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A deletion in the N gene may cause diagnostic escape in SARS-CoV-2 samples

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ABSTRACT

Five SARS-CoV-2-positive samples showed N-gene drop-out with a RT-PCR multiplex test. WGS found all samples to harbor a deletion in the same region of the N gene, which is likely to impair the efficiency of amplification. This highlights the need for a continued surveillance of viral evolution and diagnostic test performance.

1. Introduction

Viruses, particularly RNA viruses, constantly change their genetic makeup through mutation. SARS-CoV-2 shows a higher fidelity in its transcription and replication process compared to other RNA viruses (Li et al., 2020; Sevajol et al., 2014); nonetheless, its evolution driven by mutation processes has been observed globally and is expected to occur with ongoing transmission (European Centre for Disease Prevention and Control (ECDC) 2021).

Some mutations, or combinations of mutations, may provide the virus with a selective advantage, such as changes in transmissibility and reinfection, or a reduced effectiveness of vaccines (vaccine escape) (Graham et al., 2021). Changes in the viral nucleic acid could also confer the ability to evade detection by diagnostics, particularly RT-PCR based assays. The high specificity of these tests means that even small mutations can impair the efficiency of oligonucleotide annealing, substantially compromising sensitivity (increased limit of detection) or inclusivity (missed detection of some strains).

This has been extensively demonstrated in SARS-CoV-2. Variants harboring the 69−70del in the Spike (S) protein, most notably associated with lineage B.1.1.7, have shown a negative result from S gene RT-PCR assays across several diagnostic platforms (Rambaut et al., 2021). This occurrence is so widespread that the European Centre for Disease Prevention and Control (ECDC) 2021 has stated that an S target drop-out in multitarget RT−PCR assays may be used as a surrogate marker for the 69−70del, and it prompts further investigation if sequencing capacity is limited (European Centre for Disease Prevention and Control (ECDC) 2021). Although mutations are more likely to occur in the S gene (Wang et al., 2020), single-nucleotide polymorphisms were also reported in the E gene to be associated with the failure of the RT-PCR (Artesi et al., 2020).

The N gene is considered generally the best target for in vitro diagnostic detection because of the conservation of its sequence, as point mutations are less likely both to occur and to affect viral function (Dutta et al., 2020). However, this target is not immune to changes in its sequence: the N gene has been reported to be affected by mutations which can interfere with detection (Ziegler et al., 2020). Here we describe five instances of SARS-CoV-2 infection detected in March 2021 in which a widely distributed RT-PCR test showed discrepant results with different RT-PCR commercial tests and whole genome sequencing (WGS) regarding the detection of the N gene.

2. Case description

In March 2021, 5 instances were reported during routinary diagnostic testing for SARS-CoV2 in which samples tested with a commercial RT-PCR kit (Allplex SARS-CoV-2 assay, Seegene Inc., Seoul,

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Table 1
Ct values for the samples tested with both the Allplex SARS-CoV-2 assay and Xpert Xpress SARS-CoV-2 assay.

Sample ID	Allplex SARS-CoV-2 result (Ct)			Xpert Xpress SARS-CoV-2 result (Ct)	
	E gene	RdRP/S gene	N gene	E gene	N gene
1	21	23	N/A	20	22
2	19	21	N/A	19	20
3	22	24	N/A	21	22
4	21	22	N/A	21	23
5	19	19	N/A	18	19

South Korea) showed discrepant results. The samples were reported as SARS-CoV-2 positive, with a cycle threshold (Ct) value below 25 for both E and RdRP/S targets (Table 1); they however tested negative for the N gene. Repetition with the same assay yielded comparable results, confirming the lack of N-target amplification in all cases.

To further investigate this, the specimens were then tested with Xpert Xpress SARS-CoV-2 assay (Cepheid Inc., Sunnyvale, United States) and Alinity m SARS-CoV-2 Assay (Abbott Molecular, Chicago, United States). However, while Xpert Xpress shows separate amplification curves for its targets (E and N2 genes), Alinity only shows a single curve of amplification, comprehensive of both its targets (RdRP and N genes).

In all instances, positivity for SARS-CoV-2 was confirmed. Xpert Xpress also showed amplification for the N target in all samples tested (Table 1).

3. Materials and methods

Whole genome sequencing (WGS) was performed on the original samples using an amplicon-based approach, in order to characterize potential mutations in the assay target region. We implemented the CleanPlex SARS-CoV-2 Panel (Paragon Genomics, Inc., Hayward, CA) for target enrichment and library preparation, which involves multiplex PCR reactions using 343 pairs of primers separated into two pools, allowing the coverage of the entire genome of SARS-CoV-2. The sequencing step was conducted on a MiSeq platform (Illumina, Inc., San Diego, CA). Data analysis was performed, according to the supplier's recommendations, with Sophia-DDM-v4, a pipeline developed by SOPHiA Genetics (Lausanne, Switzerland), including a primers trimming step.

To describe the genetic lineages of the sequences, we utilized Phylogenetic Assignment of Named Global Outbreak LINeages (Pangolin) (O'Toole et al., 2021). The Pangolin tool follows the 'Pango' nomenclature system for classifying SARS-CoV-2 genomic sequences (Rambaut et al., 2020).

4. Results and discussion

Lineage assignment categorized 4 of the specimens as a B.1.1.7 lineage. The GISAID Accession IDs for these samples are EPI_ISL_1448024, EPI_ISL_1448510, EPI_ISL_1616834 and EPI_ISL_1617338.

Among the mutations found, all showed the same 6-nucleotide deletion (207-208del, corresponding to positions 619-624 of the N gene cDNA, genome position 28889, reference genome NC_045512.2). Coverage depth of this mutation ranged from 7374 to 20016.

The fifth sample was categorized as a different lineage (B.1.1.318), characterized by the presence of an E484K Spike mutation (Accession ID EPI_ISL_1617339). However, a 3-nucleotide deletion was found on the N gene, overlapping the one reported in the previous 4 samples (208-209indel, 623-625 of the N gene cDNA, genome position 28895), with a coverage depth of 7668.

Although not all specimens were categorized as the same lineage, in all instances WGS identified a deletion in the same region of the N gene. As yet, this mutation was never reported in any sample sequenced in our laboratory (n = 534).

Information on the proprietary primers and probes is unavailable, however it is likely that the deletions we described impair the efficiency of oligonucleotide annealing, preventing efficient amplification or detection. Up to date, deletions 207-208 and 208-209 were reported on GISAID 365 and 809 times respectively, over around 1.300.000 deposited sequences.

Four of the samples we tested were classified as lineages B.1.1.7, which is characterized by a 69-70del on the S gene, among other mutations. It is important to note the Allplex SARS-CoV-2 assay is also affected by the 69-70del; while this does not lead to the drop-out of the RdRP/S target, changes in the amplification curve slope and shape are noticeable and useful as a pre-screening (Ibba et al., 2021). The concurrence of these mutations may however cause issues in the correct identification of SARS-CoV-2-positive samples when the viral load is low.

Although the beginning of the COVID-19 pandemic showed little viral diversity (Mavian et al., 2020), positive selection has now been documented, providing the virus with advantages such as increased transmission rates.

Virological, immunological, and social factors can influence the epidemiological trajectory of SARS-CoV-2. Co-evolution of coronaviruses with their hosts is driven by genetic diversity, prompted by evolutionary pressures. Mutations that confer greater fitness are selected for, leading to antigenic drift. This is most frequently observed in surface proteins, which are highly exposed to the selective pressures of the immune system (Singh et al., 2021). Nonetheless, somewhat extensive deletions have been reported in non-structural coding regions, such as ORF7a (Holland et al., 2020) and ORF1ab (Bal et al., 2020; Benedetti et al., 2020; Islam et al., 2020; Phan, 2020), as far back as the beginning of the pandemic. This significant evolution of the SARS-CoV-2 genome, leading to virus-host adaptation, is highlighted by the emergence of novel variants following the rapid dissemination of clade G and its derivatives, such as B.1.1.7, B.1.351, P.1, and B.1.617.2 (Singh et al., 2021).

In addition to evolutionary advantages, changes in the viral nucleic acid can also confer the ability to evade detection by diagnostics, particularly RT-PCR based assays. The use of multitarget assays is crucial in preventing detection escape and improving sensitivity and specificity. However, as new variants of SARS-CoV-2 are identified, there is a need for continued surveillance of both viral evolution and diagnostic assay performance. It is critical that diagnostic tests for the virus are regularly reconfigured, as the emergence of variants that are no longer detectable by certain tests is a real possibility.

Author contributions

Conceptualization: G.D., V.S., S.Z. - Data curation: G.D., S.Z. - Investigation: S.Z., F.T., G.G., I.P. - Resources: A.D., V.A., M.M., A.M., A.B. - Supervision: G.D., V.S. - Writing - original draft: S.Z. - Writing - review & editing: G.D., F.T., G.G., I.P

Ethical statement

Ethical approval or informed consent were not required because the study has been performed using exclusively anonymized, leftover samples deriving from the routine diagnostic procedures. The anonymization was achieved by using the current procedure (AVR-PPC P09, rev.2) checked by the local Ethical Board.

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Declaration of competing interest

The authors report no conflicts of interest relevant to this article

References

Artesi M, Bontems S, Göbbels P, Franckh M, Maes P, Boreux R, et al. A recurrent mutation at position 26340 of SARS-CoV-2 is associated with failure of the E gene quantitative reverse transcription-PCR utilized in a commercial dual-target diagnostic assay. J Clin Microbiol 2020;58(10) e01598-20.

Bal A, Destras G, Gaymard A, Bouscambert-Duchamp M, Valette M, Escuret V, et al. Molecular characterization of SARS-CoV-2 in the first COVID-19 cluster in France reveals an amino acid deletion in nsp2 (Asp268del). Clin Microbiol Infect 2020;26(7):960–2.

Benedetti F, Snyder GA, Giovanetti M, Angeletti S, Gallo RC, Ciccozzi M, et al. Emerging of a SARS-CoV-2 viral strain with a deletion in nsp1. J Transl Med 2020;18(1):329.

Dutta NK, Mazumdar K, Gordy JT. The nucleocapsid protein of SARS–CoV-2: a target for vaccine development. J Virol 2020;94(13) e00647-20.

European Centre for Disease Prevention and Control (ECDC). Threat assessment brief: rapid increase of a SARS-CoV-2 variant with multiple spike protein mutations observed in the United Kingdom. Available at: https://www.ecdc.europa.eu/en/publications-data/threat-assessment-brief-rapid-increase-sars-cov-2-variant-united-kingdom. 2021

Graham MS, Sudre CH, May A, Antonelli M, Murray B, Varsavsky T, et al. Changes in symptomatology, reinfection, and transmissibility associated with the SARS-CoV-2 variant B.1.1.7: an ecological study. Lancet Public Health 2021;6(5):e335–45.

Holland LA, et al. An 81-nucleotide deletion in SARS-CoV-2 ORF7a identified from sentinel surveillance in Arizona (January to March 2020). J Virol 2020;94:14.

Ibba G, Sau R, Angioj F, Abbondio M, Rubino S, Uzzau S. A straightforward molecular strategy to retrospectively investigate the spread of SARS-CoV-2 VOC202012/01 B.1.1.7 variant. J Infect Dev Ctries 2021;15(2):242–6.

Islam MR, Hoque MN, Rahman MS, et al. Genome-wide analysis of SARSCoV-2 virus strains circulating worldwide implicates heterogeneity. Sci Rep 2020;10:14004.

Li X, Zai J, Zhao Q, Nie Q, Li Y, Foley BT. Evolutionary history, potential intermediate animal host, and cross-species analyses of SARS-CoV-2. J Med Virol 2020;92(6):602–11.

Mavian C, Marini S, Manes C, Capua I, Prosperi M, Salemi M. Regaining perspective on SARS-CoV-2 molecular tracing and its implications. medRxiv 2020 Available at https://www.medrxiv.org/content/10.1101/2020.03.16.20034470v1.

O'Toole, Á, McCrone JT, Scher E. Phylogenetic ssignment of named global outbreak LINeages (Pangolin COVID-19 lineage assigner). 2021 Available at https://pangolin.cog-uk.io/

Phan T. Genetic diversity and evolution of SARS-CoV-2. Infect Genet Evol 2020;81:104260.

Rambaut A, Holmes EC, O'Toole A, Hill V, McCrone JT, Ruis C, et al. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat Microbiol 2020;5(11):1403–7.

Rambaut A, Loman N, Pybus O, Barclay W, Barrett J, Carabelli A et al. Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. 2021 Available at https://virological.org/t/preliminary-genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novel-set-of-spike-mutations/563

Sevajol M, Subissi L, Decroly E, Canard B, Imbert I. Insights into RNA synthesis, capping, and proofreading mechanisms of SARS-coronavirus. Virus Res 2014;194:90–9.

Singh J, Pandit P, McArthur AG, Banerjee A, Mossman K. Evolutionary trajectory of SARS-CoV-2 and emerging variants. Virol J 2021;18(1):166.

Wang R, Hozumi Y, Yin C, Wei GW. Mutations on COVID-19 diagnostic targets. Genomics 2020;112(6):5204–13.

Ziegler K, Steininger P, Ziegler R, Steinmann J, Korn K, Ensser A. SARS-CoV-2 samples may escape detection because of a single point mutation in the N gene. Euro Surveill 2020;25(39) 2001650.