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Letter to the editor

Diagnosis of infection by SARS-COV-2: Is one molecular test enough?

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Dear Editor,

A pandemic phase of SARS-CoV-2 infection is currently ongoing, with a particular diffusion in Italy [1]. Accurate diagnosis of asymptomatic carriers of the virus as well as of patients with acute infection is crucial to limit viral spreading and assure adequate patient care.

Molecular testing by real-time reverse transcriptase polymerase chain reaction (RT-PCR) of nasopharyngeal swab or other respiratory specimens is the gold standard for the detection of SARS-CoV-2 infection [2]. An early study in 425 cases in Wuhan showed that the mean incubation period for COVID-19 was 5.2 days, although it varied widely between individuals [3,4]. Virus shedding patterns are not yet well understood as it is the case for the timing, compartmentalization, and quantity of viral shedding to inform optimal time for specimen collection. The need for repeated molecular testing for the rule-in of SARS-CoV-2 infection is debated [2]. WHO claimed for new studies aimed at improving current existing knowledge in these issues. This study was aimed at assessing whether molecular testing from a single respiratory specimen is enough for the diagnosis of SARS-CoV-2 infection. This study was performed in the Umbria region, in Italy. Consecutive in- or out-patients with SARS-CoV-2 infection diagnosed by RT-PCR at the Medical Microbiology, Department of Medicine, University of Perugia were included in the study. The decision to test was based on clinical judgment and epidemiological factors, and on an assessment of the likelihood of infection [2]. Subjects with negative SARS-CoV-2 infection work-up were excluded from the study. In all patients, respiratory specimens were collected. Upper respiratory specimens were represented by nasopharyngeal flocked swab in UTM^{TM} viral transport medium (Copan, Italy); lower respiratory specimens were endotracheal aspirate or bronchoalveolar lavage fluid, collected in selected patients with more severe respiratory disease and negative testing by upper respiratory specimens. All samples were assessed at the Medical Microbiology of the University of Perugia. Collection of upper respiratory specimens (swab) was performed at patient home by dedicated personnel or at the hospitals for all admitted patients. Swab specimens were tested according to the protocol described by Corman et al. Briefly, RNA was extracted from respiratory specimens with the Qiasymphony DSP Virus/Pathogen midi kit (QIAGEN, Hilden, Germany) and RT-PCR was performed targeting the SARS–CoV-2 RNA-dependent RNA polymerase (RdRP) and envelope (E) genes [5]. For all patients the number and timing of molecular testing performed before the first positive test was collected. Study patients were divided into three categories: asymptomatic, having mild symptoms (no respiratory failure) or having moderate to severe symptoms (respiratory failure). Moreover, an analysis was performed in patients receiving the first swab at home or at the hospital. Continuous variables are reported as mean and standard deviation in case of normal distribution or as median in interquartile interval in case of non-normal distribution. Categorical variables are expressed as proportions. The Fisher's exact test or the Chi^2 test were used to compare categorical variables. Continuous variables found not to follow a normal distribution were compared using the unpaired Mann-Whitney-U test.

Overall, 1206 patients were diagnosed with SARS-CoV-2 infection from February 27th through April 14th 2020 and were included in the study. The main characteristics of the patients at baseline are reported in the Table. Of note, the study population included two babies aged less than 1 year (five months and 2 months old, respectively) and 30 children aged between 1 and 10 years. The first molecular test was negative for SARS-CoV-2 infection in 66 subjects (5.5%, 95% CI 4.2-6.8). In 56 patients, the diagnosis of SARS-CoV-2 infection was made by a second test, after a median timeframe of 2.5 days from the first negative test (range 1 - 9 days), in 6 patients by a third test, after a median timeframe of 4 days (range 3 - 9 days), and in 4 patients the diagnosis of SARS-CoV-2 infection required four or more molecular testing. A first negative molecular test was more common in men as compared to women (RR 1.29, 95% CI 1.06-1.60). The first test was sampled at patient home in more than 80% of the patients. The proportion of first negative test did not differ between patients tested at home or at the hospital (5.4% vs 6.6%, OR 0.81, 95% CI 0.40-1.67).

For a set of 916 patients, data on the clinical status at the time of first test were available (Table). Severe symptoms (respiratory failure) were present in 30.7% of patients, mild symptoms with no need of oxygen therapy in 62.2% of the patients. In the remaining subjects, the reason for testing was close contact with confirmed cases, or another high-risk

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condition. Among patients with moderate to severe symptoms requiring hospitalization, 21 had a first negative molecular test (7.5%, 95% CI 4.4-10.5). Among patients with mild symptoms, 29 had a first negative molecular test (5.1%, 95% CI 3.3-6.9). Similar rates were observed among patients without symptoms (4.6%, 95% CI 0-9.7).

Our study shows that a single negative molecular test cannot safely exclude SARS-CoV-2 infection, also in patients with new onset of respiratory failure. Multiple molecular testing of respiratory samples can be necessary to correctly rule-in of SARS-CoV-2 infection.

Molecular testing able to detect genetic strands of SARS-CoV-2 by RT-PCR is the current gold standard and is recommended by the WHO and International Societies for the identification of both asymptomatic carriers of the virus and patients with acute infection. Accurate diagnosis is crucial for both: limiting diffusion and diagnosing of SARS-CoV-2 infection. However, the sensitivity of molecular testing has been reported to be as low as 30-60% [2,6-8]. This can be due to several factors. Poor quality of the specimens, containing little patient material as shown by the finding of low human DNA in samples with no evidence of SARS-CoV-2 genetic material; timing of sampling as the viral load in specimens collected too late or very early in the infection can be too low for detection or even absent [9]; inappropriate handling and/or shipping of the specimens [2]. All these being considered, it is conceivable that one negative molecular test result does not rule out the possibility of COVID-19 virus infection.

This study shows that about 5% of patients with infection (upper limit of the 95% CI about 7%) have negative results at first molecular testing either in the out-of-hospital or in-hospital setting and in patients with or without severe infection. These results have several clinical implications. Admitting a symptomatic patient to a no-COVID department or discharging at home based on a single negative molecular test may harm the community and prolong diffusion of SARS-CoV-2; in these patients, missing diagnosis may prevent proper patients' therapy. The high rate of first negative test in patients with severe symptoms represents a warning for physicians. When clinical judgement poses a high pre-test suspicion, repeated testing is required. Our data support the WHO statement that in patients with a high index of suspicion for SARS-CoV-2 infection and a negative molecular test, particularly when only upper respiratory tract specimens were collected, additional specimens should be collected and tested, including those from the lower respiratory tract [10]. In asymptomatic subjects, missing a positive test results means avoiding the possibility to limit the diffusion of infection. It is well known, in fact, that asymptotic carriers of the virus are an important source of infection. Clinical studies tailoring multiple testing based on pre-test clinical probability (signs and symptoms but also exposure to infection) are needed.

At the beginning of the epidemic, Italian National guidelines established that, in the case of a first negative sample, a subsequent sample should be analyzed by RT-PCR. Recently, guidelines have been modified, claiming that a single test may be sufficient. Our findings show that, during the epidemic phase, testing of a single sample is not able to safely exclude SARS-CoV-2 infection. Indeed, 55 patients (4.6%) were identified as positive at a second molecular testing performed about 2.5 days apart, and for other 11 patients even multiple testing were required.

Our results are highly related to the current epidemiologic phase of SARS-CoV-2 diffusion. Once the prevalence of acute SARS-CoV-2 acute disease will decrease, the specific value of molecular over serological testing can change. As for the state of disease in Italy, molecular testing should still be considered the gold standard for diagnosis and surveillance.

Our study has some limits. The limited proportion of asymptomatic subjects included in the study does not allow any firm conclusion on the need for repeated testing in this setting. The clinical presentation was not available in the full set of included patients, although it is reported for almost 1,000 subjects. However, our study has also some strengths. This is one of the largest studies reporting on the results of serial testing in patients diagnosed with SARS-CoV-2 infection, allowing estimation of strategies for accurate diagnosis.

In brief, according to current epidemiologic status and prevalence of disease, a single negative RT-PCR molecular test is insufficient to rule out COVID-19. Whether the need for multiple assessments can be tailored on clinical setting needs to be further evaluated.

Author contributions

Antonella Mencacci and Cecilia Becattini contributed equally to this manuscript.

Study concept and design: AM, CB

Data analysis and interpretation: AM, MCV, BC, EC, CB

Drafting manuscript: AM, CB

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Table. 1

Table. 1
Demographic characteristics of 1206 patients diagnosed with SARS-CoV-2 infection from February 27th through April 14th 2020, at the Medical Microbiology of the University of Perugia and molecular test results based on clinical setting.

	Overall population	First positive test	First negative test
Patients' Characteristics	(N= 1206)	(N=1140)	(N=66)
Age (years), mean±SD (range)	53±20(0-103)	53±20(0-103)	54±19(5-91)
Male sex, n (%)	588 (48.8)	550 (48.2)	41 (62.1)
Setting of sampling and disease severity			
Setting of first evaluation	(N=892)	(N=842)	(N=50)
Home	741	701	40 (5.4%)
Hospital	151	141	10 (6.6%)
Indication for testing, n (%)	(N=916)	(N=863)	(N=53)
Moderate to Severe symptoms Mild Symptoms Other (contacts, high risk subjects)	281 570 65	260 541 62	21 (7.5%) 29 (5.1%) 3 (4.6%)

Declaration of Competing Interest

The authors declare they have no conflict of interest relevant to the present study.

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