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Multiple assays in a real-time RT-PCR SARS-CoV-2 panel can mitigate the risk of loss of sensitivity by new genomic variants during the COVID-19 outbreak

Luis Peñarrubia[a], Maria Ruiz[a], Roberto Porco[a], Sonia N. Rao[b], Martí Juanola-Falgarona[a], Davide Manissero[c], Marta López-Fontanals[a,*], Josep Pareja[a,*]

[a] STAT-Dx Life, S.L. (a QIAGEN Company), Baldiri Reixac, 4-8, 08028 Barcelona, Spain
[b] QIAGEN, Inc., 19300 Germantown Road, Germantown, MD 20874, USA
[c] QIAGEN Manchester Ltd, Skelton House, Lloyd Street North, Manchester, M15 6SH, UK

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ABSTRACT

Objectives: In this study, five SARS-CoV-2 PCR assay panels were evaluated against the accumulated genetic variability of the virus to assess the effect on sensitivity of the individual assays.
Design or methods: As of week 21, 2020, the complete set of available SARS-CoV-2 genomes from GISAID and GenBank databases were used in this study. SARS-CoV-2 primer sequences from publicly available panels (WHO, CDC, NMDC, and HKU) and QIAstat-Dx were included in the alignment, and accumulated genetic variability affecting any oligonucleotide annealing was annotated.
Results: A total of 11,627 (34.38%) genomes included single mutations affecting annealing of any PCR assay. Variations in 8,773 (25.94%) genomes were considered as high risk, whereas additional 2,854 (8.43%) genomes presented low frequent single mutations and were predicted to yield no impact on sensitivity. In case of the QIAstat-Dx SARS-CoV-2 Panel, 99.11% of the genomes matched with a 100% coverage all oligonucleotides, and critical variations were tested *in vitro* corroborating no loss of sensitivity.
Conclusions: This analysis stresses the importance of targeting more than one region in the viral genome for SARS-CoV-2 detection to mitigate the risk of loss of sensitivity due to the unknown mutation rate during this SARS-CoV-2 outbreak.

Introduction

Since the beginning of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) outbreak in December 2019 (Zhu et al., 2020), the number of confirmed cases has been rising dramatically (The Open Science Prize, 2020). According to data from the World Health Organization (WHO), Coronavirus Disease 2019 or COVID-19 (Gorbalenya et al., 2020) has been diagnosed in over 6,057,853 people from more than 200 countries or territories and caused over 371,166 deaths worldwide as of 1 June 2020 (World Health Organization, 2020a).

SARS-CoV-2 is the seventh coronavirus known to infect humans and it appears to have its origin from zoonotic transmission similar to SARS-CoV (2002) and MERS-CoV (2012) (Andersen et al., 2020). SARS-CoV-2 is hypothesised to be the product of recombination or mutation from a genetically related SARS-CoV species hosted by bats (Sun et al., 2020). Unlike seasonal human coronaviruses HKU1, NL63, OC43 and 229E which are associated with mild symptoms (Corman et al., 2018), SARS-CoV-2 can cause pneumonia and severe acute respiratory syndrome (Andersen et al., 2020). All coronaviruses are single, positive-stranded RNA viruses with similar genomic ~30 kb size and structure. These genes encode non-structural proteins (ORF1a and ORF1b) involved in replication at the 5′-end and multiple structural proteins (spike (S), envelope (E), membrane (M) and nucleocapsid (N)) downstream that make up the virus particle (Chan et al., 2020; Wu et al., 2020).

During novel RNA viral outbreaks, detection of the virus relies on real-time reverse transcription polymerase chain reaction (real-time RT-PCR) for detection of the RNA of the virus. In the case of SARS-CoV-2, RT-PCR is used for confirmatory diagnosis of symptomatic patients as well as, increasingly, screening of

* Corresponding authors.
E-mail addresses: marta.lopez@qiagen.com (M. López-Fontanals), josep.pareja@qiagen.com (J. Pareja).

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asymptomatic contacts and subjects at risk. The unprecedented outbreak posed the challenge of evaluating the different diagnostic RT-PCR assays' sensitivity and specificity without a previously established gold standard.

Since the availability of the first SARS-CoV-2 genomic sequence (Wu et al., 2020), several studies have reported a rapid genetic evolution of SARS-CoV-2 through a phylogenetic tree tracking the geographical spread of the virus (Andersen et al., 2020; The Open Science Prize, 2020; Wu et al., 2020; Yi, 2020). As previously established for RNA viruses, the nucleotide mutation rate of SARS-CoV-2 is estimated to be 8E-04 substitutions per site per year as a consequence of the lack of proofreading activity of polymerases (Lauring and Andino, 2010; The Open Science Prize, 2020). It evinces that new genetic variations of SARS-CoV-2 that will most likely occur during this evolving outbreak could compromise the sensitivity and specificity of RT-PCR detection (Lippi et al., 2020).

Specifically, both natural mutation rate of a virus (Lauring and Andino, 2010) and active viral recombination (Yi, 2020) could potentially impair the efficiency of oligonucleotide annealing (Lippi et al., 2020), affecting sensitivity (increasing the limit of detection) or inclusivity (missed detection of some strains due to genomic changes). We hypothesize that in order to minimize the risk of sensitivity loss of RT-PCR for detecting novel viruses with unknown genetic variability like SARS-CoV-2, published RT-PCR design strategies for SARS-CoV-2 detection should target more than one region in the viral genome. This was the general approach followed to obtain the earliest available SARS-CoV-2 RT-PCR assays in panels used worldwide (Centers for Disease Control and Prevention, 2020a, 2020b; Chu et al., 2020; Corman et al., 2020; National Microbiology Data Center, 2020; QIAstat-Dx Respiratory SARS-CoV-2 Panel, 2020; The University of Hong Kong, 2020; World Health Organization, 2020b), including the syndromic multiplex QIAstat-Dx Respiratory SARS-CoV-2 Panel (Table 1). The selected conserved target regions from available SARS-CoV-2 genomes minimize the potential cross-reactivity with other coronaviruses (Gorbalenya et al., 2020; Zhu et al., 2020), including human coronaviruses (Wu et al., 2020) and bat-SARS-like coronaviruses (Chan et al., 2020).

The goal of this study is to assess how the genetic variability observed in the SARS-CoV-2 genome as of week 21, 2020 has affected sensitivity of publicly available SARS-CoV-2 real-time RT-PCR panel assays. Additionally, a detailed characterization and evaluation of the impact on performance of the SARS-CoV-2 assays in the QIAstat-Dx Respiratory SARS-CoV-2 Panel was carried out.

Methods

The complete set of available SARS-CoV-2 genomes larger than 26,000 nucleotides from GISAID (https://www.gisaid.org/) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases were used in this study. As of week 21, 2020, a total of 30,090 and 3,729 available genomes (GISAID and GenBank respectively, Table S1) were aligned using ClustalW algorithm implemented in Geneious software v.10.2.6. (http://www.geneious.com), with a gap open and extent cost of 5 and 3 respectively. The output alignment was curated manually.

Table 1
Description of earliest available SARS-CoV-2 RT-PCR assays.

RT-qPCR panel	Assays included	Target genes	Description[f]	References
WHO panel[a]	3	*RdRp*, *E*, *N*	the E gene assay is used as the firstline screening tool, then followed by confirmatory testing with an RdRp gene assay. The N gene assay can eventually be analyzed as an additional confirmatory assay.	Corman et al. (2020), World Health Organization (2020b)
CDC (US) panel[b]	2	*N*	This panel contains two monoplex assays (N1, N2) designed for specific detection of SARS-CoV-2. A specimen is considered positive for SARS-CoV-2 if both assays are positive, whereas if only one assay resulted positive the result is inconclusive and it should be retested.	Centers for Disease Control and Prevention (2020a, 2020b)
NMDC panel[c]	2	*RdRp*, *N*	This panel contains two monoplex assays designed for specific detection of SARS-CoV-2. A specimen is considered positive for SARS-CoV-2 if both assays are positive.	National Microbiology Data Center (2020)
HKU panel[d]	2	*RdRp*, *N*	The N gene RT-PCR is recommended as a screening assay and the Orf1b assay as a confirmatory one (the N gene assay is about 10 times more sensitive than the ORF-1b gene assay in detecting positive clinical specimens).	Chu et al. (2020), The University of Hong Kong (2020)
QIAstat-Dx Panel[e]	2	*RdRp*, *E*	This panel contains a duplex assay in the same optical channel to report an additive performance (including CT and endpoint fluorescence values) of the two PCR assays and it discriminates SARS-CoV-2 detection from more than other 20 viruses and bacteria responsible for upper respiratory infections including other endemic coronaviruses	QIAstat-Dx Respiratory SARS-CoV-2 Panel (2020)

RdRp: RNA-dependent RNA polymerase gene (inside the Orf1ab polyprotein gene), E: envelop gene, N: Nucleocaspide gene.

[a] This panel was originally proposed by the Charité-Universitätsmedizin Berlin Institute of Virology (Corman et al., 2020), and then endorsed by the WHO (World Health Organization, 2020b).

[b] Initial CDC panel contained one additional assay (N3) designed for universal detection of SARS-like coronaviruses (Centers for Disease Control and Prevention, 2020a) but has been removed due to inconclusive results based on low performance (Centers for Disease Control and Prevention, 2020b).

[c] National Microbiology Data Center (NMDC) works in collaboration with National Institute for Viral Disease Control and Prevention under Chinese Center for Disease Control and Prevention.

[d] The School of Public Health of the University of Hong Kong (HKU) developed the assay (Chu et al., 2020) and it was later established as official protocol under the WHO Network Laboratories (The University of Hong Kong, 2020).

[e] QIAstat-Dx Panel is a syndromic Point-of-Care system incorporating sample purification and real time RT-qPCR amplification for more than 20 viral and bacterial respiratory pathogens, including SARS-CoV-2.

[f] WHO and HKU assays are reactive with coronaviruses under the subgenus *Sarbecovirus* that includes SARS-CoV-2, SARS-CoV and bat SARS-like coronaviruses. Since SARS was eliminated in humans since 2004, individuals with samples that are positive in these RT-PCR assays should be infected by the 2019-nCoV or its related animal coronaviruses.

Table 2
Summary of genomic variants detected in complementary binding regions of earliest available SARS-CoV-2 RT-PCR assays.

RT-qPCR panel	Assay	Type	Oligonucleotide name	Number of genomes (%) with non-critical single variations	Number of genomes (%) with high risk single variations
WHO panel	E	Fw	E_Sarbeco_F	10 (0.030%)	2 (0.006%)[a]
		P	E_Sarbeco_P1	34 (0.101%)	9 (0.027%)[b]
		Rev	E_Sarbeco_R	14 (0.4041%)	–
	RdRp	Fw	RdRp_SARSr-F	95 (0.281%)	5 (0.015%)[c]
		P	RdRp_SARSr-P1	126 (0.373%)	2 (0.006%)[d]
		P	RdRp_SARSr-P2	15 (0.044%)	–
		Rev	RdRp_SARSr-R	128 (0.378%)	–
	N	Fw	N_Sarbeco_F	70 (0.207%)	4 (0.012%)[e]
		P	N_Sarbeco_P1	69 (0.204%)	11 (0.033%)[f]
		Rev	N_Sarbeco_R	119 (0.352%)	1 (0.003%)[g]
CDC (US) panel	N1	Fw	2019-nCoV_N1-F	40 (0.118%)	3 (0.009%)[h]
		P	2019-nCoV_N1-P	53 (0.157%)	353 (1.044%)[i]
		Rev	2019-nCoV_N1-R	168 (0.497%)	4 (0.012%)[j]
	N2	Fw	2019-nCoV_N2-F	58 (0.172%)	–
		P	2019-nCoV_N2-P	102 (0.302%)	41 (0.121%)[k]
		Rev	2019-nCoV_N2-R	41 (0.121%)	–
	N3	Fw	2019-nCoV_N3-F	344 (1.017%)[l]	–
		P	2019-nCoV_N3-P	107 (0.316%)	6 (0.018%)[m]
		Rev	2019-nCoV_N3-R	86 (0.254%)	3 (0.009%)[n]
NMDC panel	RdRp	Fw	Forward primer	23 (0.068%)	–
		P	Fluorescent probe	48 (0.142%)	10 (0.030%)[o]
		Rev	Reverse primer	30 (0.089%)	1 (0.003%)[p]
	N	Fw	Forward primer	337 (0.996%)	8257 (24.415%)[q]
		P	Fluorescent probe	15 (0.044%)	3 (0.009%)[r]
		Rev	Reverse primer	56 (0.166%)	5 (0.015%)[s]
HKU panel	RdRp	Fw	HKU-ORF1b-nsp14F	84 (0.248%)	5 (0.015%)[t]
		P	HKU-ORF1b-nsp14P	45 (0.133%)	–
		Rev	HKU-ORF1b-nsp14R	62 (0.183%)	2 (0.006%)[u]
	N	Fw	HKU-NF	73 (0.216%)	3 (0.009%)[v]
		P	HKU-NP	53 (0.157%)	6 (0.018%)[w]
		Rev	HKU-NR	83 (0.245%)	2 (0.006%)[x]
QIAstat-Dx Panel	RdRp	Fw	Forward primer	15 (0.044%)	1 (0.003%)[y]
		P	Fluorescent probe	133 (0.393%)	12 (0.035%)[z]
		Rev	Reverse primer	27 (0.080%)	3 (0.009%)[aa]
	E	Fw	Forward primer	57 (0.169%)	11 (0.032%)[bb]
		P	Fluorescent probe	10 (0.030%)	1 (0.003%)[cc]
		Rev	Reverse primer	24 (0.071%)	7 (0.021%)[dd]
Totals				2,854 (8.434%)	8,773 (25,941%)
Accumulated total				11,627 (34.380%)	

Fw: Forward primer, P: Probe, Rev: Reverse primer.
[a] Two genome sequences with a mismatch in in the three last positions of the 3′-end (U to C and G to respectively).
[b] Nine genomes with a mismatch in the last three positions of the 5′-end (five A to G, and four C to U).
[c] Five genomes with a mismatch in the last three positions of the 3′-end (G to A, C to U, or G to U).
[d] Two genomes with multiple mismatches in the binding position of this probe (positions 1 (C to U), 7 (G to A), 10 (A to G), 12 (R to C), 21 (U to A) of the 5′-end)).
[e] Four genomes with a mismatch in any of the three last positions at the 3′-end (C to U, or U to C).
[f] Eleven genomes with a mismatch in position 2 of the 5′-end (C to U).
[g] One genome with a mismatch in the first position of the 3′-end (G to C).
[h] Three genomes with a mismatch in the second position of the 3′-end (A to C/G).
[i] A total of 353 genomes containing a mismatch in any of the last three positions of the 5′-end (C to A/U, C to U/G).
[j] Four genomes with a mismatch in the third position of the 3′-end (C to A).
[k] A total of 41 genomes with a mismatch in the first position of the 5′-end (A to U).
[l] A total of 292 genomes present the same mismatch in position 15 of the 3′-end (U to C).
[m] Six genomes with a mismatch in second (Y to G) or third (C to U) positions of the 5′-end.
[n] Three genomes with a mismatch in first (G to A) or second (U to A) positions of the 3′-end.
[o] Ten genomes with any mismatch in the last three positions of the 5′-end (C to A, C to U, G to A).
[p] A single genome with a mismatch in the second position of the 3′-end (G to A).
[q] A total of 8251 genomes with multiple three consecutive mismatches in positions 20,21,22 of the 3′-end, corresponding to positions 1, 2, 3 of the 5′-end (GGG to AAC). Six additional genomes with mismatches in any of the three last positions of the 3′-end.
[r] Three genomes with a mismatch in the second (U to C) or third (G to U) positions of the 5′-end.
[s] Five genomes with a mismatch in the first (G to A) or third (C to A/U) positions of the 3′-end.
[t] Five genomes with a mismatch in the second (C to U) or third (C to U) positions of the 3′-end.
[u] Two genomes with a mismatch in the second (U to C) or third (C to U) positions of the 3′-end.
[v] Three genomes with a mismatch in the third (U to C) position of the 3′-end.
[w] Six genomes with a mismatch in the second (C to A) or third (A to G) positions of the 5′-end.
[x] Two genomes with a mismatch in the second (U to C) or third (A to G) positions of the 3′-end.
[y] One mismatch in the third position of the 3′-end.
[z] Twelve genomes with a single mismatch in any of the three last positions of the 5′-end.
[aa] Three genomes with a mismatch in the second position of the 3′-end.
[bb] Seven genomes with a mismatch in the last position of the 3′-end, together with four additional genomes with a mismatch in the second or third positions.
[cc] A single genome with a mismatch in the second position of the 5′-end.
[dd] Six genomes with a mismatch in third position of the 3′-end, and one additional genome with a double mismatch in position 1.

SARS-CoV-2 primer sequences from publicly available panels (WHO, CDC, NMDC, and HKU) and QIAstat-Dx (Table 1) were included in the alignment, and accumulated genetic variability affecting any oligonucleotide annealing was annotated (Table S2). Variations were considered as high risk when they were placed in the three last nucleotides of the 3′-end, affecting the PCR elongation step (Stadhouders et al., 2010; Whiley and Sloots, 2005) or in the 5′-end of the probe affecting the natural 5′- exonuclease activity of the polymerases (Smith et al., 2002). In addition, also considered as high risk were those variations with a significant presence (>1%) among all published genomes that could become more extended worldwide in the current or potential future spread episodes.

Results

Results showed large nucleotide insertions (EPI_ISL_416672, EPI_ISL_418061, EPI_ISL_418066, EPI_ISL_416720, EPI_ISL_423027, EPI_ISL_424274, EPI_ISL_424327, EPI_ISL_426134, EPI_ISL_427267, EPI_ISL_429646, EPI_ISL_430896, EPI_ISL_434554, EPI_ISL_437867, EPI_ISL_445217) or fragment gene inversions (EPI_ISL_427289, EPI_ISL_427291) mainly in the polyprotein Orf1ab gene, typically indicating genetic recombination common in coronavirus genomes (Sun et al., 2020; Yi, 2020). Since none of them were placed in binding regions of any of the PCR assays, recombination analysis was considered out of the scope of this current study.

A total of 11,627 (34.38%) genomes included single mutations affecting annealing of any PCR assay. Variations in 8,773 (25.94%) genomes were considered as high risk: a) a trinucleotide variation (GGG to AAC) present in 8,251 of the 8,257 (24.41%) genomes affecting the binding of the *N* gene NMDC assay forward primer; b) different single variations in the last three positions of the 3′-end or 5′-end (primers and probes respectively).

Genetic variations affecting annealing of more than one oligonucleotide of the same assay were not frequent: (a) a total of 126 out of the 128 genomes with a non-critical mismatch in Reverse primer of the WHO RdRp assay (Table 2) presented any other variation in the Probe (RdRp_SARSr-P2) or in the Forward primer binding regions, (b) 17 out of the 8,251 genomes with multiple three consecutive mismatches at the 5′-end (GGG to AAC) of the NMDC N assay presented additional single variations in the Probe or Reverse primer binding regions; (c) five genome sequences (EPI_ISL_444736 for the WHO N assay; EPI_ISL_437536, EPI_ISL_422983 and MT293178 for the CDC N1 assay; and EPI_ISL_446918 for the CDC N2 assay) presented combinations of non-critical mismatches among different oligonucleotides of the same set.

An additional 2,854 (8.44%) genomes presented low frequent single mutations considered to be low risk (Table 2) and were predicted to yield no impact on sensitivity. However, the effect in every PCR performance must be evaluated individually based on the corresponding PCR thermal profile conditions. The impact of non-3 terminal mismatches should not be underestimated, especially in a diagnostic environment (Stadhouders et al., 2010; Whiley and Sloots, 2005).

A large number of genomes with variations affecting the CDC N3 assay were also found (546 variations, 1.61%). Although they cannot be classified as critical based on the parameters defined in the present study, this data supports why the initial N3 CDC assay was removed from the official panel due to inconclusive results (Centers for Disease Control and Prevention, 2020a, 2020b).

This same method of evaluation was carried out with the QIAstat-Dx SARS-CoV-2 Panel oligonucleotide sequences. The SARS-CoV-2 assay was originally developed as a real-time RT-PCR duplex assay covering two highly conserved target genes (*RdRp* and *E*) reported with the same fluorophore and showing a verified combined limit of detection (LoD) of 500 copies/mL in clinical samples (QIAstat-Dx Respiratory SARS-CoV-2 Panel, 2020). A total of 33,518 (99.11 %) of 33,819 genomes matched with a 100% coverage with all oligonucleotides included in the SARS-CoV-2 assays of the QIAstat-Dx Respiratory SARS-CoV-2 Panel. In contrast, 301 (0.89%) genomes showed a single variation affecting annealing for any oligonucleotide, and 35 (0.10%) of those 301 genomes contained a variation in any critical position of any primer or probe end. Among these latter ones, the most frequent variation consisted of a mismatch in the last position of the 3′-end of one of the primers (7 genomes, 0.02%). Because a mismatch in this position might impair the elongation PCR step (Stadhouders et al., 2010; Whiley and Sloots, 2005), this genomic mutation was further tested *in vitro* re-evaluating the LoD. A total of twenty replicates of quantified synthetic dsDNA reproducing the sequence containing this mutation were run at the LoD concentration (500 copies/mL) described in the publicly available QIAstat-Dx Respiratory SARS-CoV-2 Panel instructions for use (QIAstat-Dx Respiratory SARS-CoV-2 Panel, 2020), and 19 out of the 20 (95%) replicates were positive (Table S3) confirming the defined LoD. Therefore, the QIAstat-Dx SARS-CoV-2 assay remains highly specific and sensitive against SARS-CoV-2 even in light of genomic variations from the first five months of the COVID-19 outbreak (Visseaux et al., 2020).

Discussion

Genetic variability observed among SARS-CoV-2 genomes available until week 21, 2020, after the onset of the outbreak showed single nucleotide mutations affecting the annealing of all SARS-CoV-2 RT-PCR panels evaluated in this study. The majority of the annotated single nucleotide variations are predicted to have no effect on sensitivity at panel level, including those placed in critical positions. This was confirmed for the QIAstat-Dx SARS-CoV-2 Panel to show no effect on the LoD of the panel.

Given that genetic variability in the SARS-CoV-2 genome is expected to increase based on the natural viral mutation and recombination rates, our results show that combination of more than one assay target in real-time RT-PCR SARS-CoV-2 panels can mitigate the risk of loss of sensitivity or specificity. In this regard, continuous monitoring of genomic variations is essential to provide a rapid response in case assay re-design is needed.

Authors contributions

LP: Planned experiments, conceptualised the laboratory work, conceptualised the overall study, wrote the manuscript draft.

MR: Planned and conducted experiments.

RP: Planned and conducted experiments.

SR: Contributed to overall study planning and review of the manuscript.

MJ: Contributed to overall study planning and review of the manuscript.

DM: Contributed to overall study conceptualization and review of the manuscript.

MLF: Contributed to overall study planning and review of the manuscript.

JP: Contributed to overall study conceptualization, co-wrote of the manuscript.

Conflict of interests

LP, RP, MR, SNR, MJF,DM,MLF and JP are QIAGEN employees.

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Appendix A. Supplementary data

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