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COVID-19 and lombardy: TESTing the impact of the first wave of the pandemic

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ABSTRACT

Background: Italy was the first western country to experience a large Coronavirus Disease 2019 (COVID-19) outbreak and the province of Bergamo experienced one of the deadliest COVID-19 outbreaks in the world. Following the peak of the epidemic in mid-March, the curve has slowly fallen thanks to the strict lockdown imposed by the Italian government on 9th March 2020.

Methods: We performed a cross-sectional study to assess the prevalence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection in 423 workers in Bergamo province who returned to the workplace after the end of the Italian lockdown on 5th May 2020. To this end, we performed an enzyme-linked immunosorbent assay (ELISA) to detect the humoral response against SARS-CoV-2 and a nasopharyngeal swab to assess the presence of SARS-CoV-2 RNA by real-time reverse transcription polymerase chain reaction (rRT-PCR). As a secondary aim of the study, we validated a lateral flow immunochromatography assay (LFIA) for the detection of anti-SARS-CoV-2 antibodies.

Findings: ELISA identified 38.5% positive subjects, of whom 51.5% were positive for both IgG and IgM, 47.3% were positive only for IgG, but only 1.2% were positive for IgM alone. Only 23 (5.4%) participants tested positive for SARS-CoV-2 by rRT-PCR, although with high cycle thresholds (between 34 and 39), indicating a very low residual viral load that was not able to infect cultured cells. All these rRT-PCR positive subjects had already experienced seroconversion. When the ELISA was used as the comparator, the estimated specificity and sensitivity of the rapid LFIA for IgG were 98% and 92%, respectively.

Interpretation: the prevalence of SARS-CoV-2 infection in the province of Bergamo reached 38.5%, significantly higher than has been reported for most other regions worldwide. Few nasopharyngeal swabs tested positive in fully recovered subjects, though with a very low SARS-CoV-2 viral load, with implications for infectivity and discharge policies for positive individuals in the post-pandemic period. The rapid LFIA used in this study is a valuable tool for rapid serologic surveillance of COVID-19 for population studies.

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1. Introduction

A novel coronavirus was identified in a cluster of patients with unexplained pneumonia in Wuhan, China, in December 2019. By January 2020, this highly infectious pathogen, named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), had been isolated and sequenced, revealing a close relationships with the coronaviruses that caused SARS and MERS in 2003 and 2012, respectively [1]. This novel coronavirus causes a severe respiratory illness that has been termed Coronavirus Disease 2019 (COVID-19), which can lead to significant morbidity and mortality in a proportion of patients.

Following the outbreak in China, Italy was the first Western country to experience a massive COVID-19 outbreak, with the first community-acquired cases reported on 20th February 2020. Since then, over 300,000 laboratory-confirmed COVID-19 cases and over 35,000 deaths have been reported. The Lombardy region was the epicenter of the Italian COVID-19 pandemic, with 105,000 reported SARS-CoV-2 infections and 17,000 fatalities as of September 25th 2020 [2,3].

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Research in context

Evidence before this study

The emergence of a novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) caused a major disease outbreak that posed a threat to public health worldwide. Among the western countries, Italy was the first to experience a vast Coronavirus Disease 2019 (COVID-19) outbreak and the province of Bergamo experienced the deadliest COVID-19 outbreak in the world. Due to the rapid diffusion of the virus, most of the health care systems were unable to cope with the high demand of testing for the identification of all the infections. Here, we have sought to perform a cross-sectional study to assess the prevalence of SARS-CoV-2 infection in 423 workers in Bergamo province who returned to the workplace after the end of the Italian lockdown on 5th May 2020.

Added value of this study

The seroprevalence in the Bergamo area is 38.5%, significantly higher than that reported for most other regions worldwide. Among all the seropositive individuals, very few tested positive for SARS-CoV-2 in nasopharyngeal swabs, with a low viral load and no infective potential in cultured cells. We also estimated the detection performance of a rapid lateral flow immunochromatography assay (LFIA), showing a specificity and sensitivity for IgG anti-SARS-CoV-2 of 98% and 92%, respectively.

Implication of all the available evidence

The cumulative seroprevalence allowed us to estimate that the actual diffusion of SARS-CoV-2 in Bergamo was 26-times higher than that reported from official data, indicating that the 96% of COVID-19 cases were underestimated. Basing on these numbers, the fatality rate of COVID-19 in the Bergamo area lays around the 1%, significantly lower than the 20% from the official data. Finding that a subset of positive subjects reported symptoms attributable to COVID-19 in the first half of February 2020, suggests that COVID-19 may already have spread widely across Lombardy before the identification of the first locally transmitted case on 20th February 2020. At the end of the first wave of the pandemic, positive nasopharyngeal swabs are potentially not contagious, with implications for human-to-human transmission of SARS-CoV-2 and the discharge policies of infected subjects. We also identified a LFIA as a valuable tool for rapid serologic surveillance of COVID-19 for population studies.

The peak of the pandemic in Lombardy was reached on March 21st, with over 3200 newly reported cases in a single day. During this peak, over 1500 patients were hospitalized in a single day and almost 150 patients were admitted to ICUs [4], creating a dire health crisis in this area [5]. Within the region, the province of Bergamo was one of the hardest hit areas with the highest single-day increase in COVID-19 cases of 715 during the peak of the pandemic [4]. Thanks to the strict lockdown imposed by the Italian government between 9th March and 5th May 2020 the curve has slowly fallen. At the time of writing, a great upsurge in SARS-CoV-2 infections was being reported across several countries in Europe, including France and Spain, which recorded over 10,000 COVID-19 cases in a single day. In Italy, the second wave of infections appears to still be rather contained, with less than 1500 daily cases as of September 25th 2020 . In the Lombardy region, the infection rate remains stable, particularly in Bergamo province where no more than 50 newly detected cases have been reported daily since early June 2020 and few patients are admitted to hospitals and even fewer to intensive care units (ICUs), suggesting the pandemic may be resolving itself in this area [6].

In contrast with the encouraging trend in Bergamo, the number of COVID-19 cases continues to rise steeply worldwide, with daily increases of over 300,000 new infections. As of September 25th, 2020, over 32 million SARS-CoV-2 infections have been reported worldwide, claiming 1 million lives. Given these numbers, there is an urgent need to assess the impact of pandemic waves on a scale of a single geographical area to anticipate the possibility of a resurgence and to limit viral transmission. The development of serological tests to detect specific antibodies against SARS-CoV-2 paved the way for the assessment of SARS-COV-2 infections in subjects who had already been infected. Despite warnings from health authorities regarding the limited diagnostic value of serological tests [7], the finding that 100% of subjects infected by SARS-CoV-2 develop specific antibodies [8] suggests that analysis of the humoral response is a valuable tool for monitoring the prevalence of SARS-CoV-2 infection across communities and populations.

Of all the available serological tests, enzyme-linked immunosorbent assay (ELISA) and chemiluminescence immunoassay (CLIA) have been most widely used for detecting antibodies against specific pathogen antigens. However, these assays are very time-consuming, given the need for blood withdrawals, serum preparation and sample analysis using dedicated laboratory equipment and specialized healthcare and laboratory workers. To overcome these limitations, faster and non-invasive tests should be developed and validated to increase diagnostic testing capacity in the short term and to extend antibody testing more widely to the general population. To this end, lateral flow immunochromatography assays (LFIA) have been developed and brought to market rapidly. In early April 2020, the US Food and Drug Administration (FDA) granted Emergency Use Authorization to over 70 rapid LFIA for COVID-19. However, the FDA did not independently verify the analytical performance of these tests and relied primarily on manufacturers' self-validation [9]. For this reason, accurate validation is needed to assess the potential specificity and sensitivity of different LFIA in order to provide accurate epidemiological data.

In this study, our primary aim was to estimate the cumulative prevalence of SARS-CoV-2 infection in Bergamo in a group of workers who returned to the workplace after the end of the Italian lockdown on 5th May 2020, almost two months after the peak of the epidemic, when the curve was already on a steady downward trajectory. To this end, we performed an ELISA to detect the humoral response against the spike and nucleocapsid proteins of SARS-CoV-2, as well as nasopharyngeal swabs to assess the presence of SARS-CoV-2 using real-time reverse transcription polymerase chain reaction (rRT-PCR). As a secondary aim, we evaluated the detection performance of a LFIA to detect anti-nucleocapsid antibodies compared to the ELISA used as a gold standard.

2. Methods

2.1. Ethics statement

Study participation was voluntary and completely free for participants. All volunteers signed an informed consent form before taking part in this study within the health surveillance program for COVID-19 funded by the Region of Lombardy.

2.2. Study design

The health surveillance screening was offered to all workers who returned to the workplace after the end of the Italian lockdown on 5th May 2020 in two companies located in the Kilometro Rosso Scientific Park in Bergamo, namely the Istituto di Ricerche

Farmacologiche Mario Negri IRCCS and Brembo S.p.A. The primary aim was to evaluate the prevalence of SARS-CoV-2 infection. Of the 172 employees working at the Istituto di Ricerche Farmacologiche Mario Negri located in Bergamo (at the Centro Anna Maria Astori and Centro di Ricerche Cliniche Aldo e Cele Daccò), 133 took part in the study, a response rate of 77%. Of the 500 workers who returned to working on-site at Brembo S.p.A, 290 agreed to take part in the study, a response rate of 58%. A total of 423 subjects were included in the study. Sample collection began on May 11th and all volunteers underwent a peripheral venous blood withdrawal to obtain serum samples for the ELISA, a capillary whole blood withdrawal by fingerstick for the LFIA and a nasopharyngeal swab for the rRT-PCR.

Additionally, we aimed to provide a descriptive data of the clinical manifestations of COVID-19 in positive subjects. To this end, subjects were asked to complete an anamnestic questionnaire that was created based on the most recent evidence in the field [10]. In this questionnaire, volunteers reported all symptoms attributable to COVID-19 that they had experienced since January 2020. They also reported co-morbidities (hypertension, cardiac diseases, diabetes, or any other relevant medical conditions), smoking habits and exposure to subjects with a laboratory-confirmed COVID-19 diagnosis or subjects with symptoms attributable to COVID-19. The survey questions are available in Supplementary Table 1.

2.3. Enzyme-linked immunosorbent assay

Anti-SARS-CoV-2 human IgG and IgM were measured using an ELISA, according to the manufacturer's instructions (DIA.PRO, Sesto San Giovanni, Milan, Italy). Briefly, serum samples were incubated on 96-microwell plates pre-coated with recombinant nucleocapsid and spike proteins of SARS-CoV-2. Captured anti-nucleocapsid and anti-spike human antibodies were detected using HRP-conjugated secondary antibodies against anti-human IgG or IgM. Results were calculated as the ratio between the optical density of the sample and the optical density of the negative control in the kit and expressed as arbitrary units (AU). The threshold for sample positivity for anti-nucleocapsid and/or anti-spike antibodies was set by the manufacturer as AU>0.9.

2.4. Lateral flow immunochromatography assay

The LFIA was provided by PRIMA Lab SA (Balerna, Switzerland). This test is a qualitative membrane-based immunoassay for the rapid detection of IgG anti-nucleocapsid in whole blood. The test consists of an IgG component in which anti-human IgG is coated in the IgG test line region. During testing, the specimen reacts to COVID-19 antigen-coated particles in the test cassette. The mixture then migrates upward on the membrane, chromatographically by capillary action, and reacts with the anti-human IgG in the IgG test line region if the specimen contains IgG antibodies to COVID-19. As a result, if the specimen contains COVID-19 IgG antibodies, a colored line will appear in the IgG test line region after 10 min. According to the manufacturer's instructions, the LFIA was compared with a leading commercial PCR and exhibited a specificity of 100% (95%CI: 86.0%~100%) and a sensitivity of 98.0% (95%CI: 89.4%~99.9%). The LFIA did not cross-react with specimens positive for antibodies against influenza A virus, influenza B virus, RSV, Adenovirus, HBsAg, Syphilis, *H. Pylori*, HIV and HCV. Triglycerides, ascorbic acid, hemoglobin, bilirubin, and total cholesterol have not been shown to interfere with LFIA.

2.5. Detection performance of the lateral flow immunochromatography assay

The secondary aim of the present study was to evaluate the detection performance of the LFIA. To this aim, a 2 × 2 table was used and the ELISA was used as a gold standard, as previously described [11]. Specificity and sensitivity were calculated as follows:

$$\text{Specificity} = T^{-}/(T^{-} + F^{+}), \text{Sensitivity} = T^{+}/(T^{+} + F^{-})$$

where T^{+} are true positives, T^{-} are true negatives, F^{-} are false negatives, and F^{+} are false positives.

2.6. Cohort for the validation of the lateral flow immunochromatography assay

An independent cohort of workers in the same geographical area was recruited in the same time period for the validation of the LFIA. The rapid serological test was offered to all the workers of the MEI System, located in Ponte San Pietro, Bergamo. Of the 160 employees (MEI cohort), 153 took part in the study, a response rate of 96%. In the event of a positive result, the worker was sent to the competent occupational physician for further molecular analyses and possible quarantine measures.

2.7. Real-time reverse transcription polymerase chain reaction

Nasopharyngeal swabs (DNA/RNA Shield™ Collection Tube, R1106, Zymo Research) were collected in 1 ml of viral transport medium (DNA/RNA Shield™). RNA was extracted starting from 200 μl of swab fluid using the Quick-RNA™ Viral Kit (R1035, Zymo Research), following the manufacturer's instructions, and eluted in 15 μl of DNase/RNase-Free water. The detection of SARS-CoV-2 RNA was carried out using the Logix Smart COVID-19 Kit (COVID-K-001, CO-DIAGNOSTICS), a multiplexed single-step real-time reverse transcription PCR test using fluorescent dye labeled CoPrimers™-probe sets specific for the RNA-dependent RNA polymerase (RdRp) gene (COVID-19–FAM) of SARS-CoV-2 and RNaseP gene (RNaseP-CF610, Internal Positive Control). According to the manufacturer, this test has been shown to have high-detection performance when compared side-by-side with the official test used by the Centers for Disease Control and Prevention (CDC). In brief, 5 μl of patient sample is mixed with 5 μl of master mix. The cycling conditions were: 15 min at 45 °C, 2 min at 95 °C, 50 cycles x [3 s at 95 °C, 32 s at 55 °C]. Samples positive for SARS-CoV-2 are characterized by a cycle threshold (Ct) value at or below 45 cycles of RdRp gene.

2.8. Cell culture and infectivity assay

Vero CCL-81 cells (ATCC, CCL-81; RRID: CVCL_0059) were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).

To evaluate the infection potential, 200 μl of 26 additional nasal positive samples (UTM™, Copan Italia) from the general population were inoculated into 24-well plates containing confluent Vero CCL-81 cells in 300 μl of EMEM, 2%FBS, and 2XP/S. Cells were incubated at 37 °C in 5% CO_2. After 7-hour incubation, inoculation medium was discarded and 500 μl of fresh EMEM, 2%FBS, and 2XP/S was added to each well. Cells were observed daily for evidence of cytopathic effects.

2.9. Sample size estimation and statistical analysis

To estimate the minimum required sample size, we used PASS 16.0.1. Based on the available data [12], we estimated that the prevalence of SARS-CoV-2 infection in the Bergamo area is about 40%. Assuming a 99% two-side confidence interval, 90% power, a 10% delta and a 10% dropout rate for non-evaluable or missing data, the minimum sample size required for this cross-sectional study is 412 [13].

Data were expressed as mean (SD) or number (%) unless otherwise specified. Comparisons of symptoms and other binary characteristics in positive *vs* negative participants were performed using

Fisher's exact test, while age and continuous levels of anti-SARS-CoV-2 IgG and IgM were compared by means of unpaired *t*-test. A similar approach was used for comparisons by sex and by type of symptoms (*i.e.* asymptomatic, paucisymptomatic and symptomatic). All analyses were carried out using SAS (Version 9.4). All *p*-values were 2-sided.

2.10. Role of the funding source

The funding sources had no role in any aspect pertinent to the manuscript.

3. Results

3.1. Seroprevalence of SARS-CoV-2 infection and subjects' characteristics according to seropositivity

Overall, 423 subjects were included in the primary cohort of the study. Baseline characteristics are reported in Table 1. Using ELISA, we identified 163 positive subjects (38.5%), of whom 84 (51.5%) were positive for both IgG and IgM, 77 (47.2%) were positive for IgG alone, and only 2 (1.2%) were positive for IgM alone. The mean age of negative subjects (43.26 ± 9.94 years) was significantly lower ($p = 0.011$) than that of positive volunteers (46.72 ± 9.92, Table 1). No differences were found in the rate of positivity between sexes (Table 1), even when IgG and IgM positivity was considered as a continuous variable (IgG titer: 5.475 ± 3.053 AU in males [95% CI: 4.884–6.066] *vs* 5.545 ± 3.372 AU in females [95% CI: 4.606–6.484]; IgM titer: 1.573 ± 1.878 AU in males [95% CI: 1.209–1.936] *vs* 1.584 ± 1.291 AU in females [95% CI: 1.155–2.013]).

While we found no difference in the workplace distribution between positive and negative subjects, we found that there were significant differences between areas of residency. Indeed, the rate of positivity (56.7%) was higher in the volunteers living in Nembro (17 positive subjects out of 30), compared to other areas in the province, such as Bergamo, in which 37.7% of inhabitants tested positive for anti-SARS-CoV-2 (49 positive subjects out of the 130 living in the area of Bergamo). The same seroprevalence as in the Bergamo area was observed in the other regions of the province (36.9%, Table 1).

Compared to negative volunteers, current smokers were significantly less prevalent in subjects who tested positive for anti-SARS-CoV-2 antibodies (14.1%, $p = 0.002$), while former smokers were significantly more represented (25.1%, $p = 0.003$) (Table 1). In this cohort, we did not find any relevant co-morbidities associated with COVID-19 seropositivity (Table 1).

Table 1
Baseline characteristics and results of serological studies.

	Overall ($n = 423$)	Negative test ($n = 260$)	Positive test ($n = 163$)	p
Age (years)*	44.26 ± 9.93	43.26 ± 9.94	46.72 ± 9.92	0.001
Male sex (%)	269 (63.6)	158 (60.8)	111 (68.1)	0.146
Smoking habits				
Current	92 (21.7)	69 (26.5)	23 (14.1)	0.002
Former	76 (18.0)	35 (13.5)	41 (25.1)	0.003
Never	255 (60.3)	156 (60.0)	99 (60.7)	0.919
Area of residence (%)				
Bergamo	130 (30.7)	81 (31.2)	49(30.1)	0.829
Nembro	30 (7.1)	13 (5.0)	17(10.4)	0.050
Other part of the province	263 (62.2)	166 (63.8)	97(59.5)	0.410
Pre-existing medical conditions (%)	237 (56.0)	145 (55.8)	92 (56.4)	0.920
Contact with patients positive for COVID-19 (%)	65 (15.4)	37 (14.2)	28 (17.2)	0.410
Contact with subjects with symptoms attributable to COVID-19 (%)	180 (42.6)	102 (39.2)	78 (47.9)	0.087

* mean±SD.

Table 2
In vitro infectivity assay of nasopharyngeal swabs on cultured cells.

Range of Ct for RdRp gene	n	CPE
From 33 to 35	8	None
From 36 to 45	18	None

Ct: cycle threshold.
CPE: cytopathic effect.

3.2. Molecular analysis of SARS-CoV-2 in nasopharyngeal swabs

Among the 423 subjects included in the study, only 23 (5.4%) had a positive nasopharyngeal swab by rRT-PCR, although cycle thresholds (Ct) were very high, ranging from 34 to 39. All 23 PCR-positive volunteers were positive for anti-SARS-CoV-2 antibodies by ELISA, with 10 subjects exhibiting only IgG reactivity, 13 with IgG and IgM anti-SARS-CoV-2, but none with IgM alone.

Recent evidence has indicated that high rRT-PCR Ct is associated with low levels of sample infectivity [14–16]. To evaluate whether this was the case in our experimental setting as well, we assessed the cytopathic effect of nasal swabs through an *in vitro* infectivity assay. As shown in Table 2, a total of 26 additional specimens that tested positive by rRT-PCR targeting the RdRp gene were obtained, with Ct values ranging from 33 to 35 ($n = 8$) and 36–45 ($n = 18$), consistent with the Ct of the 23 subjects found in our cohort study. When inoculated in cultured Vero CCL-81 cells, none of the 26 samples led to a detectable cytopathic effect, suggesting that the range of rRT-PCR positivity (33–45 Ct) lacks potential infectivity (Table 2).

3.3. Evaluation of the clinical manifestations of COVID-19

By analyzing the anamnestic questionnaires completed by volunteers, we found that 54% of positive subjects reported needing assistance from their general practitioner, a number that was significantly higher ($p<0.001$) than for negative subjects (Table 3). Only one patient in the positive group reported having needed hospitalization in the previous 2 months due to disease complications (Table 3), suggesting that COVID-19 mostly presented as a mild disease in this cohort. The symptoms most frequently associated with anti-SARS-CoV-2 antibody positivity were fever, anosmia, and ageusia (Table 3). Additionally, the most commonly reported symptoms in positive subjects were fatigue, muscular pain and headaches (Table 3). Among the subjects who reported symptoms ($n = 134$), 14 experienced them in the first half of February, 64 in the first two weeks of March, and 14 in the last two weeks of March (42 missing). We found that the symptoms that dated furthest back had occurred on 6th February 2020, although most of the self-reported symptoms clustered in the first two weeks of March 2020. Finally, we found that the number of asymptomatic positive subjects was 17.8%, with 29 positive subjects reporting no symptoms at all (Table 3).

When positive volunteers ($n = 163$) were divided according to disease severity [10], we found that subjects experiencing more than four different symptoms (symptomatic) exhibited higher levels of anti-SARS-CoV-2 ($n = 67$; AU, mean ± SE: 6.39 ± 0.36), which was statistically significant compared to paucisymptomatic subjects ($n = 67$; AU, mean ± SE: 4.95 ± 0.38, $p = 0.025$) and asymptomatic subjects ($n = 29$; AU, mean ± SE: 4.86 ± 0.58, $p = 0.007$). In patients with different degrees of symptoms, no differences were found in anti-SARS-CoV-2 IgM levels.

Table 3
Comparison between the symptoms developed in subjects with negative and positive test.

Symptoms (%)	Negative test (n = 260)	Positive test (n = 163)	P
Required assistance from the general practitioner (%)	44 (16.9)	88 (54.0)	<0.001
Required hospitalization (%)	1 (0.4)	1 (0.6)	0.738
Fever	45 (17.3)	87 (53.4)	<0.001
Shivering	29 (11.1)	40 (24.5)	<0.001
Headache	42 (16.1)	58 (35.6)	<0.001
Shortness of breath	17 (6.5)	22 (13.5)	0.024
Chest pain	17 (6.5)	9 (5.5)	0.836
Dry cough	33 (12.7)	50 (30.7)	<0.001
Productive cough	16 (6.1)	17 (10.4)	0.136
Sore throat	35 (13.5)	25 (15.3)	0.668
Cold	44 (16.9)	30 (18.4)	0.695
Rhinorrhoea	36 (13.8)	21 (12.9)	0.884
Anosmia	6 (2.3)	56 (34.3)	<0.001
Ageusia	6 (2.3)	55 (33.7)	<0.001
Conjunctivitis	5 (1.9)	8 (4.9)	0.145
Muscular pain	28 (10.8)	53 (32.5)	<0.001
Fatigue	37 (14.2)	63 (38.6)	<0.001
Diarrhea	19 (7.3)	29 (17.8)	0.001
Nausea	10 (3.8)	18 (11.0)	0.005
Vomiting	5 (1.9)	8 (4.9)	0.145
Lack of appetite	5 (1.9)	23 (14.1)	<0.001
Other	13 (5.0)	16 (9.8)	0.074
No symptoms	111 (42.7)	29 (17.8)	<0.001

3.4. Comparison between ELISA and LFIA

In order to evaluate the analytical performance of LFIA, the 423 subjects included in the study underwent capillary whole blood withdrawal by finger-stick. With the rapid LFIA, 153 subjects (36.2%) were found to be positive for IgG against SARS-CoV-2. When the ELISA was used as the comparator, 5 false positives and 13 false negatives were identified. Based on all these results (Supplementary Table 3), the estimated specificity and sensitivity of the rapid LFIA for IgG were 98% and 92%, respectively.

Given this high-performance detection, we elected to further validate the ability to assess the seroprevalence of SARS-CoV-2 infection using the rapid test alone. To this end, we offered a serological survey by LFIA to 153 volunteers (MEI cohort) in the same geographical area, recruited in the same time period. The baseline characteristics of these subjects are reported in Supplementary Table 2. In this independent cohort of workers, 61 volunteers (39.9%) were found to be positive for IgG against SARS-CoV-2, while 92 (60.1%) tested negative (Supplementary Table 2), confirming the high seroprevalence found in the primary cohort of the study.

4. Discussion

Our cross-sectional study reports a comprehensive analysis of the prevalence of SARS-CoV-2 infection in the population of the Bergamo province, one of the areas that experienced the earliest and most diffuse spread of SARS-CoV-2 infection in Italy. Our main finding is that specific anti-SARS-CoV-2 antibodies were found in the 38.5% of workers who went back to work after the strict lockdown imposed by the Italian government. This overwhelming prevalence is similar to that found in some hotspots in India and Iran [17], but far exceeds that reported for the other hardest hit areas in the world [18], including New York, with 19.9% seroprevalence, London with 17.5%, and Madrid with 11.3%. Even in Wuhan, where SARS-CoV-2 emerged, a recent study suggested that healthcare workers only exhibited 1.8% seroprevalence [19]. Moreover, very recent studies have consistently identified the presence of T cells against SARS-CoV-2 in 40–60% of individuals not exposed to SARS-CoV-2 [20,21], possibly suggesting that a large subset of subjects may have T-cell protective immunity against SARS-CoV-2 even without detectable levels of circulating antibodies. Collectively these data would tend to indicate that the cumulative prevalence could have been even higher, suggesting that the Bergamo province may be already heading towards natural herd immunity [22,23]. These findings could have major implications for second waves of infection in this region during the post-pandemic period [24,25].

Assuming that the 38.5% cumulative prevalence found in the present study applies to the general population of the Bergamo province – 1.1 million inhabitants – one should infer an actual number of 420,000 SARS-CoV-2 infections. This is much higher than those reported in the official data, which report 16,000 cases as of 25th September 2020. Within the limitation of this approach, our esteem suggests that 96% of infections went undetected by the healthcare system. The official data also indicate that the fatality rate of COVID-19 in Bergamo province is 20%, based on around 3100 deaths over 16,000 reported cases [3]. If the actual number of COVID-19 cases is 420,000, the crude infection fatality rate in this area can be estimated to be approximately 1%, similar to that recently calculated across different countries [26].

In our cohort, we demonstrate that COVID-19 mostly presented as a mild disease, with fever, anosmia and ageusia, and muscular pain and fatigue being the most commonly experienced symptoms in positive patients. Of these symptoms, anosmia and ageusia were the strongest pathognomonic signs of SARS-CoV-2 infection, as is increasingly recognized [27]. Though the analysis of backdated symptoms in our cohort revealed that most patients positive for SARS-COV-2 experienced symptoms in the first two weeks of March, a subset of positive subjects reported symptoms attributable to COVID-19 in early February 2020. The fact that the first Italian case of COVID-19 infection was reported on 20th February 2020 in a municipality of Lodi province, which is 100 km away from Bergamo, suggests that COVID-19 may already have spread widely across Lombardy before the first cases were officially reported and multiple, independent outbreaks had already occurred within the region. These data are consistent with recent findings across Europe, which document that SARS-CoV-2 was already circulating at least a month before the epidemic started between February and March 2020 [28,29].

Regarding humoral response to SARS-CoV-2, we found that the highest seropositive rates were observed for IgG while only 2 individuals tested positive for IgM alone at ELISA. This is in line with previous findings, which have shown that IgG seroconversion may occur concomitantly with IgM or even earlier, even in the absence of an IgM response [8]. Considering that all the subjects in our study exhibited symptoms between February and early March 2020, it is conceivable that the humoral response reflects the true prevalence of positive subjects at the time of assay performance in early May, approximately two to three months after symptom onset. Indeed, recent evidence suggested that the human humoral response to SARS-CoV-2 peaks at 2 months and remains at a plateau at 4 months [30], although the longevity of the anti-SARS-CoV-2 antibodies is transient with decay 4 months after symptom onset, particularly in mild COVID-19 cases [31–33].

Our data also suggests that the rapid LFIA test used in this study for serological screening has an estimated specificity and sensitivity of 98% and 92%, which are significantly higher than those estimated in other studies with different LFIA [34–37]. Specificity and sensitivity are lower than that reported by the manufacturer (100% specificity and 98.0% sensitivity). A possible explanation for the slight discrepancy in sensitivity is probably attributable to the sample size which was considerably higher than that considered by the manufacturer, as well as to the divergent selection of tested subjects. The difference in sensitivity is likely due to the fact that the ELISA measures anti-spike and anti-nucleocapsid antibodies, while the LFIA only detects antibodies against the nucleocapside protein. A future effort of creating a rapid test capable of simultaneously evaluating IgG anti-

nucleocapsid and anti-spike protein antibodies by LFIA is warranted [38]. Altogether our present data suggest the LFIA could be an extremely valuable tool for rapid and widespread serologic surveillance of COVID-19 in general populations in different geographic locations.

Our study has been conducted at the end of the first wave of the COVID-19 pandemic in Italy and shows that very few individuals who tested positive for SARS-CoV-2 antibodies had positive swabs with high Ct number indicating that the viral load in these subjects was low. These data are of considerable interest in the context of studies documenting that SARS-CoV-2 obtained from swabs with 33−34 Ct, corresponding to less than 100,000 copies of RNA/ml, are not cytotoxic *in vitro* [14−16], suggesting no infectivity for these samples. Here we have repeated those experiments and found that, indeed, swabs with Ct ranging from 33 to 45 were not able to infect cultured cells. In addition, all rRT-PCR positive subjects had reported symptoms attributable to COVID-19 approximatively two months before being tested, suggesting that the low viral load observed in these subjects was possibly due to residual SARS-CoV-2 genetic material rather than active viral replication. Collectively, the above *in vitro* findings also have implications for human-to-human transmission of SARS-CoV-2, as suggested by a report from the Korea Centers for Disease Control and Prevention that showed there were no cases of infection in the 790 contacts of 285 subjects who re-tested positive for SARS-CoV-2 RNA with a nasopharyngeal swab after being discharged from isolation [39]. Adding further complexity to the picture is the observation that in addition to viral load, genome integrity is another important criterion for evaluating the infectivity of clinical specimens [40]. In this regard, van Kampen and colleagues estimated that the 95% positivity to SARS-CoV-2 in swab samples taken 15 days after onset of symptoms was due to viral fragments rather than infective, replication-competent virus [41,42]. Altogether these findings reinforce the appropriateness of the new international criteria for discharging patients from quarantine 10 days after symptom onset without molecular retesting [42,43] and should encourage policy makers in countries that have not yet adopted these new directives, including Italy, to take into account the importance of viral load, rather than swab positivity *per se*, in order to match discharge policies to current scientific evidence.

Limitations of the study: as a cross-sectional study, the primary limitation is that no evidence of a temporal relationship between exposure and outcome could be provided, as exposure and outcome were assessed simultaneously. Additionally, due to the mean response rate of 65% this study is susceptible to selection biases and our study population may not be representative of the general population. Lastly, the self-reported data in the anamnestic questionnaire cannot be independently verified and, therefore, may be subject to biases, including selective memory, exaggeration or minimization of symptoms, as well as erroneous memories about backdated symptoms.

In summary, our results demonstrate that: 1) seroprevalence in the Bergamo area is one of the highest reported so far, 2) nasopharyngeal swabs found to be positive at the end of the first wave of the pandemic have a very low SARS-CoV-2 viral load and no infective potential, 3) the rapid LFIA is a valuable tool for serologic surveillance that allowed us to show that 96% of COVID-19 cases were went unrecorded.

Data sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of interests

The authors have no conflicts of interest to declare.

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Contributors

L.P. and S.T., data collection, data analysis, data interpretation, writing of the first draft; T.P. and A.Perna, statistical analysis; A.Pezzotta, data collection; G.R. and A.B., study design, data analysis, data interpretation, approval of the final version of the manuscript. All authors read and approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103069.

References

[1] Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. Nat. Med. 2020;26(4):450–2.
[2] EpiCentro. COVID-19 integrated surveillance: key national data. https://www.epicentro.iss.it/en/coronavirus/sars-cov-2-integrated-surveillance-data (Accessed 25 September 2020).
[3] COVID-19 ITALIA - Desktop. http://opendatadpc.maps.arcgis.com/apps/opsdashboard/index.html#/b0c68bce2cce478eaac82fe38d4138b1 (Accessed 25 September 2020).
[4] Covid-19 Regione Lombardia. https://experience.arcgis.com/experience/0a5dfcc103d0468bbb6b14e713ec1e30/ (Accessed 25 September 2020).
[5] Remuzzi A, Remuzzi G. COVID-19 and Italy: what next? Lancet 2020;395(10231):1225–8.
[6] Alicandro G, Remuzzi G, Vecchia CL. Italy's first wave of the COVID-19 pandemic has ended: no excess mortality in May 2020. Lancet 2020;396(10253):e27–8.
[7] Important Information on the Use of Serological (Antibody) Tests for COVID-19 - Letter to Health Care Providers. FDA 2020 https://www.fda.gov/medical-devices/letters-health-care-providers/important-information-use-serological-antibody-tests-covid-19-letter-health-care-providers. Accessed June 16, 2020.
[8] Long QX, Liu BZ, Deng HJ, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nat. Med. 2020;26(6):845–8.
[9] Abbasi J. The Promise and Peril of Antibody Testing for COVID-19. JAMA 2020;323(19):1881–3.
[10] Drew DA, Nguyen LH, Steves CJ, et al. Rapid implementation of mobile technology for real-time epidemiology of COVID-19. Science 2020;368(6497):1362–7.
[11] Krüttgen A, Cornelissen CG, Dreher M, Hornef M, Imöhl M, Kleines M. Comparison of four new commercial serologic assays for determination of SARS-CoV-2 IgG. J Clin Virol 2020;128:104394.
[12] Sandri MT, Azzolini E, Torri V, et al. IgG serology in health care and administrative staff populations from 7 hospital representative of different exposures to SARS-CoV-2 in Lombardy, Italy. medRxiv 2020. https://doi.org/10.1101/2020.05.24.20111245.
[13] Kelsey C of the D of E at the S of MSUJL, Kelsey JL, Whittemore AS, et al. Methods in observational epidemiology. Oxford University Press; 1996.
[14] La Scola B, Le Bideau M, Andreani J, et al. Viral RNA load as determined by cell culture as a management tool for discharge of SARS-CoV-2 patients from infectious disease wards. Eur J Clin Microbiol Infect Dis 2020;39(6):1059–61.
[15] Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. Nature 2020;581(7809):465–9.
[16] Bullard J, Dust K, Funk D, et al. Predicting infectious SARS-CoV-2 from diagnostic samples. Clin Infect Dis 2020. doi: 10.1093/cid/ciaa638.
[17] Jamkh S, ikar. Over Half of People in Mumbai's Slums Probably Infected With Coronavirus: survey. The Wire Science 2020 published online July 30 https://science.thewire.in/health/over-half-of-people-in-mumbais-slums-probably-infected-with-coronavirus-survey/. Accessed September 1, 2020.

[18] Popovich N, Sanger-Katz M. The World Is Still Far From Herd Immunity for Coronavirus. The New York Times 2020 published online May 28. https://www.nytimes.com/interactive/2020/05/28/upshot/coronavirus-herd-immunity.html. Accessed June 19, 2020.
[19] Xu X, Sun J, Nie S, et al. Seroprevalence of immunoglobulin M and G antibodies against SARS-CoV-2 in China. Nat. Med. 2020;26(8):1193–5.
[20] Grifoni A, Weiskopf D, Ramirez SI, et al. Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. Cell 2020;181(7):1489–501 e15.
[21] Le Bert N, Tan AT, Kunasegaran K, et al. SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls. Nature 2020;584 (7821):457–62.
[22] Modi C, Boehm V, Ferraro S, Stein G, Seljak U. How deadly is COVID-19? A rigorous analysis of excess mortality and age-dependent fatality rates in Italy. medRxiv 2020. https://doi.org/10.1101/2020.04.15.20067074.
[23] Italy Nears Herd Immunity As Over Half of Its Covid-19 Epicenter's Population Tested Positive for Antibodies. Science Times 2020. https://www.sciencetimes.com/articles/26009/20200610/over-50-citizens-tested-positive-coronavirus-antibodies-italys-epicenter-what.htm. Accessed July 23, 2020.
[24] Randolph HE, Barreiro LB. Herd Immunity: understanding COVID-19. Immunity 2020;52(5):737–41.
[25] Kissler SM, Tedijanto C, Goldstein E, Grad YH, Lipsitch M. Projecting the transmission dynamics of SARS-CoV-2 through the postpandemic period. Science 2020;368(6493):860–8.
[26] Mallapaty S. How deadly is the coronavirus? Scientists are close to an answer. Nature 2020;582(7813):467–8.
[27] Mehraeen E, Behnezhad F, Salehi MA, Noori T, Harandi H, SeyedAlinaghi S. Olfactory and gustatory dysfunctions due to the coronavirus disease (COVID-19): a review of current evidence. Eur Arch Otorhinolaryngol 2020. doi: 10.1007/s00405-020-06120-6.
[28] Chavarria-Miró G, Anfruns-Estrada E, Guix S, et al. Sentinel surveillance of SARS-CoV-2 in wastewater anticipates the occurrence of COVID-19 cases. medRxiv 2020. https://doi.org/10.1101/2020.06.13.20129627.
[29] Rosa GL, Iaconelli M, Mancini P, et al. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci Total Environ 2020;736:139652.
[30] Gudbjartsson DF, Norddahl GL, Melsted P, et al. Humoral Immune Response to SARS-CoV-2 in Iceland. N Engl J Med 2020. doi: 10.1056/NEJMoa2026116.
[31] Long QX, Tang XJ, Shi QL, et al. Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. Nat. Med. 2020;26(8):1200–4.
[32] Seow J, Graham C, Merrick B, et al. Longitudinal evaluation and decline of antibody responses in SARS-CoV-2 infection. medRxiv 2020 https://doi.org/10.1101/2020.07.09.20148429.
[33] Ibarrondo FJ, Fulcher JA, Goodman-Meza D, et al. Rapid Decay of Anti–SARS-CoV-2 Antibodies in Persons with Mild Covid-19. New England J. Medicine 2020;383 (11):e74.
[34] Candel FJ, Viñuela-Prieto JM, González Del Castillo J, et al. Utility of lateral flow tests in SARS-CoV-2 infection monitorization. Rev Esp Quimioter 2020;33 (4):258–66.
[35] Van Elslande J, Houben E, Depypere M, et al. Diagnostic performance of seven rapid IgG/IgM antibody tests and the Euroimmun IgA/IgG ELISA in COVID-19 patients. Clin Microbiol Infect 2020;26(8):1082–7.
[36] Pérez-García F, Pérez-Tanoira R, Romanyk J, Arroyo T, Gómez-Herruz P, Cuadros-González J. Alltest rapid lateral flow immunoassays is reliable in diagnosing SARS-CoV-2 infection from 14 days after symptom onset: a prospective single-center study. J Clin Virol 2020;129:104473.
[37] Kontou PI, Braliou GG, Dimou NL, Nikolopoulos G, Bagos PG. Antibody Tests in Detecting SARS-CoV-2 Infection: a Meta-Analysis. Diagnostics 2020;10 (5):319.
[38] Rosadas C, Randell P, Khan M, McClure MO, Tedder RS. Testing for responses to the wrong SARS-CoV-2 antigen. Lancet 2020;396(10252):e23.
[39] KCDC. KCDC. KCDC. http://www.cdc.go.kr (Accessed 24 June 2020).
[40] Huang CG, Lee KM, Hsiao MJ, et al. Culture-based virus isolation to evaluate potential infectivity of clinical specimens tested for COVID-19. J. Clin. Microbiol. 2020;58(8) e01068-20.
[41] van Kampen Jeroen JA, van de Vijver David AMC, Fraaij Pieter LA, et al. Shedding of infectious virus in hospitalized patients with coronavirus disease-2019 (COVID-19): duration and key determinants. medRxiv 2020 https://doi.org/10.1101/2020.06.08.20125310.
[42] CDC. Coronavirus Disease 2019 (COVID-19). Centers for Disease Control and Prevention. 2020; https://www.cdc.gov/coronavirus/2019-ncov/hcp/duration-isolation.html (Accessed 23 July 2020).
[43] Criteria for releasing COVID-19 patients from isolation. https://www.who.int/news-room/commentaries/detail/criteria-for-releasing-covid-19-patients-from-isolation (Accessed 4 September , 2020).