Ethics Committee of Verona and Rovigo provinces on April 15, 2020 (internal protocol number 2641CESC).

Key words

SARS-CoV-2, COVID-19, ELISA serological test, Real-time PCR, Survey Random Sample, Latent Class Analysis

Article Summary section

Strengths and limitations

Study based on 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 response rate

Patient and Public Involvement statement:

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 to patient groups.

INTRODUCTION

As of 30th 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 world in terms of number of cases. The epidemic was posing an extremely difficult challenge to health care 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[2,3,4].

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 lab, although more rapid molecular test 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 IgM 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 corona viruses[8]. A recent publication has evaluated the median seroconversion time for antibody 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 antibody was independently associated with a worse clinical classification ($p=0.006$)[9]. Therefore, the aims of this study are:

i. to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona;

ii. 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 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 old, and provide consent for the participation to the study and for the donation of biological samples for study purposes.
Subjects will be selected at random 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 25th 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.
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 (see annex 1) will also be administered and all information related to the logistics and implementation of the

study will be provided (e.g. mask and gloves use, methods of sample collection, etc.). 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 care giver), while 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 (PI) 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 anti-contagion equipment.

Auxiliary staff will monitor the movement of people externally and internally 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 twenty-four hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital. Blood samples will be immediately stored there upon reception at -80°C, until their processing and analysis that will be carried out in the following weeks, while swabs are processed upon reception, and then also stored at -80°C.

Sample collection procedures will take place with 10-minute 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, 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 cases 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 (e-CRF) using the platform OpenClinica.

Measurements

This protocol refers to 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, that will be also the basis for the estimation of prevalence.

Enzyme-linked immunosorbent assay (ELISA) 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 Inc, USA) is a chemiluminescent microparticle immunoassay (CMIA) 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 Euroimmun test were found to be 78.3% and 96.7% for IgG, and 86.7% and 82.7% for IgA; for 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. This 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 polymerase chain reaction (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 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 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, 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 latent class models[20]. Latent class analysis (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 lab personnel. Lab 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 that are aged at least 10 years - source ISTAT), a prevalence of asymptomatic SARS-CoV-2 positive individuals of 10.0% and an alpha value (first species error) of 5%, then a random sample of 1527 subjects is required to obtain a standard error of no more than 1.5%, i.e. a 95% confidence interval 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 sample 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, tests assumptions and sample dimension. All parameters will be reported with 95% confidence intervals. 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 contingence tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% confidence intervals.

For the latent class analysis, we use latent class models (LCM) 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 probability 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 1,300 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 2 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 upon 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 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 presentation at national and international conferences and publications in peer-reviewed journals.

Acknowledgements

The authors thank Nicoletta De Santis for the editing, Elinor Julie Rae Anderson for language editing and Elvia Malo that contributed to the preparation of documents for ethical approval.

Author Contributions

MG CP conceived the study, MG CP ZB AP CM contributed to study design; MG CP ZB contributed to the preparation of the manuscript. MG CP ZB contributed to the preparation of documents for ethical approval including study materials (questionnaire, participant information statement, etc). All authors have contributed to the review of manuscript and are directly involved in the study scientific committee.

Funding. This work was supported by the Italian Ministry of Health “Fondi Ricerca Corrente - Linea 3” to IRCCS Sacro Cuore Don Calabria Hospital.

Patient consent. Required.

Competing interests. None declared

Ethics approval. This study was reviewed and approved by the CESC (Comitato Etico per le Sperimentazioni Cliniche – approval n° 28, 17/04/2020).

Provenance and peer review. Not commissioned; externally peer reviewed.

Word count: 2,807.

Data statement section: The authors confirm that they will have full access to all data and as such, take responsibility for the integrity of said data and the accuracy of all data analysis. Data will be publish in a data repository

References

1. World Health Organization's COVID-19 situation report 64- 24/03/2020 https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200324-sitrep-64-covid-19.pdf?sfvrsn=703b2c40_2 accessed 25th March 2020

2. Chan JF, Yuan S, Kok KH, *et al*. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 2020; 395:514

3. Mizumoto K, Kagaya K, Zarebski A, Chowell G . Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. Euro Surveill. 2020;25(10):pii=2000180. https://doi.org/10.2807/1560-7917.

4. Wei M, Yuan J, Liu Y, *et al.* Novel Coronavirus Infection in Hospitalized Infants Under 1 Year of Age in China. *JAMA* 2020 doi:10.1001/jama.2020.2131

5. Field Briefing: Diamond Princess COVID-19 Cases, 20 Feb 2020 Update, https://www.niid.go.jp/niid/en/2019-ncov-e/9417-covid-dp-fe-02.html

6. Nishiura H, Kobayashi T, Miyama T, Suzuki A, Jung S, Hayashi K *et al*. Estimation of the asymptomatic ratio of novel coronavirus infections (COVID-19). medRxiv 2020; www.eurosurveillance.org https://doi.org/http://dx.doi.org/10.1101/2020.02.03.20020248

7. Kimball A, Hatfield KM, Arons M *et al.* Asymptomatic and Presymptomatic SARS-CoV-2 Infections in Residents of a Long-Term Care Skilled Nursing Facility — King County, Washington , *MMWR Morb Mortal Wkly Rep* 2020

8. Lee HK, Lee BH, Seok SH *et al*. Production of specific antibodies against SARS-coronavirus nucleocapsid protein without cross reactivity with human coronaviruses 229E and OC43. *J Vet Sci.* 2010;11(2):165-7

9. Zhao J, Yuan Q, Wang H *et al.* Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis*. 2020 ; doi:10.1093/cid/ciaa344

10. Azienda Zero – Regione del Veneto, SARS-CoV-2 in Veneto [Accessed May 2020]. Available from: https://www.azero.veneto.it/-/emergenza-coronavirus

11. Cohen JF *et al.* STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. BMJ Open 2016 ; 6(11): e012799. doi: 10.1136/bmjopen-2016- 012799

12. Buonfrate D et al. Accuracy of Five Serologic Tests for the Follow up of Strongyloides stercoralis Infection. *PLOS Neglected Tropical Diseases* 2015;9(2):e0003491. doi: 10.1371/journal.pntd.0003491

13. Beltrame A , et al. Accuracy of parasitological and immunological tests for the screening of human schistosomiasis in immigrants and refugees from African countries: an approach with latent class analysis. *PLOS Neglected Tropical Diseases* 2017;11(6):e0005593. doi: 10.1371/journal.pntd.0005593

14. Okba N MA *et al.* SARS-CoV-2 specific antibody responses in COVID-19 patients. *Emerging Infectious Diseases* 2020; doi: 10.3201/eid2607.200841

15. Nicol T, Lefeuvre C, Serri O, et al. Assessment of SARS-CoV-2 serological tests for the diagnosis of COVID-19 through the evaluation of three immunoassays: Two automated immunoassays (Euroimmun and Abbott) and one rapid lateral flow immunoassay (NG Biotech). Journal of Clinical Virology: the Official Publication of the Pan American Society for Clinical Virology. 2020 Aug;129:104511. DOI: 10.1016/j.jcv.2020.104511

16. Strömer A, Grobe O, Rose R, Fickenscher H, Lorentz T, Krumbholz A Diagnostic accuracy of six commercial SARS-CoV-2 IgG/total antibody assays and identification of SARS-CoV-2 neutralizing antibodies in convalescent sera, medRxiv 2020.06.15.20131672; doi: https://doi.org/10.1101/2020.06.15.20131672

17. Rutjes AW, Reitsma JB, Coomarasamy A, et al. Evaluation of diagnostic tests when there is no gold standard. A review of methods. *Health technology assessment* 2007;11(50):iii, ix-51

18. Reitsma JB, Rutjes AW, Khan KS, et al. A review of solutions for diagnostic accuracy studies with an imperfect or missing reference standard. *Journal of clinical epidemiology* 2009;62(8):797-806

19. Dendukuri N, Schiller I, de Groot J, Libman M, Moons K, Reitsma J, van Smeden M. Concerns About Composite Reference Standards in Diagnostic Research *BMJ* 2018;360:j5779. doi: 10.1136/bmj.j5779

20. Goetghebeur E, Liinev J, Boelaert M, Van der Stuyft P. Diagnostic test analyses in search of their gold standard: latent class analyses with random effects. *Stat Methods Med Res* 2000;9(3):231-48. doi: 10.1177/096228020000900304

21. La Vecchia C. Doxa: potrebbero essere oltre 5 milioni gli italiani infettati da COVID-19 a marzo 2020. 2020; *La Statale News Università degli Studi di Milano*; lastatalenews.unimi.it/doxa-potrebbero-essere-oltre-5-milioni-italiani-infettati-covid-19-marzo-2020

22. Giuseppe De Natale, The COVID-19 infection in Italy: a statistical study of an abnormally severe 2 disease. pre print, *medRxiv* https://www.medrxiv.org/content/10.1101/2020.03.28.20046243v1.full.pdf

ANNEX 1

QUESTIONNAIRE
Estimate of the prevalence of asymptomatic subjects affected by COVID-19 in residents of the municipality of Verona

1. Subject Id _____

2. Issuing informed consent
1Yes 0No

3. Enrollment date ____ / ____ / _____

4. Date of birth ____ / ____ / _____

5. Sex
1 Male 0 Female

7. Have you already been diagnosed with Covid-19 positivity?
0=No 1=Yes, with swab 2=Yes, with rapid test 3=Yes, with venous sampling
7.1 If yes, on what date or how many days ago? _____

8. How often are you vaccinated for seasonal flu?
1=Regularly 2=Occasionally 3=Never

9. Presence of comorbidities
1Yes 0No

9.1 If yes, which diseases are these?
1=Pulmonary 2=Cardiological 3=Hypertension 4=Oncological 5=Renal 6=Immunological
7=Metabolic 8=Rheumatological 9=Hepatic 10=Depression and / or anxiety 11=Other

In the past two weeks
10. Have you had loss of taste and / or smell?
1Yes 0No

11. Did you experience burning or feeling of sand in your eyes (conjunctivitis)?
1Yes 0No

12. Have you had a fever (> 37.5)?
1Yes 0No

13. Have you had dry cough and / or productive cough (phlegm)?
1Yes 0No

14. Have you suffered from general muscle pain?
1Yes 0No

15. Have you had an unjustified feeling of tiredness / general asthenia?

1Yes 0No

16. Have you had a headache?
1Yes 0No

17. Have you had a sore throat?
1Yes 0No

18. Did you have chills?
1Yes 0No

19. Have you had diarrhea?
1Yes 0No

20. Have you suffered from shortness of breath / or dyspnea?
1Yes 0No

21. Have you had nausea / vomiting?
1Yes 0No

From 10 March 2020

22. With whom do you live at home (or other residential facility)?
1=alone 2=with another person 3=with more than one other person

23. For any reason, did you leave the house (or other residential facility)?
1Yes 0No

24. Have you had direct contacts (for at least 15 continuous minutes) with people other than your potential cohabitants?
1=Yes 2=No 3=I don't have cohabitants

25. Did any of your cohabitants, if any, leave the house / other facility?
1=Yes 2=No 3=I don't have cohabitants

26. Are you aware that you have been in contact with a positive person at COVID-19?
1=Yes 2=No 3=I am not aware of it

27. Did you use protective equipment during the emergency?
1=Yes, masks and gloves 2=Yes, only masks 3=No

28. Do you currently use protective equipment?
1=Yes, masks and gloves 2=Yes, only masks 3=No

BMJ Open

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.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-040036.R2
Article Type:	Protocol
Date Submitted by the Author:	09-Sep-2020
Complete List of Authors:	Guerriero, Massimo; University of Verona, Bisoffi, Zeno; University of Verona, Department of Diagnostics and Public Health; IRCCS Sacro Cuore Don Calabria Hospital, Department of Infectious - Tropical Diseases and Microbiology Poli, Albino; University of Verona, Department of Diagnostics and Public Health Micheletto, Claudio ; Azienda Ospedaliera Universitaria Integrata Verona, Pomari, Carlo; IRCCS Sacro Cuore Don Calabria Hospital, Department of Internal Medicine, Unit of Pneumology
Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Immunology (including allergy), Epidemiology, Diagnostics
Keywords:	Public health < INFECTIOUS DISEASES, Diagnostic microbiology < INFECTIOUS DISEASES, EPIDEMIOLOGY, Molecular diagnostics < INFECTIOUS DISEASES

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Journal

BMJ Open

Title

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.

Authors

Massimo Guerriero, PhD, Clinical Research Unit, IRCCS Sacro Cuore Don Calabria Hospital, Verona and University of Verona, massimoal.guerriero@gmail.com

Zeno Bisoffi, MD, PhD, Department Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital and Department of Diagnostics and Public Health, Section of Infectious Diseases, University of Verona, zeno.bisoffi@sacrocuore.it

Albino Poli, Department of Diagnostics and Public Health, Section of Hygiene and Preventive, Environmental and Occupational Medicine, University of Verona, albino.poli@univr.it

Claudio Micheletto, MD, Pneumology Unit, Cardio-Thoracic Department, University Hospital Verona, claudio.micheletto@univr.it

Carlo Pomari, MD, Unit of Pneumology, IRCCS Sacro Cuore Don Calabria Hospital, carlo.pomari@sacrocuore.it

Correspondence to:

Prof. Zeno Bisoffi

Department of Infectious-Tropical Diseases and Microbiology, IRCCS Sacro Cuore Don Calabria Hospital and Department of Diagnostics and Public Health, Section of Infectious Diseases, University of Verona,

Via Don Sempreboni, 5

37024 Negrar, Italy

Phone: +39 45 6013326

Fax: +39 45 6013694

e-mail: zeno.bisoffi@sacrocuore.it

ABSTRACT

Introduction

As of 30th April 2020, the novel betacoronavirus SARS-CoV-2 (**Severe Acute Respiratory Syndrome Coronavirus 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 number of cases.

Therefore, the aims of this study are: i) to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona; ii) 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 old 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, that 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 April 15, 2020 (internal protocol number 2641CESC). The study results will be submitted for publication in international, peer reviewed journals and the complete data set 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.

Key words

SARS-CoV-2, COVID-19, ELISA serological test, Real-time PCR, Survey Random Sample, Latent Class Analysis

Article Summary section

Strengths and limitations

Study based on 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 response rate

Patient and Public Involvement statement:

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 to patient groups.

INTRODUCTION

As of 30th 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 world in terms of number of cases. The epidemic was posing an extremely difficult challenge to health care 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[2,3,4].

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 lab, although more rapid molecular test 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 IgM 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 corona viruses[8]. A recent publication has evaluated the median seroconversion time for antibody 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 antibody was independently associated with a worse clinical classification ($p=0.006$)[9]. Therefore, the aims of this study are:

i. to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona;

ii. 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 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 old, and provide consent for the participation to the study and for the donation of biological samples for study purposes.

Subjects will be selected at random 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 25th 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.

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 (see annex 1) will also be administered and all information related to the logistics and implementation of the study will be provided (e.g. mask and gloves use, methods of sample collection, etc.). 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 care giver), while 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 (PI) 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 anti-contagion 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 twenty-four hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital. Blood samples will be immediately stored there upon reception at -80°C, until their processing and analysis that will be carried out in the following weeks, while swabs are processed upon reception, and then also stored at -80°C.

Sample collection procedures will take place with 10-minute 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, 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 (e-CRF) using the platform OpenClinica.

Measurements

This protocol refers to 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, that will be also the basis for the estimation of prevalence.

Enzyme-linked immunosorbent assay (ELISA) 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 Inc, USA) is a chemiluminescent microparticle immunoassay (CMIA) 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 Euroimmun test were found to be 78.3% and 96.7% for IgG, and 86.7% and 82.7% for IgA; for 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 polymerase chain reaction (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 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 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, 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 latent class models[20]. Latent class analysis (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 lab personnel. Lab 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 that are aged at least 10 years - source 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 standard error of no more than 1.5%, i.e. a 95% confidence interval 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, tests assumptions and sample dimension. All parameters will be reported with 95% confidence intervals. 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 contingence tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% confidence intervals.

For the latent class analysis, we will use latent class models (LCM) 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 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 1,300 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 2 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 upon 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 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 presentation at national and international conferences and publications in peer-reviewed journals.

Acknowledgements

The authors thank Nicoletta De Santis for the editing, Elinor Julie Rae Anderson for language editing and Elvia Malo that contributed to the preparation of documents for ethical approval.

Author Contributions

MG CP conceived the study, MG CP ZB AP CM contributed to study design; MG CP ZB contributed to the preparation of the manuscript. MG CP ZB contributed to the preparation of documents for ethical approval including study materials (questionnaire, participant information statement, etc). All authors have contributed to the review of manuscript and are directly involved in the study scientific committee.

Funding. This work was supported by the Italian Ministry of Health “Fondi Ricerca Corrente - Linea 3” to IRCCS Sacro Cuore Don Calabria Hospital.

Patient consent. Required.

Competing interests. None declared

Ethics approval. This study was reviewed and approved by the CESC (Comitato Etico per le Sperimentazioni Cliniche – approval n° 28, 17/04/2020).

Provenance and peer review. Not commissioned; externally peer reviewed.

Word count: 2,796.

Data statement section: The authors confirm that they will have full access to all data and as such, take responsibility for the integrity of said data and the accuracy of all data analysis. Data will be published in a data repository

References

1. World Health Organization's COVID-19 situation report 64- 24/03/2020 https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200324-sitrep-64-covid-19.pdf?sfvrsn=703b2c40_2 accessed 25th March 2020

2. Chan JF, Yuan S, Kok KH, *et al*. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 2020; 395:514

3. Mizumoto K, Kagaya K, Zarebski A, Chowell G . Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. Euro Surveill. 2020;25(10):pii=2000180. https://doi.org/10.2807/1560-7917.

4. Wei M, Yuan J, Liu Y, *et al.* Novel Coronavirus Infection in Hospitalized Infants Under 1 Year of Age in China. *JAMA* 2020 doi:10.1001/jama.2020.2131

5. Field Briefing: Diamond Princess COVID-19 Cases, 20 Feb 2020 Update, https://www.niid.go.jp/niid/en/2019-ncov-e/9417-covid-dp-fe-02.html

6. Nishiura H, Kobayashi T, Miyama T, Suzuki A, Jung S, Hayashi K *et al*. Estimation of the asymptomatic ratio of novel coronavirus infections (COVID-19). medRxiv 2020; www.eurosurveillance.org https://doi.org/http://dx.doi.org/10.1101/2020.02.03.20020248

7. Kimball A, Hatfield KM, Arons M *et al.* Asymptomatic and Presymptomatic SARS-CoV-2 Infections in Residents of a Long-Term Care Skilled Nursing Facility — King County, Washington , *MMWR Morb Mortal Wkly Rep* 2020

8. Lee HK, Lee BH, Seok SH *et al*. Production of specific antibodies against SARS-coronavirus nucleocapsid protein without cross reactivity with human coronaviruses 229E and OC43. *J Vet Sci.* 2010;11(2):165-7

9. Zhao J, Yuan Q, Wang H *et al.* Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis*. 2020 ; doi:10.1093/cid/ciaa344

10. Azienda Zero – Regione del Veneto, SARS-CoV-2 in Veneto [Accessed May 2020]. Available from: https://www.azero.veneto.it/-/emergenza-coronavirus

11. Cohen JF *et al.* STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. BMJ Open 2016 ; 6(11): e012799. doi: 10.1136/bmjopen-2016- 012799

12. Buonfrate D et al. Accuracy of Five Serologic Tests for the Follow up of Strongyloides stercoralis Infection. *PLOS Neglected Tropical Diseases* 2015;9(2):e0003491. doi: 10.1371/journal.pntd.0003491

13. Beltrame A , et al. Accuracy of parasitological and immunological tests for the screening of human schistosomiasis in immigrants and refugees from African countries: an approach with latent class analysis. *PLOS Neglected Tropical Diseases* 2017;11(6):e0005593. doi: 10.1371/journal.pntd.0005593

14. Okba N MA *et al.* SARS-CoV-2 specific antibody responses in COVID-19 patients. *Emerging Infectious Diseases* 2020; doi: 10.3201/eid2607.200841

15. Nicol T, Lefeuvre C, Serri O, et al. Assessment of SARS-CoV-2 serological tests for the diagnosis of COVID-19 through the evaluation of three immunoassays: Two automated immunoassays (Euroimmun and Abbott) and one rapid lateral flow immunoassay (NG Biotech). Journal of Clinical Virology: the Official Publication of the Pan American Society for Clinical Virology. 2020 Aug;129:104511. DOI: 10.1016/j.jcv.2020.104511

16. Strömer A, Grobe O, Rose R, Fickenscher H, Lorentz T, Krumbholz A Diagnostic accuracy of six commercial SARS-CoV-2 IgG/total antibody assays and identification of SARS-CoV-2 neutralizing antibodies in convalescent sera, medRxiv 2020.06.15.20131672; doi: https://doi.org/10.1101/2020.06.15.20131672

17. Rutjes AW, Reitsma JB, Coomarasamy A, et al. Evaluation of diagnostic tests when there is no gold standard. A review of methods. *Health technology assessment* 2007;11(50):iii, ix-51

18. Reitsma JB, Rutjes AW, Khan KS, et al. A review of solutions for diagnostic accuracy studies with an imperfect or missing reference standard. *Journal of clinical epidemiology* 2009;62(8):797-806

19. Dendukuri N, Schiller I, de Groot J, Libman M, Moons K, Reitsma J, van Smeden M. Concerns About Composite Reference Standards in Diagnostic Research *BMJ* 2018;360:j5779. doi: 10.1136/bmj.j5779

20. Goetghebeur E, Liinev J, Boelaert M, Van der Stuyft P. Diagnostic test analyses in search of their gold standard: latent class analyses with random effects. *Stat Methods Med Res* 2000;9(3):231-48. doi: 10.1177/096228020000900304

21. La Vecchia C. Doxa: potrebbero essere oltre 5 milioni gli italiani infettati da COVID-19 a marzo 2020. 2020; *La Statale News Università degli Studi di Milano*; lastatalenews.unimi.it/doxa-potrebbero-essere-oltre-5-milioni-italiani-infettati-covid-19-marzo-2020

22. Giuseppe De Natale, The COVID-19 infection in Italy: a statistical study of an abnormally severe 2 disease. pre print, *medRxiv* https://www.medrxiv.org/content/10.1101/2020.03.28.20046243v1.full.pdf

ANNEX 1

QUESTIONNAIRE
Estimate of the prevalence of asymptomatic subjects affected by COVID-19 in residents of the municipality of Verona

1. Subject Id _____

2. Issuing informed consent
1Yes 0No

3. Enrollment date ____ / ____ / _____

4. Date of birth ____ / ____ / _____

5. Sex
1 Male 0 Female

7. Have you already been diagnosed with Covid-19 positivity?
0=No 1=Yes, with swab 2=Yes, with rapid test 3=Yes, with venous sampling
7.1 If yes, on what date or how many days ago? _____

8. How often are you vaccinated for seasonal flu?
1=Regularly 2=Occasionally 3=Never

9. Presence of comorbidities
1Yes 0No

9.1 If yes, which diseases are these?
1=Pulmonary 2=Cardiological 3=Hypertension 4=Oncological 5=Renal 6=Immunological
7=Metabolic 8=Rheumatological 9=Hepatic 10=Depression and / or anxiety 11=Other

In the past two weeks
10. Have you had loss of taste and / or smell?
1Yes 0No

11. Did you experience burning or feeling of sand in your eyes (conjunctivitis)?
1Yes 0No

12. Have you had a fever (> 37.5)?
1Yes 0No

13. Have you had dry cough and / or productive cough (phlegm)?
1Yes 0No

14. Have you suffered from general muscle pain?
1Yes 0No

15. Have you had an unjustified feeling of tiredness / general asthenia?

1Yes 0No

16. Have you had a headache?
1Yes 0No

17. Have you had a sore throat?
1Yes 0No

18. Did you have chills?
1Yes 0No

19. Have you had diarrhea?
1Yes 0No

20. Have you suffered from shortness of breath / or dyspnea?
1Yes 0No

21. Have you had nausea / vomiting?
1Yes 0No

From 10 March 2020

22. With whom do you live at home (or other residential facility)?
1=alone 2=with another person 3=with more than one other person

23. For any reason, did you leave the house (or other residential facility)?
1Yes 0No

24. Have you had direct contacts (for at least 15 continuous minutes) with people other than your potential cohabitants?
1=Yes 2=No 3=I don't have cohabitants

25. Did any of your cohabitants, if any, leave the house / other facility?
1=Yes 2=No 3=I don't have cohabitants

26. Are you aware that you have been in contact with a positive person at COVID-19?
1=Yes 2=No 3=I am not aware of it

27. Did you use protective equipment during the emergency?
1=Yes, masks and gloves 2=Yes, only masks 3=No

28. Do you currently use protective equipment?
1=Yes, masks and gloves 2=Yes, only masks 3=No