In silico evaluation of the impact of the Omicron variant on the sensitivity of RT-qPCR assays for SARS-CoV-2 detection using whole genome sequencing

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Abstract

Background

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variant of concern (VoC) Omicron (B.1.1.529) has rapidly spread around the world presenting a new threat to global public human health. Due to the large number of mutations possessed by Omicron, concerns have emerged over potentially reduced diagnostic accuracy of reverse transcription polymerase chain reaction (RT-qPCR), the gold standard diagnostic test for SARS-CoV-2. Here, we aimed to assess the impact of Omicron on the integrity and sensitivity of RT-qPCR assays used for coronavirus disease-2019 (COVID-19) diagnosis via *in silico* analysis employing whole genome sequencing data and evaluated the potential for false negatives or test failure due to mismatches between primers/probes and viral genome.

Methods

In silico sensitivity of 12 RT-qPCR tests (containing 30 primers and probe sets) developed for detection of SARS-CoV-2 reported by the World Health Organization (WHO) or available in the literature, was assessed for use in detecting SARS-CoV-2 Omicron BA.1 and BA.2 sublineages, obtained after removing redundancy from publicly available genomes from National Center for Biotechnology Information (NCBI) and Global Initiative on Sharing Avian Influenza Data (GISAID) databases. The mismatches between the amplicon regions of the SARS-CoV-2 Omicron VoC and primers and probe sets were evaluated, and the clustering analysis of the corresponding amplicon sequences was carried out.

Results

From the 232 representative SARS-CoV-2 BA.1 Omicron sublineage genomes analyzed, 229 showed substitutions at the forward primer annealing site for assay China-CDC N, 226 showed mismatches in the reverse primer annealing site for assay Thai N, and all 232 had substitution at the 3’ end of the reverse primer annealing site for assay HKUniv RdRp/Hel. Therefore, the lowest sensitivity was observed for assay ChinaCDC N, Thai N and HKUniv RdRp/Hel for SARS-CoV-2 BA.1 sublineage genomes. For 5 SARS-CoV-2 BA.2 Omicron sublineage genomes, false negative results were observed for assays ChinaCDC N, Thai N, HKUniv RdRp/Hel, SigmAldr S5, SigmAldr S6 and HKUniv S.

Conclusion

In this study, we observed three (25%) assays (ChinaCDC N, Thai N, and HKUniv RdRp/Hel) demonstrated potential for false negatives for the SARS-CoV-2 Omicron BA.1 sublineage, while four (33.3%) assays (ChinaCDC N, Thai N, HKUniv RdRp/Hel, HKUniv S, SigmAldr S5 and SigmAldr S6) demonstrated potential false negative results for the for SARS-CoV-2 Omicron BA.2 sublineage, which also has the potential for Spike (S) gene dropout despite lacking 69-70 deletion in the S gene. Further, amplicon clustering and additional substitutions analysis along with the sensitivity analysis could be used for modification and development of RT-qPCR assays for detection of SARS-CoV-2 Omicron VoC lineages.

1. Introduction

The emergence of the newly discovered severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) B.1.1.529 lineage presents a new threat to global public human health and coronavirus disease-2019 (COVID-19) pandemic containment efforts.[1] Due to an unprecedented number of mutations, the high potential for immune escape, and the rapid global spread and increasing number of cases, the World Health Organization (WHO) labeled B.1.1.529 as Omicron Variant of Concern (VoC) and has called for immediate global action in response.[2]

SARS-CoV-2 is a positive-sense, single stranded RNA virus, with a genome of ~30,000 base pairs in length.[3] As officially recommended by the WHO[4] and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)[5] the current gold standard diagnostic assay for detecting SARS-CoV-2 in human specimens is a Nucleic Acid Amplification Test (NAAT); more specifically, a quantitative reverse transcription polymerase chain reaction (RT-qPCR), to detect viral RNA in clinical specimens, most commonly, from nasopharyngeal or oropharyngeal swabs. RT-qPCR employs 3 oligonucleotides: a forward primer, a reverse primer, and a probe, which specifically hybridize with respective sequence targets in the SARS-CoV-2 genome between where the forward and reverse primers recognize.[6] Subsequent amplification of the viral genome and production of a fluorescence indicator by the probe during repeated application cycles, leads to identification and possible quantification of SARS-CoV-2 genetic material.[6] Given the high accuracy and rapid turnaround times, NAAT are the most widely used tests by clinical laboratories for COVID-19 diagnostics.[5] However, this accuracy is threatened by the rapid evolution of SARS-CoV-2, in which some RT-qPCR assays may lose sensitivity due to mutations in the viral genome of newly evolving SARS-CoV-2 VoC.[6,7]

The most likely sources of RT-qPCR false negative results are due to pre-analytical errors.[8] These errors can be in specimen collection (e.g. swab), handling, transport and storage, inappropriate and/or inadequate swab quality, and/or volume.[8] Other analytical errors, such as testing carried out outside of the proper diagnostic window, as well as mismatches between primers/probes and the viral genome due to mutations, remain a threat to diagnostic accuracy in the context of novel SARS-CoV-2 VoC.[8] Mutations located in regions hybridizing with the 3’ end of primers are the most sensitive, with a single mismatch in an annealing site leading to inhibition of amplification and potential reduced diagnostic accuracy and/or false negative results.[9–13] Mutations located in regions hybridizing with 5’ end of primers or other annealing segments of the oligonucleotide may have a more variable impact; however 2-3 mismatches in such regions may result in reduced RT-qPCR performance.[9–13]

Give that Omicron displays a high mutational profile, evaluation of all diagnostic assays (molecular and antigen immunoassays) should be urgently performed to ensure diagnostic accuracy and proper identification of COVID-19 cases, enabling contact tracing and quarantine procedures to limit community transmission. Indeed, the United States (U.S.) Food and Drug Administration (FDA) has already noted potential issues with several diagnostic assays in the context of the Omicron VoC.[14] With respect to RT-qPCR, previous *in silico* studies evaluating less mutated variants has demonstrated several mismatches, including mutations located in oligonucleotide annealing sites, which may reduce the sensitivity for some NAAT.[7,15,16]

RT-qPCR primers and probe are generally designed to hybridize with relatively conserved sequences in the SARS-CoV-2 RNA genome, with most assays targeting one of more of the following: Spike (S), Envelope (E), and Nucleocapsid (N) structural proteins and Open Reading Frame (ORF) ORF1ab, which encodes the RNA-dependent RNA polymerase (RdRp).[5–7,15,16] Mutations in all of the above proteins are observed in the Omicron VoC, including over 30 nonsynonymous mutations in the S, a nonsynonymous mutation in the E, multiple amino acid substitution and a deletion in the N, and multiple nonsynonymous mutations in ORF1ab, including the nonstructural protein (nsp)12, the RdRp, a defined target for multiple anti-viral drugs, such as molnupiravir[17] and remdesivir.[18] An overview of the mutations found in the sublineages of Omicron VoC are provided in Table 1.[19]

Table 1: Mutations in SARS-CoV-2 Omicron (B.1.1.529) VoC BA.1 and BA.2 sublineage .

Gene	Nucleotide mutation	Amino acid mutation
E	C26270T	T9I
M	A26530G	D3G
	C26577G	Q19E
	G26709A	A63T
N	C28311T	P13L
	28362_28370del	ERS31del
	G28881A, G28882A, G28883C	RG203KR
	A29510C	S413R
nucleotide	C3037T	-
	T5386G	
	C4321T	
	A9424G	
	C10198T	
	G10447A	
	C12880T	
	C15240T	
	C15714T	
	A20055G	
	C25000T	
	C25584T	
	C26858T	
	A27259C	
	C27807T	
	A28271T	
ORF1ab	T670G	S135R
	C2790T	T842I
	A2832G	K856R
	G4184A	G1307S
	6513_6515del	SL2083I
	G8393A	A2710T
	C9344T	L3027F
	C9534T	T3090I
	C9866T	L3201F
	C10029T	T3255I
	C10449A	P3395H
	11288_11296del*	SGF3675del*
	T11296G#	F3677L#
	G11287T#	L3674F#
	A11537G	I3758V
	C14408T	P4715L
	C17410T	R5716C
	A18163G	I5967V
	C19955T	T6564I
ORF3a	C26060T	T223I
ORF6	G27382C, A27383T, T27384C	D61L
S	C21618T	T19I
	21633_21641del	LPPA24S
	C21762T	A67V
	21765_21770del	HV69del
	C21846T	T95I
	G21987A (outlier); 21987_21995 (main)	G142D
	21987_21995	VYY143del
	22194_22196del	NL211I
	T22200G	V213G
	22205GAGCCAGAAins	215EPEins
	G22578A	G339D
	C22674T	S371F
	T22673C, C22674T	S371L
	T22679C	S373P
	C22686T	S375F
	A22688G	T376A
	G22775A	D405N
	A22786T	R408S
	G22813T	K417N
	T22882G	N440K
	G22898A	G446S
	G22992A	S477N
	C22995A	T478K
	A23013C	E484A
	A23040G	Q493R
	G23048A	G496S

	A23055G	Q498R
	A23063T	N501Y
	T23075C	Y505H
	C23202A	T547K
	A23403G	D614G
	C23525T	H655Y
	T23599G	N679K
	C23604A	P681H
	C23854A	N764K
	G23948T	D796Y
	C24130A	N856K
	A24424T	Q954H
	T24469A	N969K
	C24503T	L981F

Notes: Blue represents mutations unique to SARS-CoV-2 Omicron BA.1 sublineage. Yellow represents mutations unique to SARS-CoV-2 Omicron BA.2 sublineage. No color represents mutations in the SARS-CoV-2 Omicron VoC. *: The exact position of this deletion is ambiguous in nucleotide coordinates. #: Position of the mutation depends on the position of the deletion.

Here, we aimed to assess the potential impact of Omicron VoC on the integrity of RT-qPCR assays currently used for COVID-19 diagnosis and evaluate the potential for false negatives or test failure due to mismatches between primers/probes and viral genome. Ideally the evaluation of diagnostic assays should be performed *in vitro* by qualified personnel at clinical and research laboratories. However, given the limited access to the Omicron VoC, including the multiple sublineages, limited availability of reagents and assays due to global laboratory shortages and lack of personnel as recently noted in a survey by the American Association of Clinical Chemistry[20] and all primers/probes needed to performed such comprehensive evaluation, a bioinformatics approach, using an *in silico* specificity (sensitivity) evaluation, as recently demonstrated by Gand *et al.*[7,15], Khan *et al.*[16] and Nayar *et al.*[6] can be a useful initial and rapid evaluation. Thus, it is critical to take full advantage of the immense efforts of the scientific community, especially in South Africa, to generate whole genome sequencing (WGS) data that are made publicly available in databases such as National Center for Biotechnology Information (NCBI) and Global Initiative on Sharing Avian Influenza Data (GISAID).

In this study, the *in silico* sensitivity of 12 RT-qPCR tests (containing 30 primers and probe sets) developed for detection of SARS-CoV-2 was assessed for use in detecting SARS-CoV-2 BA.1 and BA.2 Omicron VoC sublineages as classified by Pangolin, obtained after removing redundancy from publicly available genomes from NCBI and GISAID databases. Mismatches between the amplicon regions of the SARS-CoV-2 Omicron VoC and primers and probe sets were evaluated and the clustering analysis of the corresponding amplicon sequences was carried out. For BA.1 Omicron sublineage, assays ChinaCDC N, Thai N, and HKUniv RdRp/Hel could not produce a theoretical positive signal, whereas, for BA.2 Omicron sublineage, theoretical positive signal could not be obtained for assays ChinaCDC N, Thai N, HKUniv RdRp/Hel, HKUniv S, SigmAldr S5 and SigmAldr S6.

2. Materials And Methods

The methodology followed in this study involves collection of whole genome sequencing data for Omicron VoC from databases and assessing the impact of RT-qPCR assays on these genomes. An overview of the methodology is presented in Figure 1.

2.1. SARS-CoV-2 Omicron VoC WGS Dataset

Whole genome sequencing data of the SARS-CoV-2 Omicron (B.1.1.529) VoC BA.1 sublineage were downloaded from GISAID EpiCoV (https://www.epicov.org, 1315 genome sequences) (**Supplementary file 1**) and NCBI virus (https://www.ncbi.nlm.nih.gov/labs/virus/, 3 genome sequences) databases on December 10th 2021. Six SARS-CoV-2 Omicron (B.1.1.529) VoC BA.2 sublineage genomes were also downloaded from GISAID EpiCoV on 12 December 12th 2021 (**Supplementary file 2**). The geographical distribution of the genome sequences is shown in Figure 2. To minimize the sequencing errors, the filters used for selecting the data from GISAID were, “Complete” which means only complete genome sequences (>29,000 nt) were selected, and “low coverage excl”, *i.e.*, entries with more than 5% unknowns (“Ns”) were excluded. For NCBI genomes, data were selected based on the filters, “SARS-CoV-2” as the virus (taxid: 2697049), “Homo sapiens” as the host (taxid: 9606), and “B.1.1.529” as the pango lineage.

2.2. Primers and Probes Dataset

Primers and probes used in this investigation consisted of 12 RT-qPCR tests targeting different regions of the SARS-CoV-2 genome that were obtained from a WHO report [4] and/or identified via literature reviews[21–25] and evaluated in previous studies.[6,7,15,16] A total of 30 primers and probes sets (24 primers and probe sets using TaqMan technology and 6 primer sets using SYBR Green technology) were used. These are part of 12 RT-qPCR tests developed for the detection of SARS-CoV-2 (Table 2 and **Supplementary file 3**). Targets of the 12 RT-qPCR tests are located in the SARS-CoV-2 genes coding for the E, N and S structural proteins, and the non-structural proteins (nsp) RdRp/helicase (Hel) and 14 (nsp14) located in the viral ORF1ab.

Table 2
Summary of assays, primers, and probes used in this investigation.

Assay	Source/Country	Target	Technology	Assay name
1	Chinese Center for Disease Control and Prevention, China[4]	ORF1b	Taqman	ChinaCDC ORF1ab
		N	Taqman	ChinaCDC N
2	Charité Hospital, Germany[4]	RdRp P1	Taqman	CoremCharite RdRp P1
		RdRp P2	Taqman	CoremCharite RdRp P2
		E	Taqman	CoremCharite E
		N	Taqman	CoremCharite N
3	Pasteur Institute, France[4]	RdRp IP2	Taqman	Pasteur RdRp IP2
		RdRp IP4	Taqman	Pasteur RdRp IP4
		E	Taqman	Pasteur E
4	Centers for Disease Control and Prevention, USA[4]	N-1	Taqman	USCDC N1
		N-2	Taqman	USCDC N2
		N-3	Taqman	USCDC N3
5	National Institute of Infectious Diseases, Japan[4]	N	Taqman	Japan N
6	Li Ka Shing Faculty of Medicine, The University of Hong Kong, China[4]	ORF1b/NSP14	Taqman	HKFacMed ORF1b/nsp14
		N	Taqman	HKFacMed N
7	Ministry of Public Health, Thailand[4]	N	Taqman	Thai N
8	University of Hong Kong/Queen Elizabeth Hospital, China[21]	RdRp/Hel	Taqman	HKUniv RdRp/Hel
		S	Taqman	HKUniv S
		N	Taqman	HKUniv N
9	Lu et al., China[22]	ORF1a	Taqman	RoujianLu ORF1a
10	Won et al., Korea[23]	RdRp	SYBR green	Won RdRP
		S	SYBR green	Won S
		E	SYBR green	Won E
		N	SYBR green	Won N
11	Sigma-Aldrich, USA[24]	N-1	SYBR green	SigmAldr N1
		N-2	SYBR green	SigmAldr N2
		ORF1a-3	Taqman	SigmAldr ORF1ab3
		ORF1a-4	Taqman	SigmAldr ORF1ab4
		S-5	Taqman	SigmAldr S5
		S-6	Taqman	SigmAldr S6
12	Huang et al., China[25]	E	Taqman	Huang E

Note: Full sequences of primers and probes are available in **Supplementary file 3**.

2.3. Selection of representative genomes by sequence identity clustering

Clustering of the Omicron VoC sequences was performed to remove redundancy in the dataset. All the downloaded sequences were clustered using CD-HIT-EST vr. 4.6.8 (https://github.com/weizhongli/cdhit) with sequence identity cut-off equal to 1.0 (other parameters were left at default settings). A total of 1,178 clusters of sequences were obtained from CD-HIT-EST for SARS-CoV-2 Omicron BA.1 sublineage and 5 clusters for BA.2 sublineage. Representative genomes of lower quality, *i.e.*, showing more than three ambiguous nucleotides (such as "N") in the genomic regions targeted by the evaluated RT-qPCR assays were removed.[7,15] Total sequences after removing the redundancy and taking high-quality sequences were 232 for BA.1 and 5 for BA.2 sublineage of Omicron VoC.

2.4. Assessment of the RT-qPCR signals using SCREENED

SCREENED (polymeraSe Chain Reaction Evaluation through largE-scale miNing of gEnomic Data) version 1.0, developed by Vanneste *et al.*[13] was employed to determine the theoretical production of RT-qPCR signals. For each PCR method to be evaluated, SCREENED first searches for the amplicon in the genomes to be assessed using BLAST and then searches in these found amplicons whether the provided forward primer, reverse primer, or the probe, will successfully

anneal.[13,15] Mismatches in the primer and the probe sequences with the genomic sequence were then evaluated for the production of a positive or negative RT-qPCR signal.

In this study, the following '*a priori*' determined settings were used to define a positive RT-qPCR signal, using SCREENED:

i. No mismatch was observed in the initial 5 nucleotides of primers' 3' end;
ii. Total number of mismatches was not more than 10% of oligonucleotides length; and,
iii. 90% or more of the oligonucleotides sequence accurately aligned with their targets.[9–13, 15]

These criteria were selected in accordance with the European Commission (EC) guidance on COVID-19 diagnostic performance criteria[26] and from scientific literature evaluating the potential for mismatches affecting the performance of PCR-like methods and were used in previous studies.[7,13,15] In our study, only 1-2 mismatches were allowed for primers and probe sets (according to EC criteria[26]), except for the forward primer of Assay HKUniv S, for which no more than 3 mismatches were allowed since it was 30 nucleotides in length. Advanced options such as the amplicon and fragment extension, and greedy clustering of the amplicon were enabled in SCREENED. Primers and probe sequences were aligned with their respective representative sequences from the clustered amplicons using MUSCLE (multiple sequence comparison by log-expectation).[27] Input files to the SCREENED were a fasta file of the genome sequences (232 for BA.1 and 5 for BA.2) and a tab-delimited text file containing primers and probes sequences to be evaluated and their corresponding amplicon sequence to be extracted in the genomes.

2.5. Evaluation of *in silico* analytical sensitivity

Sensitivity was used to evaluate the RT-qPCR tests used. We considered a theoretical RT-qPCR positive signal if the assay followed the criteria used by SCREENED. The sensitivity, known as the potential of a method to detect varying targets by a positive relation, could be referred to as inclusivity, which is used to evaluate performance of the assay.[28] The *in silico* sensitivity of an assay is more qualitative than quantitative as it signifies whether the genome is detected and could relate with the diagnostic sensitivity of the assay.[29] The *in silico* sensitivity (*i.e.* inclusivity) was calculated as the ratio of number of genomes detected, *i.e.*, producing a positive RT-qPCR signal to the total number of genomes analyzed.[13]

The *in silico* sensitivity was evaluated as shown in Equation 1.

$$Sensitivity(\%) = \left(\frac{SARS-CoV-2 genomes detected}{SARS-CoV-2 genomes analyzed}\right) * 100$$

1

3. Results

SCREENED was used for investigating the *in silico* sensitivity of 30 primers and probe sets from 12 RT-qPCR assays targeting different regions of the SARS-CoV-2 genome. We used 232 representative SARS-CoV-2 genomes from BA.1 sublineage and 5 from BA.2 sublineage SARS-CoV-2 Omicron VoC genomes obtained after data preprocessing -.

3.1. Determination of sensitivity for RT-qPCR assays

3.1.1. For SARS-CoV-2 Omicron BA.1 sublineage

Our results in Table 3 indicate that all of the 232 SARS-CoV-2 Omicron BA.1 sublineage genomes produced a false negative (genome could not be amplified *in silico* by the RT-qPCR assay) result for HKUniv RdRp/Hel assay because of a substitution in the first five nucleotide of the reverse primer's 3' end (C to T) resulting in 0% sensitivity of this test. A sensitivity of 1.29% was observed for ChinaCDC N assay owing to the substitution in the forward primer's 5' end (3-nucleotide substitution, GGG to AAC) and resulted in false negative result for 229/232 genomes. For Thai N assay, 226/232 genomes could not be detected correctly, which resulted in a 2.59% sensitivity because of 10 nucleotide mismatches in the reverse primer. A 99.57% sensitivity was obtained for assays CoremCharite E, Pasteur E, RoujianLu ORF1a, SigmAldr N2, SigmAldr S5, SigmAldr S6, and Huang E. All other assays showed 100% sensitivity as presented in Table 3, which means that these assays resulted in *in silico* amplification of all SARS-CoV-2 Omicron BA.1 sublineage genomes included in our analysis.

Table 3
Assessment of the sensitivity of RT-qPCR assays using SCREENED for SARS-CoV-2 Omicron BA.1 sublineage genomes.

Assay	Target gene	Assay name	Mismatches in the First Five Nucleotides of the Primer's 3' End		>10% Mismatches in the Annealing Sites of Primers/Probes		False negati results
			No. of genomes	Substitutions in genome	No. of genomes	Substitutions in genome	*
1	N	ChinaCDC N	0	-	229	Fw AACGAACTTCTCCTGCTAGAAT	229
1	ORF1ab	ChinaCDC ORF1ab	0	-	0	-	0
2	RdRp P1	CoremCharite RdRp P1	0	-	0	-	0
2	RdRp P2	CoremCharite RdRp P2	0	-	0	-	0
2	E	CoremCharite E	1	Rv TAATAGCGTACTTCTTTTTCTT	1	Rv TAATAGCGTACTTCTTTTTCTT	1
2	N	CoremCharite N	0	-	0	-	0
3	RdRp IP2	Pasteur RdRp IP2	0	-	0	-	0
3	RdRp IP4	Pasteur RdRp IP4	0	-	0	-	0
3	E	Pasteur E	1	Rv TAATAGCGTACTTCTTTTTCTT	1	Rv TAATAGCGTACTTCTTTTTCTT	1
4	N-1	USCDC N1	0	-	0	-	0
4	N-2	USCDC N2	0	-	0	-	0
4	N-3	USCDC N3	0	-	0	-	0
5	N	Japan N	0	-	0	-	0
6	ORF1b/NSP14	HKFacMed ORF1b/nsp14	0	-	0	-	0
6	N	HKFacMed N	0	-	0	-	0
7	N	Thai N	0	-	226	Rv AGTAACCAGAATGGTGGGG	226
8	RdRp/Hel	HKUniv RdRp/Hel	232	Rv GACCATGTCATATCAACATCACAT	0	-	232
8	S	HKUniv S	0	-	0	-	0
8	N	HKUniv N	0	-	0	-	0
9	ORF1a	RoujianLu ORF1a	1	Fw AGAAGATTGGTTAGATGATGGTAGT	0	-	1
10	RdRp	Won RdRP	0	-	0	-	0
10	S	Won S	0	-	0	-	0
10	E	Won E	0	-	0		0
10	N	Won N	0	-	0	-	0
11	N-1	SigmAldr N1	0	-	0	-	0
11	N-2	SigmAldr N2	1	Rv GAATGGCTGGCAATGGCTG	0	-	1
11	ORF1a-3	SigmAldr ORF1ab3	0	-	0	-	0
11	ORF1a-4	SigmAldr ORF1ab4	0	-	0	-	0
11	S-5	SigmAldr S5	0	-	1	Pb AGACTAAGTCTCATTGGCGGGCACG	1

Note: Results were obtained from 232 SARS-CoV-2 Omicron BA.1 sublineage genomes. The mismatches are shown in bold and underline. The forward (Fw) a primers, and probe (Pb) sequences are directed from 5'-3'. The Rv primer sequences are reverse complemented.

"-" represents that no mismatches are obtained by SCREENED.

*: False negative results represents that the primer/probe could not amplify the target *in silico*.

Assay	Target gene	Assay name	Mismatches in the First Five Nucleotides of the Primer's 3' End		>10% Mismatches in the Annealing Sites of Primers/Probes		False negati results
			No. of genomes	Substitutions in genome	No. of genomes	Substitutions in genome	*
11	S-6	SigmAldr S6	0	-	1	Pb AGACTAAGTCTCATTGGCGGGCACG	1
12	E	Huang E	0	-	1	Rv CTAGCCATCCTTACTGCGCN	1
Note: Results were obtained from 232 SARS-CoV-2 Omicron BA.1 sublineage genomes. The mismatches are shown in bold and underline. The forward (Fw) a primers, and probe (Pb) sequences are directed from 5'-3'. The Rv primer sequences are reverse complemented.							
"-" represents that no mismatches are obtained by SCREENED.							
*: False negative results represents that the primer/probe could not amplify the target *in silico*.							

3.1.2. For SARS-CoV-2 Omicron BA.2 sublineage

A total of only 5 genomes of SARS-CoV-2 Omicron BA.2 sublineage were analyzed for the *in silico* evaluation of the RT-qPCR assays; results are presented in Table 4. Assays ChinaCDC N and Thai N showed a sensitivity of 0%, as all of the 5 genomic sequences produced false negative results. A 0% sensitivity was obtained for assay HKUniv RdRp/Hel because of a substitution in the first five nucleotides of the reverse primer's 3' end (C to T). Assays SigmAldr S5 and SigmAldr S6 showed a sensitivity of 20% as these could not correctly detect four out of five sequences and had mismatches in the reverse primers. Assay HKUniv S showed 12 mismatches between the reverse primer sequence and the annealing sequence in the amplicon for three genomes, resulted in a 40% sensitivity. All other assays showed 100% sensitivity and could correctly amplify *in silico* all SARS-CoV-2 Omicron BA.2 sublineage genomes (Table 4).

Table 4
Assessment of the sensitivity of RT-qPCR assays using SCREENED for SARS-CoV-2 Omicron BA.2 sublineage genomes.

Assay	Target gene	Assay name	Mismatches in the First Five Nucleotides of the Primer's 3' End		>10% Mismatches in the Annealing Sites of Primers and Probes		False negative results
			No. of genomes	Substitutions in genome	No. of genome	Substitutions in genome	*
1	N	ChinaCDC N	0	-	5	Fw AACGAACTTCTCCTGCTAGAAT	5
1	ORF1ab	ChinaCDC ORF1ab	0	-	0	-	0
2	RdRp P1	CoremCharite RdRp P1	0	-	0	-	0
2	RdRp P2	CoremCharite RdRp P2	0	-	0	-	0
2	E	CoremCharite E	0	-	0	-	0
2	N	CoremCharite N	0	-	0	-	0
3	RdRp IP2	Pasteur RdRp IP2	0	-	0	-	0
3	RdRp IP4	Pasteur RdRp IP4	0	-	0	-	0
3	E	Pasteur E	0	-	0	-	0
4	N-1	USCDC N1	0	-	0	-	0
4	N-2	USCDC N2	0	-	0	-	0
4	N-3	USCDC N3	0	-	0	-	0
5	N	Japan N	0	-	0	-	0
6	ORF1b/NSP14	HKFacMed ORF1b/nsp14	0	-	0	-	0
6	N	HKFacMed N	0	-	0	-	0
7	N	Thai N	0	-	5	Rv AGTAACCAGAATGGTGGGG	5
8	RdRp/Hel	HKUniv RdRp/Hel	5	Rv GACCATGTCATATCAACATCACAT	0	-	5
8	S	HKUniv S	3	Rv TGATCTCTGCTTTACTAATGT	3	Rv TGATCTCTGCTTTACTAATGT	3
8	N	HKUniv N	0	-	0	-	0
9	ORF1a	RoujianLu ORF1a	0	-	0	-	0
10	RdRp	Won RdRP	0	-	0	-	0
10	S	Won S	0	-	0	-	0
10	E	Won E	0	-	0	-	0
10	N	Won N	0	-	0	-	0
11	N-1	SigmAldr N1	0	-	0	-	0
11	N-2	SigmAldr N2	0	-	0	-	0
11	ORF1a-3	SigmAldr ORF1ab3	0	-	0	-	0
11	ORF1a-4	SigmAldr ORF1ab4	0	-	0	-	0
11	S-5	SigmAldr S5	4	Rv TCAGACTCAGACTAAGTCTCATCG	4	Rv TCAGACTCAGACTAAGTCTCATCG	4

Note: The results were obtained from 232 SARS-CoV-2 Omicron BA.2 sublineage genomes. The mismatches are shown in bold and underline. The forward (F reverse (Rv) primers, and probe (Pb) sequences are directed from 5'-3'. The Rv primer sequences are reverse complemented.

"-" represents that no mismatches are obtained by SCREENED.

*: False negative results represents that the primer/probe could not amplify the target *in silico*.

Assay	Target gene	Assay name	Mismatches in the First Five Nucleotides of the Primer's 3' End		>10% Mismatches in the Annealing Sites of Primers and Probes		False negative results
			No. of genomes	Substitutions in genome	No. of genome	Substitutions in genome	*
11	S-6	SigmAldr S6	4	Rv GTTATCAGACTCAGACTAAGTCTCA	4	Rv GTTATCAGACTCAGACTAAGTCTCA	4
12	E	Huang E	0	-	0	-	0

Note: The results were obtained from 232 SARS-CoV-2 Omicron BA.2 sublineage genomes. The mismatches are shown in bold and underline. The forward (F reverse (Rv) primers, and probe (Pb) sequences are directed from 5'-3'. The Rv primer sequences are reverse complemented.

"-" represents that no mismatches are obtained by SCREENED.

*: False negative results represents that the primer/probe could not amplify the target *in silico*.

3.2. Analysis of the amplicon clusters and additional substitutions in the primer and probe sequences

SCREENED also clustered the amplicon sequences from all genomes analyzed in our study targeted by the evaluated primers/probes. For each RT-qPCR assay, genomes were clustered based on the identical amplicon sequence, where the greater the number of clusters represents a higher diversity of the genomic region. The total number of clusters obtained for each assay and the redistribution of genomes in the top three clusters (from largest to smallest) are shown in Table 5. The largest cluster of the corresponding amplicon sequences for Pasteur RdRp IP2, USCDC N1, HKUniv N, HKUniv S, and SigmAldr S6 assays, targeting RdRp, N and S genes, was present in 90 to 97% of the genomes but for most of the assays, above 97% of the amplicon sequences could be clustered in one large cluster. The USCDC N1 assay with seven amplicon clusters and SigmAldr S6 assay with six amplicon clusters showed higher number of amplicon clusters consisting of one large cluster and other smaller clusters indicating higher diversity in these regions of the SARS-CoV-2 Omicron BA.1 sublineage genome. Intriguingly, USCDC N1 and SigmAldr S6 assays sensitivity was determined to be more than 99%, despite showing the largest amplicon diversity. For SARS-CoV-2 Omicron VoC BA.2 sublineage, the amplicon sequences for corresponding primers/probes could be clustered in only one cluster, this could be due to a very limited number of genome sequences available for this Omicron VoC BA.2 sublineage.

Table 5
Amplicon clusters among the SARS-CoV-2 Omicron BA.1 sublineage sequences amplified by the evaluated primers and probes and percentage of genomes within largest 3 clusters.

Assay	Target gene	Assay name	No. of clusters	Cluster I (%)	Cluster II (%)	Cluster III (%)
1	N	ChinaCDC N	5	97.8	0.9	0.4
1	ORF1ab	ChinaCDC ORF1ab	2	99.6	0.4	
2	E	CoremCharite E	4	98.3	0.9	0.4
2	RdRp P1	CoremCharite RdRp P1	3	98.7	0.9	0.4
2	RdRp P2	CoremCharite RdRp P2	3	98.7	0.9	0.4
2	N	CoremCharite N	1	100	-	-
3	RdRp IP2	Pasteur RdRp IP2	4	93.1	5.2	1.3
3	E	Pasteur RdRp E	4	98.3	0.9	0.4
3	RdRp IP4	Pasteur RdRp IP4	1	100	-	-
4	N-1	USCDC N1	7	90.5	5.2	2.6
4	N-2	USCDC N2	2	98.3	1.7	-
4	N-3	USCDC N3	2	99.6	0.4	-
5	N	Japan N	2	98.3	1.7	-
6	N	HKFacMed N	2	98.3	1.7	-
6	ORF1b/NSP14	HKFacMed ORF1b/nsp14	1	100	-	-
7	N	Thai N	2	97.4	2.6	-
8	S	HKUniv S	4	90.1	6	3.4
8	N	HKUniv N	4	91.8	6	1.7
8	RdRp/Hel	HKUniv RdRp/Hel	1	100	-	-
9	ORF1a	RoujianLu ORF1a	3	99.1	0.4	0.4
10	E	Won E	4	98.3	0.9	0.4
10	RdRp	Won RdRp	3	98.7	0.9	0.4
10	S	Won S	2	99.6	0.4	-
10	N	Won N	1	100	-	-
11	S-6	SigmAldr S6	6	96.5	1.7	0.4
11	N-1	SigmAldr N1	5	97.8	0.9	0.4
11	N-2	SigmAldr N2	5	97.8	0.9	0.4
11	S-5	SigmAldr S5	4	98.7	0.4	0.4
11	ORF1a-3	SigmAldr ORF1ab3	2	99.6	0.4	-
11	ORF1a-4	SigmAldr ORF1ab4	2	99.6	0.4	-
12	E	Huang E	2	99.1	0.9	-

Sequence alignment of the amplicons present in the largest cluster with the corresponding primer/probe sequences were performed to explore the additional substitutions or mismatches present in majority of amplicons of the genomes, apart from the ones that prevent the *in silico* amplification by the primers/probes obtained by SCREENED. This approach was also adopted by Gand *et al.*[7,15] Additional substitutions were present in the sequences of almost all the RT-qPCR assays (Table 6) except in ChinaCDC ORF1ab, CoremCharite N, Pasteur RdRp IP2, Pasteur RdRp IP4, HKUniv S, Won S, SigmAldr N1, and Huang E assays. These substitutions were observed in the first and the largest clusters of the respective primers and probe sets, which contained >90% of the genomes investigated. These substitutions along with mismatches in primers and probe sequences determined by SCREENED could be considered for development of new primers and probes sets for the RT-qPCR assays used to detect the SARS-CoV-2 Omicron VoC.

Table 6
Substitutions in the genomes of the largest cluster of respective primer and probes for SARS-CoV-2 Omicron BA.1 lineage obtained from multiple sequence alignments.

Assay	Target gene	Substitutions	No. of genomes (%)
1	N	Fw AACGAACTTCTCCTGCTAGAAT	227 (97.84)
		Rv AACGAACTTCTCCTGCTAGAAT	
		Pb AACGAACTTCTCCTGCTAGAAT	
2	E	Fw ATAGGTACGTTAATAGTTAATAGCGT	228 (98.28)
2	RdRp P1	Fw GTGAAATGGTCATGTGTGGCGG	229 (98.71)
		Pb CCAGGTGGAACCTCATCAGGAGATGC	
2	RdRp P2	Fw GTGAAATGGTCATGTGTGGCGG	229 (98.71)
3	E	Fw ATAGGTACGTTAATAGTTAATAGCGT	228 (98.28)
4	N-1	Rv CCGCATTACGTTTGGTGGACCCTC	210 (90.52)
		Pb ACTCCGCATTACGTTTGGTGGACC	
4	N-2	Rv GCACAATTTGCCCCCAGC	228 (98.28)
4	N-3	Rv TCACATTGGCACCCGCAATCCTG	231 (99.57)
		Pb ATCACATTGGCACCCGCAATCCTG	
5	N	Fw GAATGTCGCGCATTGGCATG	228 (98.28)
6	ORF1b/NSP14	Fw TGGGGTTTTACAGGTAACCT	232 (100)
		Rv ATGGGGTTTTACAGGTAACCT	
		Pb TAGTTGTGATGCAATCATGACTAG	
6	N	Rv GCAAATTGCACAATTTGCC	228 (98.28)
		Pb GCAAATTGCACAATTTGCCC	
7	N	Fw CAACTGGCAGTAACCAGAAT	226 (97.41)
8	RdRp/Hel	Fw CGCATACAGTCTTACAGGCT	232 (100)
8	N	Rv CGGGAACGTGGTTGACCTACACA	213 (91.81)
		Pb AACGTGGTTGACCTACACAGGT	
9	ORF1a	Rv TTCAAACAATTGTTGAGGTTCAACC	230 (99.14)
		Pb TTCAAACAATTGTTGAGGTTCAACC	
10	E	Fw TTCGGAAGAGATAGGTACGTT	228 (98.28)
10	N	Rv GCTGCAATCGTGCTACAACT	232 (100)
10	RdRp	Rv TGTGTGGCGGTTCACTATAT	229 (98.71)
11	N-2	Rv CAGCCTCTTCTCGTTCCTC	227 (97.84)
11	ORF1a-3	Pb AAGGATCACCGCAAGGTTCTTCTTC	231 (99.57)
11	ORF1a-4	Pb AAGGCTTACCGCAAGGTTCTTCTTC	231 (99.57)
11	S-5	Pb AGACTAAGTCTCATCGGCGGGCACG	229 (98.71)
11	S-6	Pb AGACTAAGTCTCATCGGCGGGCACG	224 (96.55)

Note: The mismatches are shown in bold and underline. The forward (Fw) and reverse (Rv) primers, and probe (Pb) sequences are directed from 5'-3'. Mismatches shown in this table were not obtained from SCREENED but from multiple sequence alignments. These mismatches did not halt amplification but if additional mismatches appear could result into false negatives.

4. Discussion

With the increasing number of mutations in the SARS-CoV-2 emerging VoC, as in B.1.1.529, also known as Omicron, the evaluation of the current RT-qPCR assays used for the detection of SARS-CoV-2 is important for correct diagnosis. Evaluation of these assays in the wet laboratory is limited in this rapidly evolving Omicron outbreak, because of the time constraint and lack of representative strains available for clinical laboratories, as previously noted.[30] Therefore, an *in silico* approach was used to evaluate the sensitivity of current RT-qPCR assays using the whole genome sequencing data of the SARS-CoV-2 Omicron VoC (particularly from the publicly available GISAID database), and employing suitable bioinformatics tools.[7,30] We evaluated the sensitivity of 30 RT-

qPCR primers and probe sets in this study using SCREENED, which produces alignment statistics and thus, number of false-negative results could be obtained. An overall summary of the results is presented in Table 7.

Table 7. Assays with potentially false negative results based on *in silico* analysis.

Assay	Source/Country	BA.1	BA.2	Both BA.1 and BA.2
1	Chinese Center for Disease Control and Prevention, China			
2	Charité Hospital, Germany			
3	Pasteur Institute, France			
4	Centers for Disease Control and Prevention, USA			
5	National Institute of Infectious Diseases, Japan			
6	Li Ka Shing Faculty of Medicine, The University of Hong Kong, China			
7	Ministry of Public Health, Thailand			
8	University of Hong Kong/Queen Elizabeth Hospital, China			
9	Lu et al., China			
10	Won et al., Korea			
11	Sigma-Aldrich, USA			
12	Huang et al., China			

Note: Red indicate risk of false negatives (low sensitivity, <99% for all primers and probes in assay). Green indicate low risk of false negative (high sensitivity, ≥99% for all primers/probes in assay)

The HKUniv RdRp/Hel assay, developed at the University of Hong Kong, showed the worst sensitivity for the reverse primer, due to presence of a substitution at the 3' end of the primer annealing site, which is present in all the 232 Omicron BA.1 sublineage genomes. The ChinaCDC N assay had second lowest sensitivity, because of the presence of three substitutions at the beginning of the forward primer annealing site. These substitutions were observed in the 5' end of the assay, which may not prevent the amplification of the genome *in vitro* but due to the criteria used by SCREENED, this assay produces false negative results *in silico* and was observed in 229 Omicron BA.1 sublineage genomes. A total of 10 substitutions were observed in the reverse primer annealing site of the Thai N assay (developed by the Ministry of Public Health, Thailand), in 226 BA.1 Omicron sublineage genomes. Therefore, for the BA.1 Omicron sublineage, ChinaCDC N, Thai N, and HKUniv RdRp/Hel assays do not meet the criteria used by SCREENED in our analysis, which is also the criteria elaborated by the EC for evaluation of primers and probe sets.[26] For the BA.1 Omicron sublineage, a mismatch in the sixth nucleotide from 3' end was also observed for USCDC N3 assay forward primer, which was not matching with our SCREENED criteria but could give a false negative result, as a single mismatch in the 3' end may result in failure or reduced performance of the test. Related to BA.1 sublineage, other primers and probe sets sensitivity was > 99% and this was in agreement with our criteria.

In comparison with previous studies evaluating the sensitivity of the RT-qPCR assays for SARS-CoV-2 genomes from April 2020 to January 2021,[7,15] ChinaCDC N assay had the lowest sensitivity, which is in line with our observations in this study with Omicron VoC. Further, we observed a sensitivity of 2.59% for the Thai N assay and 0% for the HKUniv RdRp/Hel assay for the Omicron VoC, while Gand *et al.*[15] for the same assays obtained a sensitivity of 99.73% and 100%, respectively, analyzing other VoC, which points towards important differences between the Omicron VoC and previous SARS-CoV-2 variants. Interestingly, CoremCharite N and Pasteur RdRp IP4 assays showed the best results in our study, as these showed the highest sensitivity (Table 7), less diversity of amplicon among the genomes (Table 7), and no substitutions in the primers and probes annealing site. The Pasteur RdRp IP4 assay was intended to be specific to SARS-CoV-2, however, the specificity of assay CoremCharite N was not communicated.[15]

For SARS-CoV-2 Omicron BA.2 sublineage, which was restricted to five genomes due to very limited number of genomes available at time of this study, ChinaCDC N, Thai N, HKUniv RdRp/Hel assays displayed the lowest sensitivity. ChinaCDC N and Thai N assays had 3 and 10 mismatches in the forward and reverse primer annealing sites respectively in all five genomes analyzed in this study. The HKUniv RdRp/Hel assay showed a substitution in the 3' end of the reverse primer for all five genomes. The second lowest sensitivity was observed for SigmAldr S5 and SigmAldr S6 assays with 13 and 12 mismatches,respectively, in the reverse primer annealing site for four genomes of SARS-CoV-2 Omicron BA.2 sublineage. The HKUniv S assay showed a 40% sensitivity with 12 mismatches in the reverse primer annealing site for three SARS-CoV-2 Omicron BA.2 sublineage genomes. Hence, ChinaCDC N, Thai N, HKUniv RdRp/Hel, HKUniv S, SigmAldr S5 and SigmAldr S6 assays do not meet the criteria for an appropriate primers and probe set for detecting BA.2 sublineage of the SARS-CoV-2 Omicron VoC. For SARS-CoV-2 Omicron BA.2 sublineage, other primers and probe sets) sensitivity was in agreement with the *a priori* criteria.

It is likely that SARS-CoV-2 Omicron BA.2 sublineage, due to lacking the deletion in S at residues 69-70, is not "identified" as likely Omicron due to no S gene target dropout on some NAAT, which is often a pretext to sequencing, thus leading to underrepresentation of this sublineage in WGS data. However, in our study with the primers and probes employed, the S gene dropout could not be explained clearly for the SARS-CoV-2 Omicron BA.1 results as HKUniv S, Won S, SigmAldr S5 and SigmAldr S6 assays have high sensitivity (Table 7), which implies that these assays could correctly detect most of the studied SARS-CoV-2 Omicron BA.1 genomes included in our analysis. Thus, care should be taken in validating the use of the S gene target dropout for each specific RT-qPCR assay as means for tracking the SARS-CoV-2 Omicron BA.1 sublineage. Interestingly, for SARS-CoV-2 Omicron BA.2 genomes, the S gene dropout could be potentially observed in HKUniv S, Won S, SigmAldr S5 and SigmAldr S6 assays, which we observed to have low sensitivity (Table 6). Conversely, the presence of the N gene dropout for SARS-CoV-2 Omicron BA.1 could potentially be observed for some assays, such as ChinaCDC N and Thai N. The presence of the N gene and/or S gene dropout on RT-qPCR are not likely to occur for the SARS-CoV-2 Delta VoC, and if detected, should alert to a high likelihood of being the Omicron VoC.[14] However, the potential for both N and S genes dropout highlights the importance of using an assay with multiple gene targets and the need for additional testing approaches in cases of suspected false negatives.

This preliminary study was limited by a small number of currently available SARS-CoV-2 Omicron VoC genomes, as well as by the rapid genetic diversification of the B.1.1.529, with multiple sublineage, including a newly classified SARS-CoV-2 Omicron BA.3.[31] However, our findings demonstrate the urgent need to evaluate NAAT for detection of SARS-CoV-2 Omicron and other potentially similar emerging VoC. We will continue to update our analysis in the weeks ahead. Importantly, the large number of RT-qPCR tests currently being performed employ commercially developed primers and probes, whose sequences are unknown and often not shared due to concerns over intellectual property. However, in these unprecedented times, false negative results can have detrimental consequences, especially early in the SARS-CoV-2 Omicron outbreak, limiting capacity for viral tracing, intervention, and interrupting the transmission chain. Thus, wet laboratory evaluation and validation of commercial assay primer and probes should be urgently performed. We amplify the call by Metzger et al.[30] for governmental bodies or neutral institutions to be tasked with *in silico* evaluation in emergency settings for global spread of a new SARS-CoV-2 VoC to inform clinical research laboratories of potential diagnostic performance issues while protecting intellectual property.

5. Conclusion

In our *in silico* analysis evaluating 12 RT-qPCR assays with a total of 30 primers and probes, for the SARS-CoV-2 Omicron BA.1 sublineage, three (25%) assays (ChinaCDC N, Thai N, and HKUniv RdRp/Hel) demonstrated potential for false negatives, while for SARS-CoV-2 Omicron BA.2 sublineage, four (33.3%) assays (ChinaCDC N, Thai N, HKUniv RdRp/Hel, HKUniv S, SigmAldr S5 and SigmAldr S6) demonstrated potential false negative results. Interestingly, we observed a greater potential for dropout in the SARS-CoV-2 Omicron BA.2 sublineage as opposed to the SARS-CoV-2 Omicron BA.1 sublineage despite lacking the 69-70 deletion in the S protein. Many mutations in the primers and probe sets of the RT-qPCR assays were seen, resulting in low sensitivity of the assays reflective of the high number of mutations in the SARS-CoV-2 Omicron VoC. Apart from the sensitivity analysis, the amplicon sequence clustering and the additional substitutions in the primer and probe sets in a large number of genomes revealed the potential new primer and probe sequences that could be used for the development of RT-qPCR tests for detecting the SARS-CoV-2 Omicron VoC sublineages. As the number of SARS-CoV-2 Omicron VoC sequences is increasing rapidly, our analysis on a larger dataset could reveal more mutations and amplicon clusters and could provide more insights on the specificity of RT-qPCR assays, particularly for SARS-CoV-2 Omicron BA.2 sublineage, as well as the newly identified SARS-CoV-2 Omicron BA.3 sublineage. The effect of mismatches in the primers and probe sets revealed in this study on the sensitivity of RT-qPCR assays could be further investigated in the wet laboratory for preparation of more specific diagnostics for SARS-CoV-2 Omicron VoC detection. Lastly, given the number of unresolved potential issues with COVID-19 diagnosis testing with respect to Omicron VoC, symptomatic patients, vulnerable patients, or those with high-risk contact with infected patients, but testing negative, should be confirmed true negative by a second assay (*i.e.* alternative RT-qPCR assay or lateral flow assay).

Declarations

Conflicts of Interests: None

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Figures

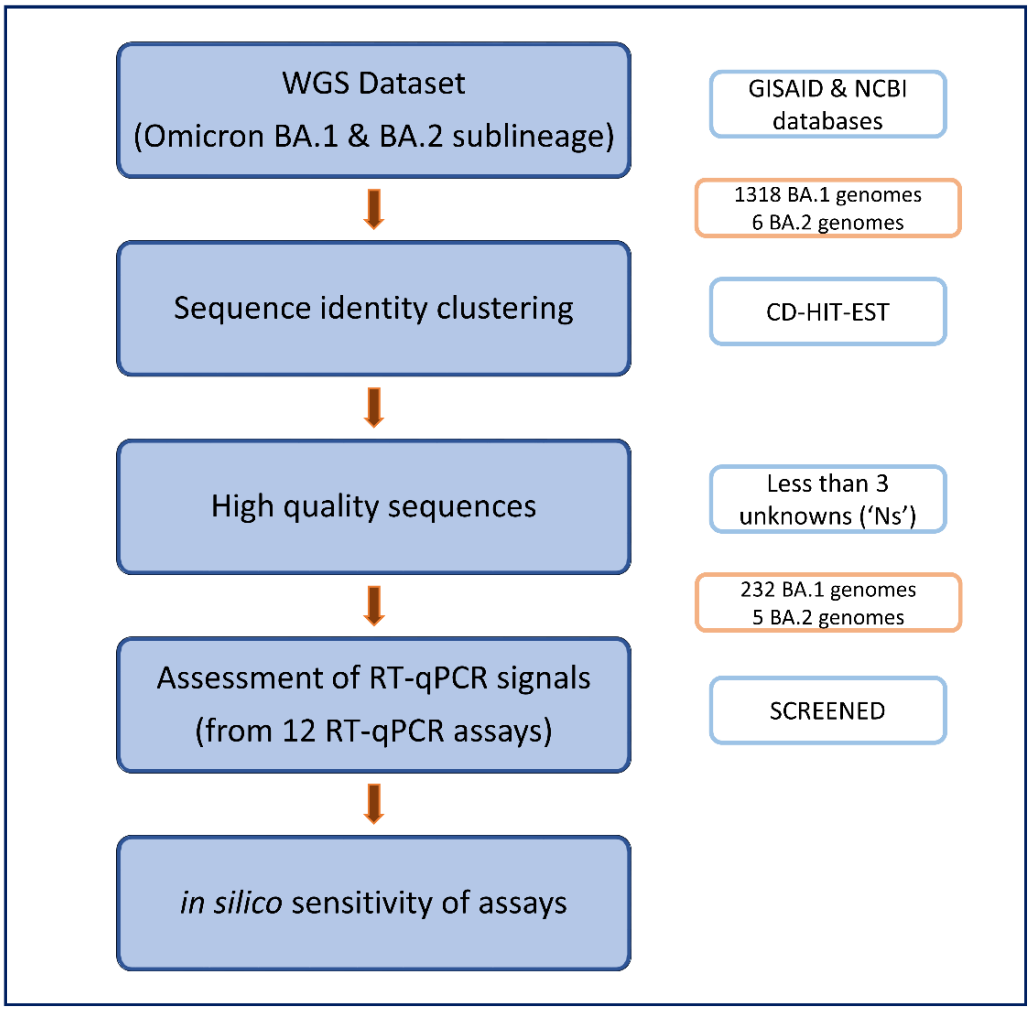

Figure 1

Overview of study methodology.

WGS – Whole Genome Sequencing; RT-qPCR - reverse transcription polymerase chain reaction; GISAID - Global Initiative on Sharing Avian Influenza Data (GISAID); NCBI - National Center for Biotechnology Information

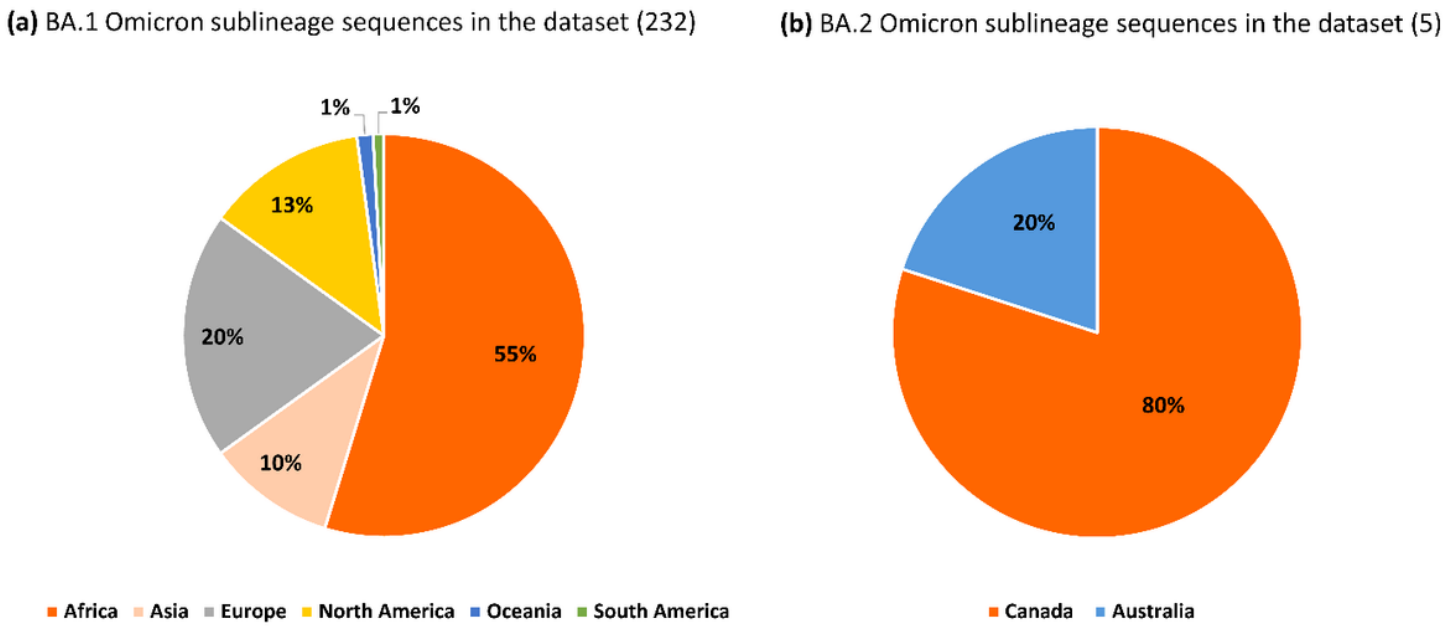

Figure 2

Geographical distribution of the SARS-CoV-2 Omicron VoC sequences analyzed in the study.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryfileS1.tsv
- SupplementaryfileS2.tsv
- SupplementaryfileS3.txt