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SARS-CoV-2 was already circulating in Italy, in early December 2019

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Abstract. – OBJECTIVE: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) identified in China, in December 2019 determines COronaVIrus Disease 19 (COVID-19). Whether or not the virus was present in Italy earlier the first autochthonous COVID-19 case was diagnosed is still uncertain. We aimed to identify anti-SARS-CoV-2 antibodies in sera collected from 4th November 2019 to 9th March 2020, in order to assess the possible spread of the virus in Italy earlier than the first official national diagnosis.

PATIENTS AND METHODS: Anti-SARS-CoV-2 antibodies were evaluated in retrospective serum samples from 234 patients with liver diseases (Hep-patients) and from 56 blood donors (BDs). We used two rapid serologic tests which were confirmed by a validated chemoluminescence assay.

RESULTS: *Via* rapid tests, we found 10/234 (4.3%) IgG-positive and 1/234 (0.4%) IgM-positive cases in the Hep-patient group. Two/56 (3.6%) IgG-positive and 2/56 (3.6%) IgM-positive cases were detected in BD group. Chemoluminescence confirmed IgG-positivity in 3 Hep-patients and 1 BD and IgM-positivity in 1 Hep-patient. RNAemia was not detected in any of the subjects, rendering the risk of transfusion transmission negligible.

CONCLUSIONS: Our results suggest an early circulation of SARS-CoV-2 in Italy, before the first COVID-19 cases were described in China. Rapid tests have multiple benefits; however, a confirmation assay is required to avoid false positive results.

Key Words:
SARS-CoV-2 infection, Anti-SARS-CoV-2 antibodies, COVID-19, Rapid tests, Chemoluminescence.

Introduction

In early January 2020, a new coronavirus named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was identified as the infectious agent that caused a viral pneumonia epidemic in Wuhan, China, where the first known cases were recorded in December 2019[1,2]. The most common symptoms of SARS-CoV-2 infection, termed COronaVIrus Disease 19 (COVID-19) include fever, dry cough, and breathing difficulties. The infection can cause severe pneumonia, severe acute respiratory distress syndrome (ARDS), kidney failure and even death[3]. The main symptoms can appear between 2 to 14 days after exposure to the virus, with an overall average of 5 days. Person-to-person transmission mainly occurs *via* respiratory droplets and from contact with asymptomatic subjects[4,5]. The World Health Organization declared COVID-19 a global pandemic on 11th March 2020 (https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020). Italy was severely and predominately affected in the North of the country during the initial stage of the virus. At present, Tuscany

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is the seventh most affected region, and the total number of cases was (http://www.salute.gov.it) updated on 19th February 2021.

The humoral response to SARS-CoV-2 infection seems to be particularly dynamic and arduous to interpret[6]. The majority of patients seroconvert within 2 weeks after symptom onset. The Immunoglobulin G (IgG) peak appears simultaneously or slightly later than Immunoglobulins M (IgMs). Although, IgMs and IgGs are usually detectable after one month, in some cases[6], seroconversion is weak or undetectable[7]. The lack of solid data and the dynamic behavior of humoral response to SARS-CoV-2 infection renders the serologic test unsuitable to confirm a diagnosis. However, it could be a useful tool in the assessment of previous infection. Different seroprevalence surveys to establish the proportion of the population infected with SARS-CoV-2 are currently ongoing in Italy and in several other countries. All these analyses illustrate the current situation, but whether or not the virus was present in Italy earlier than the first autochthonous COVID-19 case was diagnosed in a small town called Codogno (Lombardy), on 21st February, is still unclear.

The aim of our study was to identify the presence of anti- SARS-CoV-2 antibodies (IgM and IgG), in retrospectively collected serum samples from patients with different liver diseases referred to the MaSVE center outpatient clinic and from blood donors. Serologic tests were used in order to assess the possible spread of the virus in the Florentine area earlier than the first case was officially diagnosed in Italy.

Patients and Methods

Study Population

Two hundred and thirty-four retrospective plasma samples from patients with different liver diseases (Hep-patients) referred to the MaSVE Center outpatient clinic, (Careggi University Hospital, Florence, Italy) and 56 plasma samples from healthy blood donors (BDs) were tested for anti-SARS-CoV-2 IgG and IgM through serologic tests. The main features of the tested subjects are reported in Table I.

The Hep-patient plasma samples were collected from 4th November 2019 to 9th March 2020 when lockdown started in Italy. The BD samples were collected from 22nd January 2020 to 9th March 2020 at the Transfusion Medicine Center at Careggi University Hospital in Florence, Italy. Samples were collected and frozen in order to perform previous studies and BDs were used as healthy controls. All of the Hep-patients and BDs signed an informed consent form prior to blood collection. An updated consent form was later provided for the purpose of performing the anti-SARS-CoV-2 IgG and IgM test and RNAemia evaluation. The study was conducted according to the Ethical Guidelines of the 1975 Declaration of Helsinki and it was approved by the Local Institutional Review Board (Comitato Etico Area Vasta Centro, AOU Careggi, Florence, Italy, study code #17886_bio).

Rapid Serologic Tests

The serologic test we used (COVID-19 IgG/IgM Rapid Test Cassette, produced by CHIL, Cigli-Izmir, Turkey) was approved by the Italian Ministry of Health. The test is a rapid chromatographic immunoassay for the qualitative detection of IgG and IgM antibodies to SARS-CoV-2 in human whole blood, serum, or plasma. The manufacturer ensures 100% sensitivity: (95% CI: 96.1%~100.0%), 99.5% specificity (95% CI: 98.1%~99.9%) and 99.6 % accuracy (95% CI: 98.4%~99.9%) for IgGs and 91.8% sensitivity (95% CI: 83.8%~96.6%), 99.2% specificity (95% CI: 97.7%~99.8%) and 97.8 % accuracy (95% CI: 96.0%~98.9%) for IgMs. The positive samples were retested three times with the same kind of assay and twice with a different rapid test (MP RAPID 2019-NCOV IgG/IgM COMBO TEST CARD produced by MP Biomedicals, Santa Ana, CA, USA).

Chemiluminescent Immunoassay

The positive samples were tested with a third validated routine test performed by Synlab Lazio Laboratory and Diagnostic Center, Rome Italy. The test was a chemiluminescent microparticle immunoassay (CMIA) used for the qualitative detection of SARS-CoV-2 IgM and IgG antibodies in human serum and plasma on the ARCHITECT i System diagnostic testing platform (Abbott Diagnostic Division-Longford, Ireland).

RNAemia Detection

Furthermore, in order to assess SARS-CoV-2 RNAemia, we followed a previously published RT-PCR protocol[8], performed on the total RNA extracted from 250 mL of plasma by TRIzol LS (Invitrogen, Carlsbad, CA, USA).

Table I. Main features of the subjects tested for anti-SARS-CoV-2 IgG and IgM.

		Total population n (%)	Hep-Patients n (%)	BDs n (%)
Total number		290	234	56
M/F		152/138	111/123	41/15
Median age		61.5	65	46.9
Rapid test				
ìIgG positivity	12/290 (4.1%)	10/234 (4.3%)	2/56 (3.6%)	
	IgM positivity	3/290 (1%)	1/234 (0.4%)	2/56 (3.6%)
Chemoluminescence*		15	11	4
	IgG positivity	4/15(26.6%)	3 /11(24.2%)	1/4 (25%)
	IgM positivity	1/15(6.6%)	1/11(9%)	0/4

*The chemoluminescence assay was performed on 15 subjects with IgG or IgM positivity after the rapid tests. *Abbreviations:* Hep-patients: Patients with liver diseases; BDs: Blood donors; M: male; F: female; IgG: Immunoglobulin G; IgM: Immunoglobulin M.

Results

The first screening, conducted using CHIL rapid test, showed a positivity to IgG in 12/290 (4.1%) cases and a positivity to IgM in 3/290 (1%) cases (Table I).

Considering the two different settings, we found 10/234 (4.3%) IgG-positive and 1/234 (0.4%) IgM-positive cases in the Hep-patient group and 2/56 (3.6%) IgG positive and 2/56 (3.6%) IgM positive cases in the BD group (Table I).

The test was repeated three times using the same brand cards and then retested twice using rapid cards from a different manufacturer, which confirmed the same results.

In addition, we decided to assess an evaluation of 15 anti-SARS-CoV-2 IgG and IgM positive cases and had Synlab Lazio Laboratory and Diagnostic Center (Rome, Italy) performing a serologic test through a validated chemoluminescence assay. It was a blinded test as we did not provide any results concerning the previous assessments. All of the serum samples were evaluated twice.

Among the 15 anti-SARS-CoV-2 antibody positive cases after rapid tests, chemoluminescence confirmed an IgG positivity in 4 serum samples, 3 in the Hep-patient group and 1 in the BD group. IgM positivity was confirmed in 1 subject from the Hep-patient group. The main features of the positive cases are detailed in Table II.

Surprisingly, subject#1 (a 56-year-old female) was IgG positive as confirmed by three different tests at the end of November 2019, which was earlier than when the first case in China was described[1, 2]. We repeated the tests (Table II) when the woman came to the Masve outpatient clinic for a scheduled check-up in July and the results were confirmed. The woman did not recall any particular symptoms apart from a mild cough during an ill-defined period after the Summer season. She also disclosed that she had spent the Summer holidays in her native country, the Philippines. She boarded a plane to Manila, with a stopover in Doha, Qatar, and returned to Italy on 14th September 2019. Her social interaction included meetings with family and friends for the duration of her trip to the Philippines.

As previously mentioned, we analyzed two metachronous serum samples from subject #1: although the chemoluminescence assay performed by SynLab provides qualitative results (it is a semi-quantitative assay), we noted that the IgMs were close to the threshold of positivity in November. In July 2020, the level was relatively low and, conversely, the level of IgGs increased from November onwards. We also tested the woman's husband with negative results.

Among the 4 subjects confirmed positive by the chemoluminescence assay, no particular risk factor seemed to be attributable to subject #3 or #12. Subject #5 is a nun who lives in a convent with other sisters and #8 had frequent contact with foreign tourists as he owns and manages a resort in Tuscany. None of the other subjects, apart from #1, reported any symptoms.

Overall, considering the chemoluminescence assay, 4/290 (1.4%) subjects, 3 Hep-patients and 1 BD were anti-SARS-CoV-2 IgG positive and 1/290 (0.34%) was IgM positive. In detail, considering the Hep-patient group 3/234 (1.28%) were IgG positive and 1/234 (0.43%) was IgM positive. One/56 (1.8%) was IgG positive in the BD group. Considering the two settings together and both, IgG and IgM positivity, we found 5/290 (1.7%) positive individuals.

Table II. Results of the confirmation analysis of the 15 positive serum samples after rapid serologic tests and the subjects' details.

ID	Sex	Age	Blood drawn date	Serologic tests				Possible risk factors
				Rapid		Chemoluminescence		
				IgG	IgM	IgG	IgM	
#1	F	56	25/11/2019	+	-	+	-	Trip to the Philippines during the Summer of 2019 / returned to Italy on 14th September 2019
#1 re-test	F	56	17/07/2020	+	-	+	-	
#2	M	56	06/12/2019	+	-	-	-	ND
#3	F	83	17/12/2019	-	+	-	+	ND
#4	F	54	09/01/2020	+	-	-	-	
#5	F	86	17/01/2020	+	-	+	-	Communitarian life: the patient is a nun who lives in a convent
#6	M	46	20/01/2020	+	-	-	-	ND
#7	F	85	04/02/2020	+	-	-	-	ND
#8	M	64	05/02/2020	+	-	+	-	Contact with foreign tourists as manager of a resort
#9	F	84	10/02/2020	+	-	-	-	ND
#10	F	75	11/02/2020	+	-	-	-	ND
#11	M	44	12/02/2020	+	-	-	-	ND
#12 BD	M	53	29/01/2020	+	-	+	-	ND
#13 BD	F	28	10/02/2020	+	-	-	-	ND
#14 BD	M	47	10/02/2020	-	+	-	-	ND
#15 BD	M	63	12/02/2020	-	+	-	-	ND

ID: Identification. *Abbreviations:* M: male; F: female; IgG: Immunoglobulin G; IgM: Immunoglobulin M; blood donor: BD; not determined: ND.

The RT-PCR analysis to assess viremia did not detect viral RNA in any of the 290 subjects.

Discussion

We evaluated the presence of anti-SARS-CoV-2 antibodies and RNAemia in serum samples collected in the Florenc area before lockdown was declared in Italy. In Tuscany, the total number of cases was 96,990, which was updated on 24th November (total population: 3.73 million). This number has recently escalated due to the second wave of the pandemic.

We examined two groups of subjects, Hep-patients and BDs with the intent of performing an epidemiologic study to assess the circulation of the virus before lockdown and before the first case was diagnosed in Lombardy. At this point, we did not intend to analyze the correlations between SARS-CoV-2 positivity and liver parameters in Hep-patients.

We used rapid serologic tests for the first screening of positive samples. The results were surprising as some subjects showed positivity to anti SARS-CoV-2 antibodies earlier than the first cases in China were described and before the virus was detected in Italy. We decided to test the positive samples through one of the most recently developed methods used by diagnostic laboratories. Chemoluminescence confirmed rapid test screening only in one third of positive individuals. This is in consonance with an African French study that demonstrated the risk of false positives using currently available rapid SARS-CoV-2 serologic tests, especially for the IgM band, even with CE-label and national health authority approval[9]. The authors adopted an identical method to confirm IgG and IgM serum positivity (CMIA on the ARCHITECT System diagnostic testing platform -Abbott Diagnostic Division)[9].

Rapid tests could be relatively beneficial in initial screening as they are inexpensive, easy to use and non-invasive as only a finger prick is required. However, our experience seems to indicate that second level screening should be performed to confirm the presence of anti-SARS-CoV-2 IgG and IgM in the serum of subjects who test positive following the administration of rapid tests.

It has been speculated that SARS-CoV-2 was silently circulating in Italy, as well as in other European countries, before the first official diagnoses (21st February in Italy). An indirect demonstration of the virus' circulation in Italy earlier than the first case was diagnosed, was identified by a study on wastewater conducted in three cities in northern Italy (Milan, Turin and Bologna)[10]. The authors found viral RNA in wastewater samples dating back to 18th December 2019 in Milan and Turin and to 29th January 2020 in Bologna[10]. Conversely, Capalbo et al[11] did not find any clinical evidence of COVID-19 among the 166 patients affected by severe acute respiratory syndrome in an academic hospital in Rome between 1st November 2019 and 1st March 2020[11]. From laboratory or radiological data analysis (using COVID-19 Reporting and Data System and COVID-19 lab-score) the authors identified about 17% of cases that were compatible with COVID-19, although all of the stored nasopharyngeal swabs tested with SARS-CoV-2 RT-PCR were negative[11].

Researchers have been trying to ascertain whether infections had occurred earlier in other countries. In France, where the outbreak was believed to have started in late January 2020, a retrospective study of a stored respiratory specimen collected from a hospitalized patient on 1st December 2019, was positive for SARS-CoV-2[12]. The authors reported that the patient had clinical signs and radiological patterns as previously observed in Chinese and Italian cases[12-14] and detected the viral RNA through two different RT-PCR methods using standardized commercial Real-Time assays[12].

Recently, an Italian group[15] reported a rather high percentage (11.6%) of SARS-CoV-2 specific antibodies in blood samples isolated from 959 asymptomatic individuals. The subjects were enrolled in a lung cancer screening between September 2019 and March 2020[15]. The authors used a homemade non-validated test as a means to assess the presence of IgG and IgM in the sera, therefore, the results, although rather interesting and in line with our findings, should be confirmed through certified and reliable methods. In point of fact, the aforementioned findings are similar to our own experience[9]. We consequently suggest the use of caution when interpreting results even after the administration of CE-labelled rapid tests as approved by national health authorities.

Interestingly, the first subject we found positive returned to Italy in mid-September 2019 after a trip to the Philippines. Although, the CMIA we used as a confirmatory test was not developed to provide complete quantitative results (it is a semi-quantitative assay), we noted IgMs close to the threshold of positivity in two metachronous serum samples in November and a relatively low level in July. Conversely, the level of IgGs increased from November onwards. We also tested the woman's husband with negative results. Interestingly, he did not accompany his wife to the Philippines. However, this is only a speculation, and we are not able to provide any demonstrative evidence. It is conceivable that subject#1 contracted the infection in her country of origin and, subsequently, she was no longer contagious following her return to Italy in September.

A further 2/5 positive subjects had possible risk factors, as subject #5 is a nun who lives in a convent with several sisters (community lifestyle) and subject #8 had frequent contact with foreign tourists as he owns and manages a resort in Tuscany.

Percivalle et al[16] performed a study on BDs recruited in the so-called "Red Zone" in Lombardy, where the first Italian case was diagnosed. Interestingly, despite an extremely high prevalence of anti-SARS-CoV-2 antibodies in those who donated blood during lockdown (28%), the percentage of IgG and IgM positive donors in the 3 weeks before the first diagnosis was 2%. The latter percentage is in line with the one obtained from our analysis of the BD group. This, apparently, complicates our understanding of the behavior of the outbreak in the two Italian regions during the first wave. However, we should consider the fact that the BD group we analyzed was smaller. This could be a statistical bias in our study as it was not designed as a prevalence analysis.

The fact that we found IgG positive subjects among the BDs could raise serious concerns relative to the safety of blood transfusions. As specific SARS-CoV-2 tests were introduced in BDs after 9th March, the issue concerning the safety of blood donated in the time frame in which the virus was circulating without our knowledge is of pertinent relevance. The analysis of previous studies pertaining to SARS-CoV-2 infection through hematic exchange together with the assessment of the admission rules of blood donation in Italy, renders the risk of transfusion transmission of SARS-CoV-2 negligible. In the previously published papers, a cautious approach prevailed. Notwithstanding, reported evidence of hematic transmission, several authors did not exclude

its theoretical risk[17-21]. Moreover, recent papers completely exclude the risk of transmission from blood and blood products after an analysis was conducted on SARS-CoV-2 positive patients who donated blood while infected without any new contagion[22].

Corman et al[23] stated that the risk of infection from transfusion should be considered negligible as no RNAemia was present in the asymptomatic subjects who tested positive after an oral swab or sputum[23]. Despite this, some authors only found RNAemia in patients with severe disease[24]. Our results confirmed these findings, however, a recently published French study showed that a rather small fraction of donors 3/311 remained asymptomatic after donation. The donors were RNAemic for SARS-CoV-2 and showed negative results following the antibody test[25]. A similar observation was reported in Chinese research describing 4 out of more than 7,400 blood donors who had an extremely low plasma viral load[26]. The discrepancy between our study and the one by Chang et al[26] could be due to the higher number of subjects they screened[26]. The disparate results obtained by the French group[25] are harder to explain, due to the fact that the overall number of subjects they evaluated is comparable to our population (Hep-patients plus BDs).

In addition, Corman et al[23] also stated that the risk of infection from transfusion should be considered negligible as people with symptoms of infectious diseases are not permitted to donate blood in Germany[23]. The same strict rules are followed in Italy and people are not allowed to donate blood even with fairly mild symptoms, such as a cough, a sneeze, a headache etc., rendering blood transfusion effectively safe before the first diagnosis of COVID-19 in our country.

As previously stated, the SARS-CoV-2 infection rate in Italy was underestimated due to the high number of asymptomatic patients[27]. In fact, we identified 5 subjects (in a small population) who were completely unaware that they had contracted the infection.

One limitation in our study was the small volume of the retrospectively collected sera that did not permit to repeat the serological tests using different platforms. In fact, at the time we performed our evaluation, the techniques to evaluate SARS-CoV-2 IgM and IgG antibodies allowed only a qualitative detection. Very recently, new platforms for SARS-CoV-2 IgM and IgG quantification became available. Regarding in particular the patient we followed up analyzing metachronous serum samples, a quantitative assay could have allowed to evaluate the behavior of IgM and IgG levels, useful to assess a more accurate contagion time.

Conclusions

Our results suggest an early circulation of SARS-CoV-2 in asymptomatic individuals in Italy, particularly in the area of Florence (Tuscany), unexpectedly before the first COVID-19 cases were described in China. Furthermore, we suggest confirming the positive results obtained from rapid tests with second level analysis even if they are CE-labelled and approved by national authorities. In fact, these tests have multiple benefits, although, in our experience 2/3 positive samples were false positives, which is a further indication that confirmation is indispensable.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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