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Short communication

# Diminished amplification of SARS-CoV-2 ORF1ab in a commercial dual-target qRT-PCR diagnostic assay

Stephanie Popping [a,*], Richard Molenkamp [a], Joachim D. Weigel [b], Pim G.N.J. Mutsaers [c], Jolanda C. Voermans [a], Sander van Boheemen [a], Marc C. Shamier [a], Reina S. Sikkema [a], David F. Nieuwenhuijse [a], Bas B. Oude Munnink [a], Marion P.G. Koopmans [a], Jeroen J. A. van Kampen [a,*]

[a] Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
[b] Department of Intensive Care, Erasmus MC, Rotterdam, the Netherlands
[c] Department of Hematology, Erasmus MC, Rotterdam, the Netherlands

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ABSTRACT

Here we describe a SARS-CoV-2 variant with diminished amplification of the ORF ORF1ab target in the Cobas® dual-target SARS-CoV-2 assay resulting in a discrepancy of Ct-values (Ct-value 20.7 for the E-gene and Ct-value 30.2 for ORF1ab). Five unique nucleotide mutations were identified in ORF1ab: C11450A (nsp10) C14178T (RdRp), G15006T (RdRp), G18394T (Hel), and G20995T (Hel). This case highlights the importance of surveillance of genomic regions used in molecular diagnostics and the importance of the public release of target regions used to update commercial and in-house developed SARS-CoV-2 PCR tests. This work underpins the importance of using dual-targets in molecular diagnostic assays to limit the change of false-negative results due to primer and/or probe mismatches.

## 1. Short communications

SARS-CoV-2 is highly contagious and has rapidly evolved into a global pandemic overburdening healthcare systems worldwide. Prompt molecular detection of SARS-CoV-2 is crucial to identify and isolate infected individuals thus preventing further SARS-CoV-2 transmission. The early availability of the SARS-CoV-2 genome sequence led to the fast development of in-house PCR protocols followed by several commercially available qRT-PCR assays such as, the widely used Cobas® dual-target SARS-CoV-2 assay (Corman et al., 2020; Iglói et al., 2020).

SARS-CoV-2 has an estimated $\sim 1 \times 10^{-3}$ substitutions per nucleotide position per year, which is comparable to SARS-CoV-1 and other coronaviruses (Duchene et al., 2020). Viral mutations in the primer or probe binding regions of SARS-CoV-2 could lead to mismatches, resulting in false negative PCR results. As example, the B.1.1.7 (alpha) variant had deletions at positions 69 and 70 which causes failure of the S-gene target used in the Thermofisher® diagnostic assay (Public Health England, 2020). Furthermore, SARS-CoV-2 variants with mutations at position 26340 in the E-gene led to target failure in one of the targets of the Cobas® dual-target assay (Artesi et al., 2020). Reporting such findings is of utmost importance to update commercial and in-house developed SARS-CoV-2 PCR tests. Here we report a patient infected with a SARS-CoV-2 variant that showed diminished amplification of the ORF1ab target of the Cobas® dual-target SARS-CoV-2 assay.

A 55-year-old woman with a recently diagnosed aggressive B-cell non-Hodgkin lymphoma (double-hit lymphoma, DHL) and pulmonary embolism (PE) was presented to the emergency department with periodic episodes of non-neutropenic fever and progressive pain in her right leg. Admission vitals were not alarming except her admission labs showed a platelet count of 48.000/μL, a leucocytosis of 25.000/μL, and a haemoglobin concentration of 4.9 mmol/L, related to intensive immunochemotherapy (DA-EPOCH-R) and granulocyte colony stimulating factor (G-CSF) given two weeks before the index hospitalization. The next day, the patient was found mottled, unresponsive, and profound hypotensive. She was immediately transferred to the intensive care unit (ICU). She was intubated, mechanically ventilated, and temporarily started on norepinephrine to control persistent hypotension to some extent. An abdominal computed tomography (CT) scan showed massive bleeding in the iliopsoas muscle, possibly multifactorial, a combination of therapeutic nadroparin, thrombocytopenia and direct lymphoma

* Corresponding authors at: Erasmus MC, Wytemaweg 80, 3015CN, Rotterdam, the Netherlands.
*E-mail addresses:* S.popping@erasmusmc.nl (S. Popping), j.vankampen@erasmusmc.nl (J.J.A. van Kampen).

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**Table 1**
Virological investigations and CT-values.

| Days from admission | 1 | 3 | 10 | 14 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Nasopharyngeal swab*** | | | | | | | | | | | |
| Cobas® E-gen | 21.2 | 19.2 | 20.7 | 19 | 21 | 21.3 | 21.1 | 23.1 | 23.1 | 20.6 | 20.3 |
| Cobas® ORF1ab | 27.4 | 28.4 | 30.2* | 29.4** | 29 | 28.4 | 27.2 | 29.5 | 29.8 | 28.9 | 30.2 |
| In-house PCR E-gene | | | 19.4 | 16.8 | | | | | | | |
| In-house PCR RdRP | | | 21.9 | 18.3 | | | | | | | |
| ***Sputum*** | | | | | | | | | | | |
| Cobas® E-gene | | | | | 21.6 | 16.8 | 17.7 | 18.8 | | 19.2 | |
| Cobas® ORF1ab | | | | | 27.9 | 24.9 | 25.6 | 27.6 | | 28 | |
| ***Virus Culture*** | | | | | | | | | | | |
| Nasopharynx | | | | | | Positive | Positive | | | Positive | |
| Sputa | | | | | | Positive | Positive | | | Positive | |

Reported are the Ct-values of the various SARS-CoV-2 PCR assays used and results (positive or negative) of the virus cultures.
*GISAID number: hCov-19/Netherlands/ZH-EMC-938 **GISAID number: hCov-19/Netherlands/ZH-EMC-939.

infiltration in her right gluteal muscle. Her hemodynamic condition was treated with fluid resuscitation including blood- and platelet transfusion. Haemodynamic stability could not be achieved until endovascular embolization was performed.

The course of her admission was further characterized in particular by respiratory distress with SARS-Cov-2 pneumonitis. At admission to the emergency department, the patient tested positive for SARS-Cov-2 using the Cobas® SARS-CoV-2 test. A lung computed tomography (CT) scan revealed the typical signs of SARS-Cov-2 pneumonitis, such as bilateral ground-glass opacities and consolidation. However, despite the application of lung-protective ventilation and additional therapies including neuromuscular blockade and high dose corticosteroids, acute respiratory distress syndrome (ARDS) with refractory hypoxaemia persisted. Prone positioning (PP) was applied to improve oxygenation (PaO2/FiO2 ratio) by recruitment of atelectasis (collapsed lung parenchyma) in dorsal lung parts creating more lung homogeneity and increased total aerated lung volume. PP was initially effective by rapidly improving blood oxygenation, but the situation promptly deteriorated probably due to ongoing massive alveolar damage. At an interval as required by our daily clinical practice, detecting SARS-CoV-2 RNA in oropharyngeal swab and lower respiratory tract samples, and serum-IgG responses to SARS-CoV-2 were performed. Results showed both a persistent SARS-CoV-2 replication without seroconversion and a large discrepancy in Ct-values between the two targets of the Cobas® SARS-CoV-2 test (Ct-value 20.7 for the E-gene and Ct-value 30.2 for ORF1ab) (Table 1). Progressive respiratory, circulatory, and renal failure were the grave consequences. Unfortunately, the patient died after a prolonged ICU stay probably due to the ongoing effects of SARS-Cov-2 infection and subsequent multiple organ failure. Written informed consent to publish this case was provided by the family.

The large discrepancy in Ct-values between the E-gene and ORF1ab of the Cobas® SARS-CoV-2 test was further investigated by performing our in-house developed Corman-based dual-target SARS-CoV-2 PCR (Corman et al., 2020), virus culture (van Kampen et al., 2021), and whole genome sequencing as described before (Table 1). The Corman-based PCR assay confirmed the presence of SARS-CoV-2 RNA and no discrepancies in Ct-values of the E-gene and ORF1ab were noted (Table 1). Using virus culture, we confirmed that this SARS-CoV-2 variant was infectious (Table 1). The full-length genome of this SARS-CoV-2 variant was recovered using amplicon-based Nanopore sequencing (GISAID number hCov-19/Netherlands/ZH-EMC-938 and hCov-19/Netherlands/ZH-EMC-939) (Table 1). Considering the importance of PCR testing to control the SARS-CoV-2 pandemic and the widespread use of the Cobas SARS-CoV-2 PCR test, we immediately notified Roche about this SARS-CoV-2 variant. However, the Cobas® primers and probe sequences were considered proprietary information and could not be disclosed. To identify the mutation(s) causing this problem, we therefore compared the sequence with 150 other full genome sequences that were generated by our laboratory. For these 150 other full genome sequences, no discrepancies were noted in the Ct-values of ORF1ab and E-gene of the Cobas® dual-target assay. Five unique nucleotide mutations were identified in ORF1ab: C11450A (nsp10) C14178T (RdRp), G15006T (RdRp), G18394T (Hel), and G20995T (Hel) (Supplemental data Fig. 1).

This case showed diminished amplification in a commercial dual-target SARS-CoV-2 assay. Moreover, this case highlights the importance and urgency of genomic SARS-CoV-2 surveillance to provide and maintain reliable diagnostics. By comparing the sequence of the SARS-CoV-2 variant with diminished amplification in the ORF1ab target to other SARS-CoV-2 sequences generated in our laboratory, we were able to identify 5 unique mutations which may have caused this amplification problem: C11450A (nsp10) C14178T (RdRp), G15006T (RdRp), G18394T (Hel), and G20995T (Hel) (Supplemental data Fig. 1). Contact with the manufacturer did not result in the release of the primer and probe sequences used for the Cobas® dual-target SARS-CoV-2 assay.

Usually, the sequences of primers and probes from commercially available PCR assays are considered propriety company information and will not be disclosed to customers. However, diagnostics companies have the obligation to perform active post-market surveillance to demonstrate that the assay continues to be “fit-for-purpose”. The EU in vitro diagnostic medical devices regulation that will take effect in May 2022 limits the use of laboratory developed tests. Especially for emerging pathogens that are prone to a high level of genetic variation this could increase the risk of missing information on primer-probe mismatches unless post-market surveillance and transparency to customers is enhanced. The number of variants jeopardizing diagnostic results is currently unclear as there is no systematic reporting system. Only estimations on currently available SARS-CoV-2 genome data can be made. Vogel et al. analysed almost 1,000 SARS-CoV-2 genomes and reported 12 primer-probe mismatches in at least two of the genomes (Vogels et al., 2020). Although in this analysis the RdRp reverse primer seemed potentially problematic, case reports also mention variants with failure of the E-gene and N-gene (Supplemental data Table 1) (Artesi et al., 2020; Li et al., 2020). Additionally, the S-gene target is failing among the widely spread B.1.1.7 alpha variant (Public Health England, 2020).

Recent publicly available primer check tools became available which check for mismatches. However, these platforms are also depending on open data and sharing of the primer sequences (Primer-Check, 2021). Current, genomic SARS-CoV-2 surveillances mostly focuses on mutations in the spike glycoprotein but should also include all primer and probe targeting areas to maintain reliable PCR detection of SARS-CoV-2. Furthermore, Ct-values should be combined with available SARS-CoV-2 data to identify target failures in publicly available data.

Reporting correct Ct-values of the SARS-CoV-2 PCR is of importance for infection prevention and control practices. As there is a clear correlation between viral load, which correlates with Ct-values, and shedding of infectious virus in COVID-19 patients, Ct-values are used to downscale or stop SARS-CoV-2 infection prevention and control measures in hospitalized COVID-19 patients (van Kampen et al., 2021;

Bullard et al., 2020; Wölfel et al., 2020). Therefore, we could highly recommend the use of dual-target PCR test in daily practice.

In conclusion, we describe a SARS-CoV-2 variant with diminished amplification of the ORF1ab target in the Cobas® dual-target SARS-CoV-2 assay, highlighting the importance of surveillance of genomic regions used in molecular diagnostics and the importance of the public release of target regions used. In addition, this case underpins the importance of using dual-targets in molecular diagnostic assays to limit the chance of false-negative results due to primer and/or probe mismatches.

## Data availability

Sequence data is published on GISAID and also available on request.

## Declaration of Competing Interest

Sequencing was funded by ZonMW (grant agreement no: 10150062010005).

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RM; RS; MK; JW; PM; BOM; DN; JvV; MS; SvB; Jvk: reported no conflict of interest.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114397.

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