

Discrepancy between PCR based SARS-CoV-2 tests suggests the need to re-evaluate diagnostic assays

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Research note

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Abstract

Objective: To investigate the possible cause of discrepancy between clinical and RT-PCR based diagnosis of patients with COVID-19.

Results: Laboratory records of ten patients with mild to severe COVID-19 were examined. Initially, respiratory samples from the patients were tested using the Roche SARS-CoV-2 Cobas assay and were found to be negative. Further investigation for other viral causes of pneumonia was conducted using the Filmarray RP2.1 assay, which identified SARS-CoV-2 as the pathogen in all ten cases. To investigate whether this could be due to discrepancies between PCR assay, additional testing was conducted using the TaqPath™ COVID19 PCR. Eight of ten samples were positive for SARS-CoV-2 on the TaqPath assay. Further, Spike gene target failures (SGTF) were identified in three of these eight cases. Discrepancy between the three PCR assays could be due to variation in PCR efficiencies of the amplification reactions or, could be due to variation in the primer binding sites. The identification of strains with SGTF identify the possible presence of new SARS-CoV-2 variant strains. Regular modification of gene targets in diagnostic assays may be necessary to maintain robustness and accuracy of SARS-CoV-2 diagnostic assays to avoid reduced case detection, under-surveillance, and missed opportunities for control.

Introduction

Robust diagnostic testing for SARS-CoV-2 is integral to disease surveillance and control of COVID-19. Reverse-transcription polymerase chain reaction (RT-PCR) based assays are the most widely used assay to detect RNA viruses. Selection of target genes for diagnostic assays is a critical and most diagnostic assays in use include two or more gene targets to maximize diagnostic accuracy (sensitivity and specificity) [1].

SARS-CoV-2 was identified in December 2019 as a beta-coronavirus of the *Sarbecovirus* family, with a positive-sense RNA genome of 29.9 kb in size, fourteen open reading frames (*orf*) encoding accessory nonstructural viral proteins, the nucleocapsid (N), membrane (M), spike (S), and envelope (E) structural proteins [2]. In order for SARS-CoV-2 testing to be reliable, it is necessary that assays that identify viral RNA should be consistent and comparable. When recommendations for SARS-CoV-2 diagnostics were first made, the E gene and RdRp were amongst recommended potential target genes [3]. The E gene is known to have higher sensitivity while the inclusion of the N, S, and RNA dependent RNA polymerase (*RdRp*) genes are recommended for higher specificity [4].

No test is 100% accurate and false-negative results with commercially available diagnostic assays have been documented since the early days of the pandemic [5]. False-negative results in the context of symptomatic COVID-19 illness may have several determinants, such as the clinical specimen type (sputum and bronchoalveolar lavage have higher detection rates than nasal and naso- or oropharyngeal specimens) [2], temporal variation in viral shedding [6], as well as diagnostic primer/probe mismatches with infecting SARS-CoV2 virus sequence [7]. However, in the context of increasing reports of SARS-CoV-2 variants seeing false negative results in diagnostic tests are of particular concern, as there is a wider implication on misidentification of asymptomatic cases as well, particularly [8, 9].

Methods

Patients were diagnosed with COVID-19 based on clinical, laboratory and radiological parameters which were used to assess the severity of disease. Clinical history was taken. Ferritin, C-reactive protein (CRP) and D-dimers were measured. Chest imaging was done by Computerised tomography (CT) scan. Initial PCR tests used the SARS-CoV-2 Cobas® (Roche diagnostics Rotkreuz, Switzerland) targeting Orf1-ab and E genes. A second PCR was conducted using the Filmarray RP2.1 test (bioMérieux, Marcy-l'Étoile, France) which includes 4 bacterial and 18 viral pathogen targets i.e. Adenovirus, influenza A viruses H1, 2009H1, H3 (FluA-H1, FluA-2009H1, FluA-H3), influenza B virus (FluB), parainfluenza virus types 1 to 4 (Para

1–4), coronaviruses 229E, HKU1, OC43, and NL63 (CoV-HKU1, NL63, 229E, OC43), MERS-CoV (MERS coronavirus), human metapneumovirus (hMPV), Respiratory Syncytial Virus (RSV), human rhinovirus/enterovirus (Rhino/Entero), *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Bordetella pertussis*, and *Bordetella parapertussis* in addition to SARS-CoV2 (S and M gene targets). A third PCR was conducted using TaqPath™ COVID19 (Thermo, Applied Biosystems, USA) assay (S, N and Orf1ab gene targets).

Results

We report on ten patients with COVID-19 from Karachi, Pakistan from 18th January – 18th February 2021, presenting to acute care at a tertiary hospital. The patients were investigated for COVID-19 based on their clinical presentation. They were mostly aged 65–74 years (60%), followed by two cases aged > 75 years and two below 54 years of age, Table 1. Patients had between five and forty days of illness with a median period of 8 days. Laboratory parameters found to be raised in all cases were: hyperferritinemia, high biomarker levels (C-reactive protein, D-dimer), details not shown. Chest imaging showed ground-glass opacities or bilateral parenchymal infiltrates, and high population prevalence in all cases. Respiratory samples tested by PCR for SARS-CoV-2 using the SARS-CoV-2 Cobas Roche assay were negative in all cases. Due to a high clinical suspicion for COVID-19, samples were further set for testing on the Filmarray RP2.1 platform. In all ten cases, Filmarray results indicated SARS-CoV-2 RNA to be present in the respiratory samples.

Table 1
Description of COVID-19 patients and their differential diagnostic PCR results

No.	Age range Δ (years)	Duration of illness at presentation (Days)	Severity	Chest imaging (radiography/CT scan)	Results - Roche SARS-CoV-2 assay (<i>orf1ab</i> and E gene targets)	Results for the Filmarray RP2.1 assay (S and M gene targets)*	Results for TaqPath 2019-nCoV assay (<i>orf1ab</i> , S and N gene targets)	Explanation for discrepancy with Roche or Filmarray assays
1	>75	7	Non-severe	CT: Bilateral ground glass opacities	NEG \boxtimes	POS	<i>orf1ab</i> , S, and N detected	Possible <i>orf1ab</i> and E gene polymorphism
2	65–74	5	Severe	CT: Bilateral patchy consolidations	NEG \boxtimes	POS $\boxtimes\boxtimes$	<i>orf1ab</i> and N detected, SGTF	Possible <i>orf1ab</i> and E gene and S gene polymorphism
3	65–74	8	Severe	CT: Bilateral ground glass opacities	NEG \boxtimes	POS	No targets detected, SGTF	Possible N, E, and <i>orf1ab</i> gene and S gene polymorphism
4	45–54	5	Severe	CT: Bilateral ground glass opacities	NEG \boxtimes	POS $\boxtimes\boxtimes$	S and N detected (<i>orf1ab</i> not detected)	Possible E and <i>orf1ab</i> polymorphism
5	65–74	8	Critical	Radiograph: bilateral infiltrates	NEG \boxtimes	POS	<i>orf1ab</i> , S, and N detected	Possible <i>orf1ab</i> and E gene polymorphism
6	65–74	10	Critical	Radiograph: predominant right infiltrates	NEG \boxtimes	POS	<i>orf1ab</i> , S, and N detected	Possible <i>orf1ab</i> and E gene polymorphism
7	65–74	40	Long COVID/Post COVID sequelae	CT: Bilateral ground glass opacities	NEG \boxtimes	POS	<i>orf1ab</i> , S and N detected	Possible <i>orf1ab</i> and E gene polymorphism

NP = not performed; D = Detected; NEG = Negative; POS = Positive; CRP = C-reactive protein (normal range 0–14 mg/L); F = Ferritin (normal range 10–291 ng/mL); D-dimer normal range <0.5mg/L FEU; \uparrow = elevated; Ct = Cycle threshold; SGTF, S gene target failure.

Δ Patient ages are given as age ranges to preserve confidentiality and anonymity

\boxtimes Results rechecked on a new sample collected within 48 hours

$\boxtimes\boxtimes$ Results rechecked on same sample

* Results negative for all other pathogens detected by Filmarray RP2.1 i.e. Adenovirus, influenza A viruses H1, 2009H1, H3 (FluA-H1, FluA-2009H1, FluA-H3), influenza B virus (FluB), parainfluenza virus types 1 to 4 (Para 1–4), coronaviruses 229E, HKU1, OC43, and NL63 (CoV-HKU1, NL63, 229E, OC43), MERS-CoV (MERS coronavirus), human metapneumovirus (hMPV), Respiratory Syncytial Virus (RSV), human rhinovirus/enterovirus (Rhino/Entero), *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Bordetella pertussis*, and *Bordetella parapertussis*.

No.	Age range Δ (years)	Duration of illness at presentation (Days)	Severity	Chest imaging (radiography/CT scan)	Results - Roche SARS-CoV-2 assay (<i>orf1ab</i> and E gene targets)	Results for the Filmarray RP2.1 assay (S and M gene targets)*	Results for TaqPath 2019-nCoV assay (<i>orf1ab</i> , S and N gene targets)	Explanation for discrepancy with Roche or Filmarray assays
8	>75	8	Critical	Radiograph: bilateral infiltrates	NEG \boxtimes	POS	<i>orf1ab</i> , S, and N detected	Possible <i>orf1ab</i> gene polymorphism
9	65–74	7	Critical	CT: Bilateral ground glass opacities	NEG \boxtimes	POS	No targets detected, SGTF	Possible N, E, <i>orf1ab</i> and S gene polymorphism
10	35–44	10	Non-severe	CT: Bilateral ground glass opacities	NEG \boxtimes	POS $\boxtimes\boxtimes$	<i>orf1ab</i> , S, and N detected	Possible <i>orf1ab</i> and E gene polymorphism
NP = not performed; D = Detected; NEG = Negative; POS = Positive; CRP = C-reactive protein (normal range 0–14 mg/L); F = Ferritin (normal range 10–291 ng/mL); D-dimer normal range < 0.5mg/L FEU; \uparrow = elevated; Ct = Cycle threshold; SGTF, S gene target failure.								
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To further investigate this, the same ten respiratory specimens tested by Filmarray test were subsequently tested for the presence of SARS-CoV-2 RNA on the TaqPath™ COVID19 assay. Here we found, eight respiratory specimens to be positive and two were negative. All three gene targets in the TaqPath assay (Orf1ab, N and S) were detected in six samples. In one sample N and S were detected but Orf1ab target amplification was absent. S gene target failure (SGTF) was identified in three specimens.

Discussion

Our data suggest show variations between diagnostic platforms for SARS-CoV-2. A summary of differential commercial RT-PCR test results and possible explanations are given in Table 1. Single nucleotide mismatch in primer/ probe, especially in the 3' binding region, may result in failure of target binding and false-negative results [6]. The *orf1ab*, N, and S regions are shown to be the most mutable in SARS-CoV-2, whilst E gene and M gene have been reported to be relatively less prone [7]. Genomic sequencing would be necessary to further delineate the assumed polymorphisms in SARS-CoV-2 *Orf1ab*, N, E, and S genes that could result in the discrepancies identified. However, as sequences of target regions in commercial assays are proprietary, a direct comparison between the binding regions of *Orf1ab* in the Roche and TaqPath assays; and of the S region in the Filmarray and TaqPath assays cannot be made.

The TaqPath COVID19 assay has been used to screen for UK Variant of Concern (B.1.1.7) with SGTF used as a surrogate marker for H69-V70 variant detection [8]. We have limited data on genomic surveillance of SARS-CoV-2 in Pakistan however, we have recently identified the introduction of B.1.1.7 lineage strains (unpublished data, sequence submitted). The variability observed in the three cases with S gene drop-out suggests the presence of new variants.

These cases we present illustrate the importance of taking into account both the quality of the diagnostic assay and its appropriate design strategy to best capture SARS-CoV-2 strains moving forward. While keeping primer and probe information proprietary is the norm, the medical and research community will benefit from requests from scientists to reveal further information, which is often not made available even upon request [10]. We propose frequent evaluation of national databases of viral genome sequences to inform standards on diagnostic assays. Such an initiative can advise on targets with low mutation frequency, such that > 99% of circulating variants are detectable using selected primers and probes. Further, in keeping with the rapidly evolving nature of the SARS-CoV-2 genome it is of particular consequence that there be a regular review of the target primers being used and an improvement of diagnostic assays to keep up the expected sensitivity and specificity required of diagnostic assays for COVID-19.

Limitations

Our results are subject to inherent limitation due to small sample size of the study. Further, as we do not have genome sequences available of the SARS-CoV-2 strains described in the study, we cannot ascertain the polymorphisms present in the genomes. Further, due to the proprietary nature of the commercial assays used it would not be possible to associate sequence variations with differential amplification frequencies of the PCR tests.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Review Committee, Aga Khan University as an Exemption Study based on retrospective review of clinical and laboratory records.

Availability of data and material

The data is available in the table here and raw data is available upon request

Consent for publication. Not applicable.

Competing interests

The authors have no competing interests to declare.

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Authors' contributions

All authors have read and approved the final version of the manuscript. ZM identified the clinical dilemmas; SS, AN, AK, IA, ZA and NM were involved in the laboratory testing of samples; ZM and SFM reviewed the clinical evaluations; SS and ZH

designed the study; ZM, SS, ZH and RH wrote the manuscript.

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