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Severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) detection pitfalls

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Abstract

The Severe Acute Respiratory Syndrome (CoVID 19) provoked by Coronavirus 2 (SARS CoV 2) require science-based responses. The aim of this work is to assess pitfalls found during the search of viral genomes due to sampling timing, swabbing, storage, heat-infectivity inactivation and further sample processing.

According to several meta-analysis, on the day of symptom onset, the median false-negative rate is estimated to be 38% and decreased to 20% on day 8 (3 days after symptom onset) then increased to 66% on day 21 suggesting that rRT-PCRs adds little information immediately after exposure.

RNA isolation from samples requires cautious handling using RNase-free solutions, pipet tips and glassware. The rRT PCR detection limits are estimated between 39 and 779 copies/mL but 3000 to 20.000 copies/ml for the antigen test.

External cross contamination by imperceptible splatting requires risk management integrating the Pharmacopoeias by processing at least 10 negative contiguous to 10 positive controls in each sennries of 100 tests. . For Ct >34 it was suggested no transmissible disease.

The detection of antibodies one month or later after clinical signs may confirm positivity. Lack of immune response in non-immune compromised asymptomatic people may invalidate positivity. False positive disrupts efficiency for containing infections and leads to societal anxiety undermining health workforce. Because spurious methods create confusion, each step of diagnosis requires quality-control and risk assessment, knowing that rRT PCRs amplify more than 10.000 million times the signal of 1 viral element.

Key words: SARS CoV2; CoVID; rRT-PCR; False positive; False negative; Antigen

1. Introduction

Emerging respiratory viral infections like the Severe Acute Respiratory Syndrome (CoVID 19) provoked by Coronavirus 2 (SARS CoV 2) require science-based responses. Policymakers are charged with taking actions to protect their population from the diseases whilst it appears that their lack of reliable information. Hence, their risk assessment of critical parameters should contribute to the highest chance of detection sources of wrong conclusions especially while performing laboratory testing [1].

The Nucleic Acid Amplification Techniques (NAATs), particularly the reverse transcriptase real-time polymerase chain reactions (rRT-PCRs) are largely used for detecting infected agents [2]. However researchers, policymakers and the public lack of arguments to explain that people with positive rRT-PCRs SARS-CoV-2 results are whether symptomatic, paucy-symptomatic (mild symptoms) or asymptomatic, and in several cases the putative infection does not trigger

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specific immune responses [3], [4],[5],[6]. On the other hand, clinical presentations of CoVID with false negative results also require a better understanding.

This work is aimed to propose a few realistic solutions to minimize false viral detection deductions.

2. Tests for detection of SARS-CoV-2

Nucleic amplification techniques (NAATs) were largely used for case identification and surveillance of SARS-CoV-2 spread (WHO-1). They were mainly developed by researchers in China, Germany, Hong Kong, Japan, Thailand, France, and USA [7], [8], [9],[10].

Among NAATs, the rRT-PCRs are considered the gold standard for detecting SARS-CoV-2 particularly in the acute phase of infection and its use out of confirmed history of viral exposure remains controversial. Moreover, the current rRT-PCRs were validated across panels of material tested under idealized situation and with hospital samples containing viral loads at different levels than those found in communities to be screened [4], [10], [11].

The loop-mediated isothermal amplification (LAMP) is a one-step isothermal amplification reaction different from the PCRs. Here, the amplification of a target sequence is conducted with 4 to 6 primers to ensure high sensitivity and specificity under isothermal conditions (63-65°C). It uses a polymerase with high strand displacement activity and several primers to identify different regions of the SARS-CoV-2 genome. For RNA viruses, LAMP also requires reverse transcription of the viral RNA. Results can be determined visually without requiring a machine [12], [13]. The rRT-LAMP has been used for the detection of various respiratory agents with detection limit of 1000 copies/ml [14].

3. Risk assessment of pre-analytical procedures

WHO recommends that adequate standard operating procedures should assure that staff is trained for appropriate specimen collection, storage, packaging, and transport, indicating that all specimens collected for laboratory investigations should be regarded as a potential risk.

Guidelines on laboratory biosafety and international recommendations stated strict adherence to physical distancing and adequate personal equipment for health care workers [15], [16]. Masks, eye shield, grown, gloves and equipment must be available during sampling as well in clinical and in laboratory settings. These mechanical barriers do not protect the samples (do not avoid viral RNA to stick on gloves or on other surfaces).

Despite continuous improvements in automation and bar codes for tubes and files to minimize risks of wrong determinations, the pre-analytical phase is still source of errors [17], [18].

For people presenting clinical signs, the SARS CoV 2 detection is affected by the sampling timing (before and after symptoms onset), the swabbing procedures, the transport solutions [19], [20] and the conditions of freezing and thawing of the samples in the transport media.

In addition, the quality and relevant abundance of viral RNA is heavily dependent on the type and site of collection (nasopharyngeal and oropharyngeal swabs samples are suitable for respiratory viruses) [21].

After sampling, the tubes containing RNA viruses require special care due to the degradation of the viral genes during shipping, freezing, storage, thawing and heat-infectivity inactivation. Numerous laboratories preform sample-infectivity inactivation by heating at 56°C. This procedures disrupt the integrity of the single-stranded viral RNA and adversely affects the quality of viral detection, especially for samples carrying low viral loads [22].

Coronaviruses genomes are single-stranded RNA exposed to RNases, a group of enzymes that degrade RNA molecules. These enzymes are abundant in the environment, including on hands and on surfaces, and it is difficult to remove/destroy RNases completely. On the above, RNA isolation procedures require extreme cautious handling according to aseptic techniques and using RNase-free solutions with RNase-free pipet tips and glassware.

The Dacron or polyester flocked swabs with mucosal secretions and with the potentially infected cells and the free viral particles should be introduced into tubes and firmly closed (with gloves that may have stick viral micro drops). Numerous units disinfect the gloves and surfaces with biocides. Nevertheless, there is no evidence showing that alcohols or any other disinfectant eliminate nucleic acids. Knowing that positive PCR results were experimentally obtained with

the eluate of gloves used for sampling [23] the traces of viral genomes, -if released into further sampling devices or tubes-, are source of false positives. Nonetheless, changing gloves after sampling each individual seems unrealistic in contexts of massive screening with restricted health budgets.

4. Laboratory pitfalls during sample preparation

The purpose of sample preparation is to extract and concentrate the viral nucleic acids to make them accessible for retro-transcription and amplification, removing potential inhibitors of the reaction. At the beginning, RNA-sequences of an internal control (IC) unrelated to the SARS-CoV-2 should be introduced into each sample. These ICs will be extracted, retrotranscribed and amplified simultaneously in the same tube of each sample, validating that the whole reactions proceeded correctly [24].

For viral nucleic acid extraction in high concentration of background, the use of silica-coated magnetic beads is suitable. Here, nucleic acids are captured on the particles and washed to remove unbound components. The bound nucleic acids are eluted from the beads and transferred to a plate for further procedures (Mayer et al).

It was shown that manual RNA-extraction accounted to be less efficient than robotic systems [21], [25].

4.1. Retro-transcription

The extracted and purified RNAs should be converted to a complementary cDNA by a reverse transcriptase of a thermostable DNA-polymerase derived from the thermophilic bacteria *Thermus thermophilus* (rTth DNA polymerase). First, the reverse primers for the SARS-CoV-2 and the IC anneal to their respective targets, and if present, they are extended. After a denaturation step, -in which the temperature of the reaction is raised above the melting point of the neo-formed double-stranded cDNA/RNA-, a second set of primers anneals to the cDNAs (of the IC and of the target) and is extended by the polymerase activity of the rTth to create a double-stranded DNA [9]. The manual procedures for extraction and retro transcription require serial pipetting into different reaction tubes with potential risks for micro drops to splatter.

4.2. The need for endogenous rRT-PCR controls

Epithelial cells that line the nasal pits and the oropharyngeal respiratory epithelium (expressing SARS CoV 2 receptors) should be present in the samples to be tested.

Most of the current tests just assess the presence of human DNA in the specimens by detecting the presence of beta actin or the cellular housekeeping ribonuclease /MRP subunit p30 (RPP30) gene. These markers were not yet calibrated with serially dilutions of epithelial cells to allow semi quantification of cell load in the samples. Nevertheless, even with their limitations, their qualitative assessment showed a strong association between positive SARS CoV 2 rRT PCR results and high levels of RPP30 gene expression. On this matter, we and others determined previously that samples containing less than 50 cells/µl of swab eluates of conjunctival scrapings from infected children with clinical signs of active trachoma produced false negative results [26], [27]. The samples with low RPP30 gene expression accounted for most contradicting results [28] and for false negative. On the above, it appears that results for specimens containing cell loads below a threshold (to be uniformized by laboratories) should be invalidated. Finally, the quantification of cells will allow uniformizing viral load denominator (viral RNA copies/ number of cells).

5. Amplification process

The SARS-CoV-2 genome is structured as a positive-sense single-stranded RNA genome with a 3′ terminus coding information for 4 structural proteins: spike (S) glycoprotein; envelope (E) protein; membrane (M) glycoprotein and nucleocapsid (N) phosphoprotein, and 8 accessory proteins. These genes are detected using synthetic probes consisting in single-stranded DNA oligonucleotides with fluorophores at the 5′ end and quenchers at the 3′ end. In the absence of target sequences, the fluorescence of the probes is quenched. In presence of target sequences, the probes hybridize to complementary sequences separating the fluorophore and the quencher, allowing fluorescent emission and detection. None of the commercially rRT-PCR kits should cross-react towards a panel of other respiratory viruses except for the SARS-CoV-1 E-gene [29], [30].

During the chain reactions, the amplificated products dissociate to single strands at high temperature during each round, allowing further primer annealing and extension as the temperature is lowered. As new copies are built, the

calibrated computer software tracks the amount of fluorescence after each cycle. When a certain level of fluorescence is surpassed, this indicated that the searched sequence is present.

The signals produced in each sample by the IC should be the same than those obtained with the negative controls. Delayed Cts for the IC reveal deficiency in the extraction and/or in the retro transcription and/or the presence of rRT-PCR inhibitors. Negativity cannot then be reported and the extracted RNA should be retested and if possible a second sample should be requested.

Positive SARS CoV 2 results require the detection of at least 2 or 3 signals (S,M,E,N) after amplifying several targets of the virus genome. Discordant results for signals from symptomatic patients, especially the S require resampling and sequencing the virus or the amplicons from the original specimen. The detection of only 1 signal is not sufficient for rRT-PCR positivity [31], [32].

6. Negative and false negative results

The rRT-PCR is a complementary diagnostic tool given its high specificity for SARS CoV2. However, some reports suggest it has a limited sensitivity. In fact, the incidence values of the rRT-PCR positive asymptomatic cases in different studies show despair levels of 16% for children in Wuhan [32] , 30.8% in Japan [33], 50% in the Diamond Princess cruise ship [34], 10.7% in Korea [35], and 56.5% in the USA [36], [37], [38]. Moreover, viral nucleic acid test results of 4880 cases suspected of having a respiratory infection in Wuhan found a sensitivity of 57% [39].

In pharyngeal swab samples the sensitivity of the rRT-PCR tests for COVID-19 was of 32% samples (taken 1–3 days after admission). Although rRT-PCR testing reports low sensitivity for SARS-CoV-2 when a pharyngeal sample was used, the greater values for RT-PCR sensitivity given appropriate sampling (i.e., sputum or nasal), compared with the biased studies, affirm biases resulted in an underestimation of the sensitivity of RT-PCR testing [28].

Moreover, seven studies provided data on rRT-PCR performance by time since symptom onset or SARS-CoV-2 exposure. rRT-PCRs were used to rule out infection among exposed people and health care workers by day since infection was estimated. On the day of symptom onset, the median false-negative rate was 38% (CI, 18% to 65%) and decreased to 20% (CI, 12% to 30%) on day 8 (3 days after symptom onset) then began to increase again, from 21% (CI, 13% to 31%) on day 9 to 66% (CI, 54% to 77%) on day 21. On the above, it appears that rRT-PCRs adds little information in the days immediately after exposure. Hence, it was suggested to consider waiting 1 to 3 days after symptom onset to minimize wrong conclusions. Hence, if clinical suspicion is high, infection should not be ruled out based on rRT-PCR results without considering the clinical and environmental conditions [40].

According to reports from China it seems that in asymptomatic people false negative rRT-PCRs may account for approximatively 40%, and indeed being negative, they may potentially spread the infection (false negative results in subjects for up to two weeks) [32],[39]. In repeat sample testing a systematic review reported false negative results between 2 and 33%. [41]. Other studies showed negative rRT PCR results in patients with pathognomonic ground glass chest images in computerized tomography that may contribute to clarify the diagnosis. However, normal chest computerized tomography were reported in positive SARS CoV-2 rRT-PCRs) [40], [42].

The analysis of the distribution of cycle threshold (CT) values for patients who tested positive or presumptive positive on the SARS-CoV-2 rRT PCR, showed viral detection limits based on dilution of a SARS-CoV-2 clinical isolates of 0.004 to 0.007 median tissue culture infectious dose, ($TCID_{50}$)/ml. This detection limit corresponds to CT values of approximately of 32.7 to 36.4. The limit of detection concentration of analyte that will be detected in 95% of replicate tests between the laboratory-based tests was estimated to 39-779 copies/mL by rRT PCR and of 3000 to 20.000 copies/ml for one of the antigen point of care tests [43], [44), [45]. Hence, for inpatients whose symptoms have resolved and two tests over 24 hours apart are either negative or close to the Ct cutoff (Ct >34) it was suggested that they likely do not have transmissible disease, and thus do not need to be retested [46]. Furthermore, it appears pertinent for clinicians to consider the Ct results in context to determine when a patient can discontinue isolation, shortening duration of isolation. Taking the Ct value into account may also justify symptom-based strategies including time-since-illness-onset and time-since-symptom-resolution based approaches [46).

From a technical point of view, special precautions are required in the lab to avoid air bubbles in the micro tips (difficult to detect by the human eye) while pipetting extremely reduced volumes (5 or 10 µl) of nucleic-acids because they may dramatically reduce the molecular patterns available for amplification.

False results should be suspected for patients experiencing a “turn positive” of SARS-CoV-2 by rRT-PCR after consecutive negative results or for recurrences or prolonged positive conversion [47].

6.1. Positive and false positive results

The current rRT-PCR lack of predictive power in terms of telling whether people will develop severe clinical complications or die. This global confusion led to the reporting of cases as a function of the number of tests conducted. Nonetheless, and irrespective of the clinical picture, several heath authorities have considered a single positive rRT-PCR result for confirmation of CoVID 19. Positive rRT-PCR results do not inform whether the person carries the whole viral particles or whether the virus is infectious. They do not rule out bacterial infections or co-infections with other viruses. A positive rRT-PCR result may not necessarily mean the person is infectious or still has meaningful diseases because the detected RNA could be from nonviable viruses. In fact, live virus is generally isolable only during the first week of symptoms but not after day 8, even if rRT PCR tests remain positive [6], [45], [47].

In asymptomatic people with rRT-PCR positive results, normal laboratory findings were reported for 55%. In these cases, lymphocytopenia as a marker of viral infection was observed only in 16%, and C-reactive protein levels were slightly above the normal range only in 14% [1], [9], [48] suggesting that rRT-PCRs positive did not always reflect the presence of active/transmissible infection. In addition, and as previously said, low cellular loads in specimens obtained from clinically suspected patients should have invalidate negative results [49].

In New York City area, the screening by rRT-PCR and the further ELISA for the detecting anti-SARS CoV-2 spike antibodies showed that of the 624 participants with clinical confirmed CoVID19 symptoms, all but three seroconverted to the SARS-CoV-2 spike protein, whereas only 37% with just “suspected SARS CoV-2 infection” seroconverted, suggesting that the rRT-PCR overestimated the presence of the virus [50].

In the Veneto Region (Italy) the rRT-PCRs conducted in health care workers who developed severe COVID-19 presentations detected specific antibodies in 100% but only in 83% of those with moderate disease. In asymptomatic subjects with rRT-PCR positive results the seroconversion rate was much lower (58%) [51].

For positive rRT-PCR asymptomatic people in close contact with COVID19 patients in Jiangsu (China, the pathognomonic ground glass chest images were observed only in 50% [40], [42], [52], [53]. Moreover, other studies reported that none of the asymptomatic rRT PCR positive patients developed severe pneumonia or died [33].

For individuals who did not seroconvert the RT-PCR positive results require carefully assessment of cross contamination risks. In the current climate, false positive results may have increased, with consequences at the health workers and society without pointing that methodological limitations generating false positive results interfere with the figures used to draw up SARS CoV 2 active infection, morbidity and death rates. On this matter, in 2020 the US Center for Disease Control and Prevention had to withdraw testing kits when they showed high rate of false positive [4], [54].

7. Antigen testing

Many companies have come up with rapid antigen tests as laboratory-based tools and point-of-care tests. These tests are based on lateral flow immunochromatographic assay for the detection of protein antigens specific to SARS-CoV-2.

A positive antigen test can be confirmatory in symptomatic patients, while a negative result should be verified with rRT PCR.

The rapid antigen tests are not reliable for testing asymptomatic people, who often carry low viral load, because the limit of detection are 100 times higher than rRT PCR (values ranging between 3000 and 20.000 copies/ml for antigen testing versus less than 40 copies/ml for rRT PCR) [43], [44], [45].

The rapid antigen testing detected between 11.1 % and 45.7 % of rRT-PCR-positive samples from COVID-19 patients, suggesting that they may serve only as adjunct to rRT-PCR because of potential for false-negative results [56]. On the above, the interim guidelines for antigen testing for SARS-CoV-2 formulated by CDC recommended a different strategy to interpret the results of antigen testing among symptomatic and asymptomatic individuals [55].

8. Contributions for technical pitfalls remediation

To prevent the spread of infections it is necessary to identify people who are infectious. Currently, diagnosis, screening, and surveillance depend on SARS-CoV-2 reverse transcriptase quantitative PCRs results reported to the ordering physician as positive or negative. However, these does provide any estimation of the viral load in the sample (reported from the cycle threshold value), particularly when testing in the absence of symptoms for CoVID-19, the Ct value could help to clinical decisions. Because viral RNA loads peak within the first infection days in the upper, and later in the lower respiratory tract, viral load testing needs to be standardized.

Nucleic acid preparation (especially if manually performed) and mix for retro transcription and amplification are frequently handled with open recipients. Here, external cross contamination risks by imperceptible splatting is not negligeable. The procedures of commercially kits indicate to run controls in each run of determinations. However, they do not mention the number of negative tests related to the number of samples to be included in each series (most recommend to test 1 positive and a 1 negative control per run).

Considering the risks of false positive rRT-PCR results during sample processing, it appears pertinent to extrapolate the procedures largely established by the Pharmacopoeia for external contamination controls. In fact, at least 10% of representative samples of each batch of 100 elements should be controlled as established by the European Pharmacopoeia [57]. According to this, a minimum of 10% controls should be processed in each run (i.e. couples of 10 negative contiguous to 10 positive controls randomly distributed in each series of 100 tests).

In addition, the location (well, tube, etc.) of positive results in a run may contribute to assess the potential cross contamination risks in contiguous samples especially for samples from asymptomatic individuals without notion of contact with infected fellows. If i.g. 2 samples produce positive results in flanking positions, and if for one the Ct >32 or <25, the clinical signs and the viral transmission context should be carefully reverified for both.

If a sample neighboring a positive result shows Ct>32 (for at least 2 viral targets) retesting should be conducted [58].

Considering that the infected subjects with viruses develop specific cellular and humoral immune responses, the detection of (at least) specific antibodies (one month or later after clinical signs) confirms the positive rRT-PCR result. The lack of immune response in non-immune compromised asymptomatic people may invalidate the positive rRT-PCR SARS CoV 2 results However, the diagnostic accuracy of antibody tests to determine if a person presenting in the community or in primary or secondary care has SARS-CoV-2 infection, or has previously had SARS-CoV-2 infection, and the accuracy of antibody tests for use in seroprevalence surveys showed for pooled results for IgG, IgM, IgA, total antibodies and IgG/IgM, low sensitivity during the first week since onset of symptoms (all less than 30.1%), rising in the second week and reaching highest levels in the third week. The combination of IgG/IgM showed a sensitivity of 30.1% for 1 to 7 days, 72.2% for 8 to 14 days, 91.4% for 15 to 21 days. For 21 to 35 days, pooled sensitivities for IgG/IgM were 96.0%. On the above, the sensitivity of antibody tests is too low in the first week since symptom onset to have a primary role for the diagnosis of COVID-19. Nevertheless, they may still have a role complementing other testing in individuals presenting later, when rRT-PCR tests are negative, or cannot be performed. Antibody testing may help for detecting Coronaviruses infections if performed 21 or more days after symptom onset. Concerns about high risk of bias and applicability make it likely that the accuracy of tests when used in clinical care will be lower than reported in the published studies [59].

Finally, the addition of controls for rRT PCR requires additional health resources because it increases the cost of each determination (by at least 20% for the reactants). Nonetheless, the false positive diagnosis possesses a threat to the public health disrupting the efficiency of emergency strategies, health policies, and preventive measures for containing known or emergent infections. They may lead to unrequired treatment and societal anxiety, undermining the health workforce [60].

9. Conclusion

Accurate testing for air borne infections followed by appropriate protective measures is paramount to prevent both, nosocomial and community transmission. However, spurious methods create confusion.

Each step of laboratory diagnosis requires constant and strict quality-control and risk assessment, knowing that rRT PCRs amplify more than 10.000 million times the signal of 1 viral element.

Keeping in mind that rRT-PCRs are tools for clinical reference and should not be the exclusive evidence for diagnosis and treatments (especially positive results for asymptomatic and not exposed populations), false results induce wrong decisions faced by governments and international organizations [61].

Any deviation of the diagnostic sensitivity and specificity will reduce the predictive values of the test. The occurrence of contaminations of commercial primers/probe sets with the SARS-CoV-2 target sequence of the rRT-PCR is an example for pitfalls during PCR diagnostics affecting diagnostic specificity.

It appears imperative to pre-test each batch of reagents extensively before use in routine diagnosis, to avoid false-positive results and low positive predictive value in low-prevalence situations. As such, contaminations may have happened more widely, and COVID-19 diagnostic results should be re-assessed retrospectively to validate the epidemiological basis for control measures [62].

Care must be taken when interpreting rRT-PCRs particularly early in the course of infection and when using these results as a basis for removing precautions intended to prevent onward transmission. In fact, false-negative results have a deleterious epidemiological effect against the global efforts facing outbreaks.

Presently, isolated RT-PCR positive results cannot be used to inform about the severity, the spread of the illness and the potential death rates and additional efforts are required to improve the quality and the predictive value of laboratory testing.

Methods lacking appropriate validation procedures are part of the uncertainty surrounding coercion and coaxing not yet sustained on a precise determination of infection. Therefore, diagnostic pitfalls require clear understanding because they may have attribute to SARS CoV 2 severe complications and deaths from other conditions [3]|, 63].

Compliance with ethical standards

Disclosure of conflict of interest

Author declares no conflict of interest.

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