TRENDS IN SCIENCES 2025; 22(9): 10253
https://doi.org/10.48048/tis.2025.10253
RESEARCH ARTICLE

Evaluation of SARS-CoV-2 Detection Efficiency by qRT-PCR Assays, Mainly on New Variants in East Java

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Received: 25 March 2025, Revised: 12 April 2025, Accepted: 19 April 2025, Published: 10 July 2025

Abstract

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the novel coronavirus, SARS-CoV-2, which led to a global pandemic. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has undergone mutations, leading to the emergence of new variants worldwide, which may affect the accuracy of the diagnostic methods. This study aimed to identify the distribution of SARS-CoV-2 variants in East Java from 2021 to 2024 and analyze the mutation positions in the primer/probe targets of quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). This study assessed the mismatches in WHO-recommended and other kits binding regions qRT-PCR assays against SARS-CoV-2 genome sequences from East Java through in-silico bioinformatics analysis. The results indicate that from 2021 to 2024, the distribution of SARS-CoV-2 variants in East Java has changed over time, including B.1.466.2, Alpha, Beta, Delta, and Omicron, along with various Omicron lineages. Primer and probe sequences from Sansure, Liferiver, US-CDC, EasyDiagnosis, and Pasteur displayed accurate matches (> 90%) with the SARS-CoV-2 sequences from East Java. On the other hand, many Omicron subvariants in East Java had high mismatches with the primer sequences from Charité-*RdRp* and CN-CDC-*N*. These findings highlight the importance of continuous surveillance of circulating variants and their mutations to ensure the effectiveness of diagnostics and response actions for emerging and re-emerging variants in the future.

Keywords: COVID-19, In silico, Molecular diagnostic, Mutations, Primer and probe mismatches, qRT-PCR, SARS-CoV-2, Variants

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified in December 2019 in Wuhan, China, and rapidly spread worldwide, with over 777 million cases reported globally by the end of December 2024. The World Health Organization (WHO) reported 81,716 new cases in 28 days up to January 26, 2025 [1]. This indicates that COVID-19 cases were still being detected and circulated. Recent studies have stated that the global threat of COVID-19 is not over, as the virus continues to mutate [2]. In Indonesia, COVID-19 cases exceeded 6.8 million by the end of December 2024, with East Java Province consistently ranking among the top 4 provinces with the highest number of cases [1].

Since its identification, SARS-CoV-2 has continually mutated, leading to many new variants. This

virus has an extraordinarily high mutational rate, and its mutational profiles are likely to change in the future [3]. The WHO has classified these variants into 3 categories based on their potential impact, including their ability to be detected by laboratory tests: variants of concern (VoC), variants of interest (VoI), and variants under monitoring (VuM) [4]. The reverse transcription polymerase chain reaction is the gold standard test for detecting SARS-CoV-2. This test is mandatory for confirming the diagnosis of COVID-19 [5]. The target genes commonly used in qRT-PCR detection kits are the *N*, *E*, or *ORF1ab*/*RdRp* genes. Some kits recommended by the WHO, such as US-CDC, CN-CDC, Pasteur, and Charité [6], are among the most widely used methods for clinical testing in various countries [7], including Indonesia. Additionally, several other kits include Daan, Easy Diagnosis, Sansure, and Liveriver [8].

Food and Drug Administration (FDA) emphasizes the importance of ongoing monitoring and evaluating the potential impact of viral mutations on COVID-19 tests, as false negative results may occur with molecular tests to detect SARS-CoV-2, particularly if a mutation occurs in the regions of the virus' genome that the test assessed [9]. Previous studies in Italy, India, and other countries performed genome sequences alignment with various qRT-PCR targets and reported numerous mutations in the target genes commonly used in qRT-PCR assays [10-12]. However, these studies were limited to the variants circulating during that time period.

Research specifically addressing the variants circulating in East Java and aligning qRT-PCR target kit sequences with recently emerging variants has been limited. The accuracy of COVID-19 diagnostic testing is essential for effective patient management and preventing further virus transmission. This study aimed to identify the distribution of SARS-CoV-2 variants in East Java from 2021 to 2024 and analyze the mutation positions in the primer/probe targets of qRT-PCR. If there are no mutations in the target regions of the assay, it would increase confidence in the test results. Conversely, the presence of mutations could inform the strategies for reassessing diagnostic assays. This study will provide crucial information for laboratory professionals and policy-makers.

Materials and methods

Study design and data collection

This study used an in-silico approach. The inclusion criteria for this study included whole genomes classified as variants of concern (VoC), variants of interest (VoI), and variants under monitoring (VuM) from East Java during the years 2021 - 2024. Partial or incomplete genomes (<29,000 base pairs) were excluded. The whole genome sequences of viruses isolated from East Java Province; Indonesia were downloaded from the Global Initiative on Sharing All Influenza Data (GISAID) EpiCoV database (https://www. gisaid.org/) [13]. Total of 4.554 sequences were included to identify SARS-CoV-2 variants in East Java. To analyze mutation locations, 439 selected SARS-CoV-2 variant sequences were examined using total sampling and simple random sampling (**Figure 1**). According to Arikunto (1998), if the number of subjects is less than 100, it is better to include all the research samples.

Selection of primers and probes of qRT-PCR

A total of 42 primer-probe sets from 8 kits targeting the *Nucleocapsid* (N = 17), *ORF1ab* (N = 11), *RdRp* (N = 9), and *Envelope* (N = 5) were selected based on publicly available [6,8], including recommendations from WHO and several other kits.

Multiple sequence alignment and mutation analysis

Multiple sequence alignment was performed using Multiple Alignment using Fast Fourier Transform (MAFFT) v.7 tool available online (https://mafft.cbrc.jp/alignment/server/). Multiple alignments were conducted with the reference sequence (NC_045512.2), the SARS-CoV-2 variant sequences, and the target primer/probe of qRT-PCR sequences. Matches/mismatches between the SARS-CoV-2 variant sequences and the primer/probe sequences were analyzed, including the type and location of the mutations.

Ethics approval

This study has been approved by the Health Research Ethics Committee with approval number 77/EC/KEPK/FKUA/2024.

Results and discussion

SARS-CoV-2 variants in East Java Province, Indonesia

Based on the collection of SARS-CoV-2 data in the GISAID, it was found that the circulation of SARS-CoV-2 variants in East Java, which are classified as VoC, VoI, and VuM according to the WHO classification began in 2021.

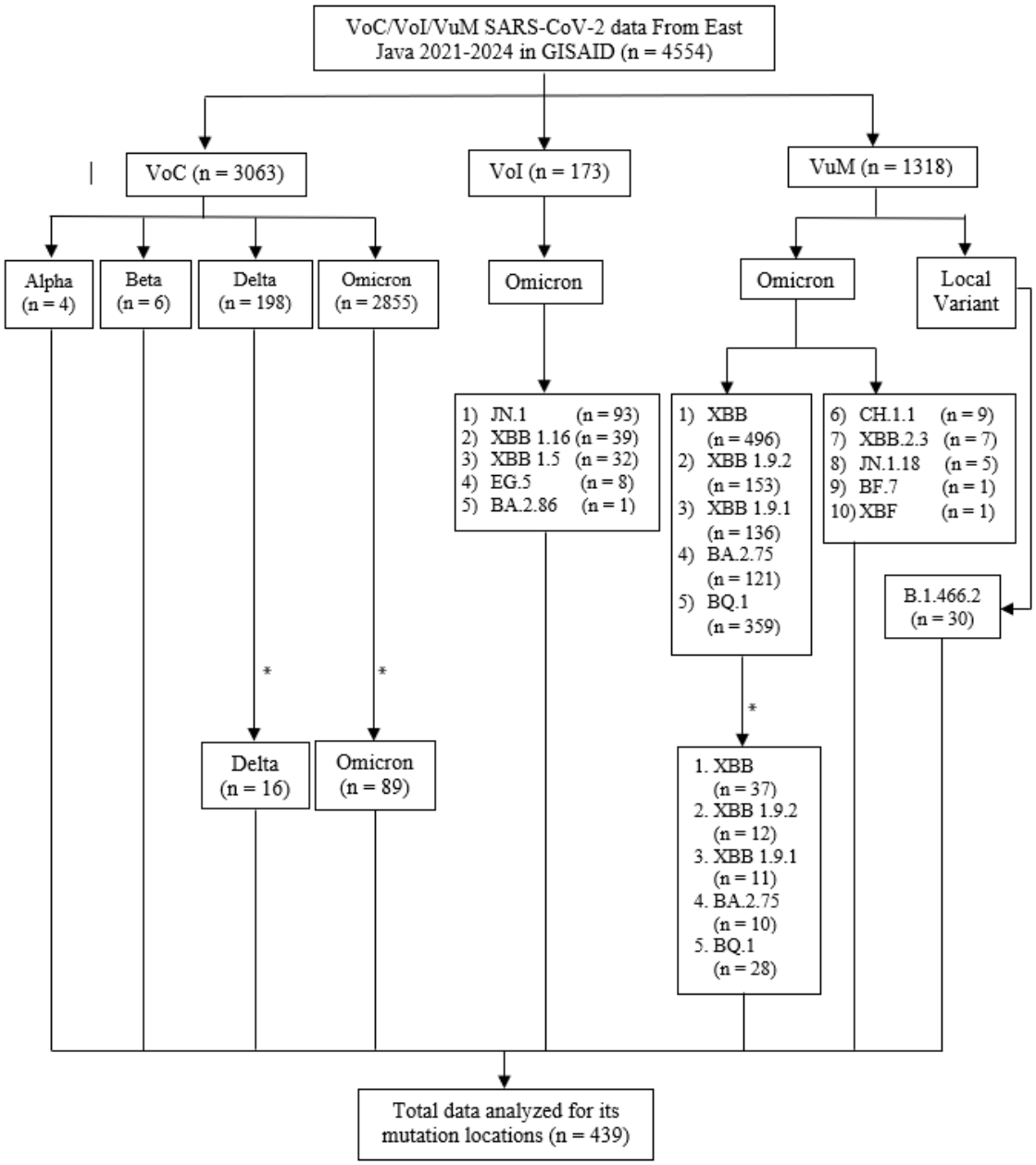

Figure 1 A flowchart of research data collection. The asterisk (*) indicates the selected variant/subvariant through simple random sampling, based on the minimum calculated using the sample size formula.

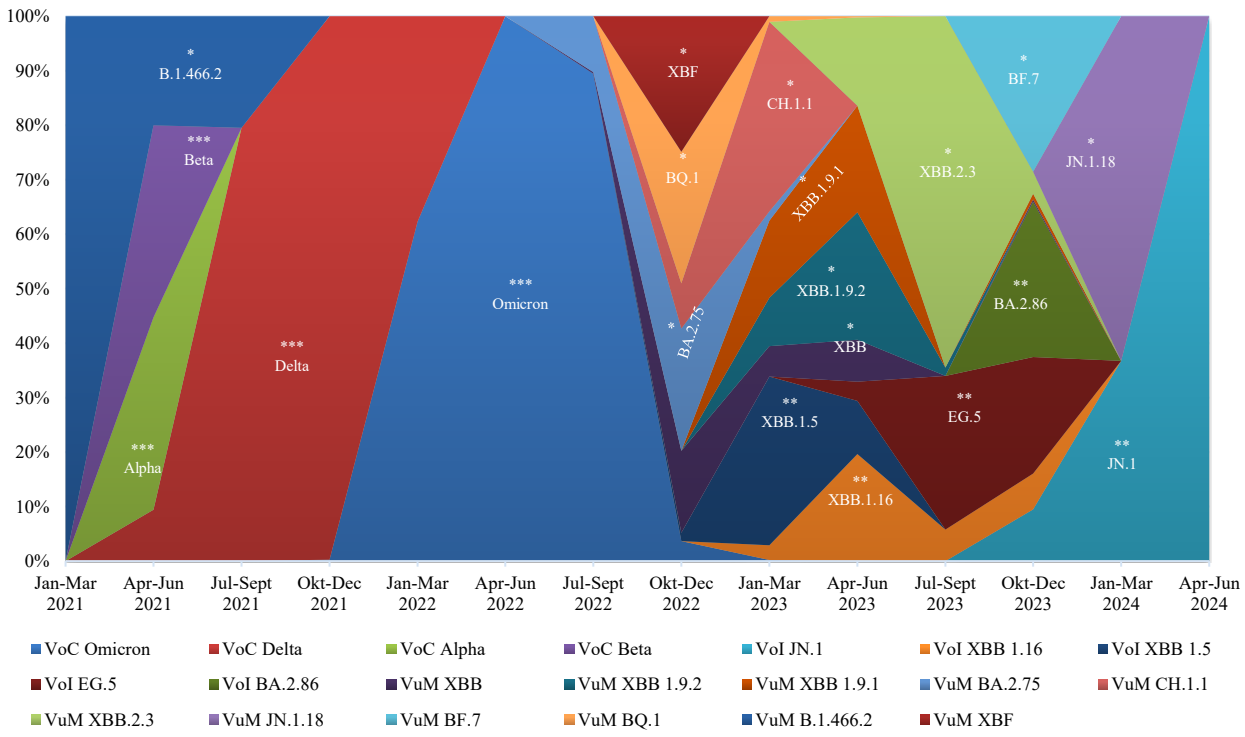

Figure 2 The distribution of SARS-CoV-2 variants in East Java every 3 months, from 2021 to June 2024. Each color represents a specific variant and the period during which it circulated. The asterisk (***) indicates VoC, (**) indicates VoI, and (*) indicates VuM. The lineages listed from the end of 2022 to 2024 are all from the Omicron lineage.

As shown in **Figure 2**, the distribution of circulating SARS-CoV-2 variants in East Java province has changed over time. Initially, the B.1.466.2 variant was predominant, consistent with previous studies indicating that this variant was the primary one during Indonesia's first wave of COVID-19 [14]. World Health Organization (WHO) has announced that the Greek letters will only be assigned to VoC [4].

Variants of Concern were first identified in East Java in April 2021, starting with the Alpha variant, followed by the emergence of the Beta and Delta variants in May 2021, and the Omicron variant in December 2021. The results indicated that the Delta variant was the dominant in East Java during 2021 (80.91%; 178/220). Reports of variant distribution show that the circulation of the Alpha and Beta variants declined in the middle of 2021, while the Delta variant rose to prominence [15-18].

The prevalence of the Delta variant began to decline in 2022. In contrast, the Omicron variant showed a significant increase in 2022 (78.15%; 2836/3629) and became the most dominant in East Java, although its prevalence decreased in the middle of 2023. Despite this decline, new sub-lineages were still emerging. This finding aligns with other studies that also indicate a shift to Omicron as the globally dominant variant, with continuous mutations leading to the emergence of several subvariants or lineages [19-21].

By the end of 2023, a new Omicron subvariant, VoI JN.1 had emerged. The circulation of this subvariant increased in 2024, with a total percentage of 94.03% (63/67) (**Figure 2**). This variant has not only made an impact in Indonesia but has also been prominently reported in Europe and the United States, where its prevalence has seen a sharp increase [22].

The analysis match/mismatch between SARS-CoV-2 variant sequences and the target primer/probe of qRT-PCR

Table 1 Summary of primer and probe mismatches in qRT-PCR assays for VoC.

qRT-PCR Assays	Gene	Sequences (5' – 3')	Primer/Probe	Mutation Location	SARS-CoV-2 Variants			
					Alpha	Beta	Delta	Omicron
US-CDC, USA	*N1*	GACCCCAAAATCAGCGAAAT	F	3' end				
		TCTGGTTACTGCCAGTTGAATCTG	R	3' end				
		ACCCCGCATTACGTTTGGTGGACC	P	middle				
	N2	TTACAAACATTGGCCGCAAA	F	3' end				
		GCGCGACATTCCGAAGAA	R	3' end			C29218T	
		ACAATTTGCCCCCAGCGCTTCAG	P	middle				G29195T
CN-CDC, China	*N*	GGGGAACTTCTCCTGCTAGAAT	F	3' end				
		CAGACATTTTGCTCTCAAGCTG	R	3' end				A28959G
		TTGCTGCTGCTTGACAGATT	P	middle				
	ORF1ab	CCCTGTGGGTTTTACACTTAA	F	3' end				
		ACGATTGTGCATCAGCTGA	R	3' end				
		ACCGTCTGCGGTATGTGGAAAGGTTATGG	P	middle				
Pasteur, Paris	*RdRp*_IP2	ATGAGCTTAGTCCTGTTG	F	3' end				
		CTCCCTTTGTTGTGTTGT	R	3' end				
		AGATGTCTTGTGCTGCCGGTA	P	middle				
	*RdRp*_IP4	GGTAACTGGTATGATTTCG	F	3' end				
		CTGGTCAAGGTTAATATAGG	R	3' end				
		TCATACAAACCACGCCAGG	P	middle				
Charite, German	*RdRp*	GTGAAATGGTCATGTGTGGCGG	F	3' end			G15451A	
		CAAATGTTAAAAACACTATTAGCATA	R	3' end				
		CAGGTGGAACCTCATCAGGAGATGC	P	middle				A15486G
	E	ACAGGTACGTTAATAGTTAATAGCGT	F	3' end				
		ATATTGCAGCAGTACGCACACA	R	3' end				
		ACACTAGCCATCCTTACTGCGCTTCG	P	middle				
Daan, China	*N*	TGGAAGTCACACCTTCGGGA	F	3' end				A29257T
		GACAAAGATCCAAATTTCAA	R	3' end		C29296T		
	ORF1ab	TGATAAAGGAGTTGCACCAG	F	3' end				
		ATTCAGATCTTAATGACTTT	R	3' end				
Easydiagnosis, China	*N*	TGATTACAAACATTGGCCGC	F	3' end				
		CGGAATGTCGCGCATTGGCA	R	3' end			C29218T	
	ORF1ab	GGTATGTGGAAAGGTTATGG	F	3' end				
		GTCAGCTGATGCACAATCGT	R	3' end				
Sansure, China	*N*	AAGCTGGACTTCCCTATGGT	F	3' end				
		GAATACACCAAAAGATCACA	R	3' end				
	ORF1ab	TTATTGTAACAGCTTTAAGG	F	3' end				
		GATGTCTTGTGCTGCCGGTA	R	3' end				
Liferiver, China	*N*	TTACAAACATTGGCCGCAAA	F	3' end				
		GTTCTTCGGAATGTCGCGCA	R	3' end				
	ORF1ab	TGTGTCAACCTATACTGTTA	F	3' end				
		CATCAACTTTTAACGTACCA	R	3' end				
	E	ACAGGTACGTTAATAGTTAA	F	3' end				
		TGTGCGTACTGCTGCAATAT	R	3' end				

Abbreviations: F = forward primer; R = reverse primer; P = probe; *N* = nucleocapsid; *RdRp* = RNA-dependent RNA polymerase; *ORF1ab* = open reading frame 1ab.

Table 2 Summary of primer and probe mismatches in qRT-PCR assays for VoI.

qRT-PCR Assays	Gene	Sequences (5' – 3')	Prime Prob	Mutatio Locatio	SARS-CoV-2 Variants				
					Omicron				
					JN.1	XBB.1.1	XBB.1.5	EG.5	BA.2.86
US-CDC, USA	*N1*	GACCCCAAAATCAGCGAAAT	F	3' end					
		TCTGGTTACTGCCAGTTGAATCTG	R	3' end					
		ACCCCGCATTACGTTTGGTGGACC	P	middle					
	N2	TTACAAACATTGGCCGCAAA	F	3' end				G29179,	
		GCGCGACATTCCGAAGAA	R	3' end	C29218′				
		ACAATTTGCCCCCAGCGCTTCAG	P	middle		A29201(			
CN-CDC, China	*N*	GGGGAACTTCTCCTGCTAGAAT	F	3' end	T28902(				
		CAGACATTTTGCTCTCAAGCTG	R	3' end	C28958,	G28960′			C28958,
		TTGCTGCTGCTTGACAGATT	P	middle	C28948′		C28948′		
	ORF1ab	CCCTGTGGGTTTTACACTTAA	F	3' end					
		ACGATTGTGCATCAGCTGA	R	3' end					
		ACCGTCTGCGGTATGTGGAAAGGTTATGG	P	middle					
Pasteur, Paris	*RdRp*_IP2	ATGAGCTTAGTCCTGTTG	F	3' end					
		CTCCCTTTGTTGTGTTGT	R	3' end	C12781′				
		AGATGTCTTGTGCTGCCGGTA	P	middle		T12730,			
	*RdRp*_IP4	GGTAACTGGTATGATTTCG	F	3' end					
		CTGGTCAAGGTTAATATAGG	R	3' end	A14170(				
		TCATACAAACCACGCCAGG	P	middle					
Charite, German	*RdRp*	GTGAAATGGTCATGTGTGGCGG	F	3' end		G15451,	G15451,	G15451,	
		CAAATGTTAAAAACACTATTAGCATA	R	3' end					
		CAGGTGGAACCTCATCAGGAGATGC	P	middle					
	E	ACAGGTACGTTAATAGTTAATAGCGT	F	3' end					
		ATATTGCAGCAGTACGCACACA	R	3' end					
		ACACTAGCCATCCTTACTGCGCTTCG	P	middle					
Daan, China	*N*	TGGAAGTCACACCTTCGGGA	F	3' end					
		GACAAAGATCCAAATTTCAA	R	3' end	C29296′				
	ORF1ab	TGATAAAGGAGTTGCACCAG	F	3' end					
		ATTCAGATCTTAATGACTTT	R	3' end					
Easydiagnosis, China	*N*	TGATTACAAACATTGGCCGC	F	3' end				G29179,	
		CGGAATGTCGCGCATTGGCA	R	3' end	C29218′				
	ORF1ab	GGTATGTGGAAAGGTTATGG	F	3' end					
		GTCAGCTGATGCACAATCGT	R	3' end					
Sansure, China	*N*	AAGCTGGACTTCCCTATGGT	F	3' end					
		GAATACACCAAAAGATCACA	R	3' end					
	ORF1ab	TTATTGTAACAGCTTTAAGG	F	3' end					
		GATGTCTTGTGCTGCCGGTA	R	3' end					
Liferiver, China	*N*	TTACAAACATTGGCCGCAAA	F	3' end				G29179,	
		GTTCTTCGGAATGTCGCGCA	R	3' end					
	ORF1ab	TGTGTCAACCTATACTGTTA	F	3' end					
		CATCAACTTTTAACGTACCA	R	3' end					
	E	ACAGGTACGTTAATAGTTAA	F	3' end					
		TGTGCGTACTGCTGCAATAT	R	3' end					

Abbreviations: F = forward primer; R = reverse primer; P = probe; *N* = nucleocapsid; *RdRp* = RNA-dependent RNA polymerase; *ORF1ab* = open reading frame 1ab.

Note: The listed lineages under "Omicron" are subvariants of the Omicron variant categorized as Variants of Interest (VoI).

Table 3 Summary of primer and probe mismatches in qRT-PCR assays for VuM.

qRT-PCR Assays	Gene	Sequences (5' – 3')	Primer/ Probe	Mutation Location	SARS-CoV-2 Variants							
					Omicron							Local
					XBB	BA.2.75	CH.1.1	JN.1.18	BF.7	BQ.1	XBF	B.1.466.2
US-CDC, USA	*N1*	GACCCCAAAATCAGCGAAAT	F	3' end								
		TCTGGTTACTGCCAGTTGAATCTG	R	3' end								
		ACCCCGCATTACGTTTGGTGGACC	P	middle								
	N2	TTACAAACATTGGCCGCAAA	F	3' end								
		GCGCGACATTCCGAAGAA	R	3' end								
		ACAATTTGCCCCCAGCGCTTCAG	P	middle								
CN-CDC, China	*N*	GGGGAACTTCTCCTGCTAGAAT	F	3' end								
		CAGACATTTTGCTCTCAAGCTG	R	3' end				C28958A				
		TTGCTGCTGCTTGACAGATT	P	middle								
	ORF1ab	CCCTGTGGGTTTTACACTTAA	F	3' end								
		ACGATTGTGCATCAGCTGA	R	3' end								
		ACCGTCTGCGGTATGTGGAAAGGTTATGG	P	middle								
Pasteur, Paris	*RdRp*_IP2	ATGAGCTTAGTCCTGTTG	F	3' end								
		CTCCCTTTGTTGTGTTGT	R	3' end								
		AGATGTCTTGTGCTGCCGGTA	P	middle								
	*RdRp*_IP4	GGTAACTGGTATGATTTCG	F	3' end								
		CTGGTCAAGGTTAATATAGG	R	3' end								
		TCATACAAACCACGCCAGG	P	middle								
Charite, German	*RdRp*	GTGAAATGGTCATGTGTGGCGG	F	3' end	G15451A * ** ***	G15451A	G15451A				G15451A	
		CAAATGTTAAAAACACTATTAGCATA	R	3' end								
		CAGGTGGAACCTCATCAGGAGATGC	P	middle								
	E	ACAGGTACGTTAATAGTTAATAGCGT	F	3' end								
		ATATTGCAGCAGTACGCACACA	R	3' end								
		ACACTAGCCATCCTTACTGCGCTTCG	P	middle								
Daan, China	*N*	TGGAAGTCACACCTTCGGGA	F	3' end	C29253T ***							
		GACAAAGATCCAAATTTCAA	R	3' end								
	ORF1ab	TGATAAAGGAGTTGCACCAG	F	3' end								
		ATTCAGATCTTAATGACTTT	R	3' end								
Easydiagnosis, China	*N*	TGATTACAAACATTGGCCGC	F	3' end								
		CGGAATGTCGCGCATTGGCA	R	3' end								
	ORF1ab	GGTATGTGGAAAGGTTATGG	F	3' end								
		GTCAGCTGATGCACAATCGT	R	3' end								
Sansure, China	*N*	AAGCTGGACTTCCCTATGGT	F	3' end								
		GAATACACCAAAAGATCACA	R	3' end								
	ORF1ab	TTATTGTAACAGCTTTAAGG	F	3' end								
		GATGTCTTGTGCTGCCGGTA	R	3' end								
Liferiver, China	*N*	TTACAAACATTGGCCGCAAA	F	3' end								
		GTTCTTCGGAATGTCGCGCA	R	3' end								
	ORF1ab	TGTGTCAACCTATACTGTTA	F	3' end								
		CATCAACTTTTAACGTACCA	R	3' end								
	E	ACAGGTACGTTAATAGTTAA	F	3' end								
		TGTGCGTACTGCTGCAATAT	R	3' end								

Abbreviations: F = forward primer; R = reverse primer; P = probe; *N* = nucleocapsid; *RdRp* = RNA-dependent RNA polymerase; *ORF1ab* = open reading frame 1ab.

Note: The lineages under "Omicron" are subvariants of the Omicron variant, and those under "Local" include an Indonesian-origin variant. All listed lineages are classified as Variants under Monitoring (VuM). The asterisk (*) indicates XBB.1.9.1, the double asterisk (**) indicates XBB.1.9.2, and the triple asterisk (***) indicates XBB.2.3.

Table 4 The percentage of match between SARS-CoV-2 variants (VoC/VoI/VuM) and the primer/probe of qRT-PCR assays.

	US-CDC, USA	CN-CDC, China	Pasteur, Paris	Charite, German	Daan, China	EasyDiagnosis, China	Sansure, China	Liferiver, China
Alpha	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
Beta	100 %	100 %	100 %	100 %	50 %	100 %	100 %	100 %
Delta	100 %	100 %	100 %	93.75 %	100 %	93.75 %	100 %	100 %
Omicron	97.58 %	75.30 %	90.07 %	59.56 %	99.27 %	99.52 %	100 %	99.76 %

Note: The percentage represents the proportion of genome sequences that match with the primer/probe, calculated as: (number of matched sequences / total sequences of the corresponding variant)×100%. The label "Omicron" in this table refers to the Variant of Concern (VoC), Variants of Interest (VoI), and Variants under Monitoring (VuM).

The results indicated that out of 439 samples analyzed, 114 contained mutations at the 3' end of the primer or in the middle of the probe (**Tables 1** to **3**). As illustrated in Table 1, mutations leading to mismatches with primer/probe targets began to emerge in the Beta, Delta and most prominently in the Omicron. **Table 2** shows that within the Omicron lineage, which is classified as a VoI, the primer/probe targets of the CN-CDC-*N* displayed the highest number of mismatch variations caused by SARS-CoV-2 mutations. Notably, **Table 3** highlights the presence of the G15451A mutation, which leads to mismatches among various Omicron lineages classified as VuM. This mutation is also present in several other variants, as illustrated in **Table 2**.

Table 4 shows that the primer/probe targets recommended by WHO (including US-CDC, CN-CDC, and Pasteur) demonstrated a 100% match with the Alpha, Beta and Delta variants. Among all the WHO recommendations, the Charité had the lowest match for Omicron variants and subvariants from East Java, with only 59.56% (246 out of 413). This was because of the presence of mutations, one of the most common being the G15451A mutation at the 3' end of the Charité-*RdRp* primer. This mismatch was observed in all isolates of EG.5; XBB.1.16; XBB.1.5; XBB; XBB.1.9.1; XBB.1.9.2; BA.2.75; CH.1.1; XBB.2.3; and XBF (**Tables 2** and **3**).

The CN-CDC matched with only 311 sequences (75.30%) of the Omicron variants/subvariants. The C28958A mutation in the *N* gene partially caused the reduced match found in all BA.2.86, JN.1, and JN.1.18 isolates in East Java. In addition, testing with other kits (Daan, EasyDiagnosis, Sansure, and Liveriver) evaluated that the Sansure kit from China showed a 100% match with all sequences from the Alpha, Beta, Delta, Omicron variants and their subvariants from East Java (439/439). Meanwhile, the Daan kit showed only a 50% match with the Beta variant. The mismatch was due to a mutation from C29296T in the N gene, observed in 3 out of 6 Beta variant isolates from East Java.

Previous studies have indicated that the *E* and *M* genes of SARS-CoV-2 are among the genomic regions that are relatively conserved [23]. The *RdRp* gene is also recognized as a conserved gene [24]. A gene is considered conserved when it undergoes minimal changes or mutations. In contrast, the genes that most frequently undergo mutations are the *S* and *N* genes [23]. Other studies indicated that the N gene has the highest number of mutations among primers and probes commonly used to diagnose COVID-19 [25,26]. In this study, we also identified mutations in the *RdRp* gene among various SARS-CoV-2 variants (**Tables 1** to **3**). While the *RdRp* gene is typically conserved, our findings reveal mutations within this gene that may have implications for diagnostic detection.

Although mutations in conserved genes are less common, previous research has noted that they can still occur. These mutations may arise from several factors, one of which is replication errors. Viruses like SARS-CoV-2 utilize enzymes to copy their genetic material, but these enzymes are not always entirely accurate during replication. This inaccuracy can lead to errors or mutations in the genetic sequence. Additionally, mutations may develop as an adaptive response of the

virus to its environment, allowing it to survive and replicate under ever-changing conditions [24].

Previous studies indicate that mismatches in the 3' region of a primer (defined as the last 5 nucleotides of the 3' end region) have significantly larger effects on primer efficiency [27,28]. Even a single mismatch at the 3' end can lead to substantial reductions in efficiency [11,28-30]. In contrast, mutations in the middle of the probe are more likely to reduce detection efficiency than near-end mutations [28,31]. Another previous study also stated that mismatches in probe binding sites can result in false-negative results during qRT-PCR assays, these mismatches are located in the middle of the probe [32]. Mismatches in the primer and probe regions of SARS-CoV-2 can hinder the binding of the DNA template during the initial stages of the PCR reaction. This issue negatively affects the stability of the primer-template duplex and reduces the efficiency of the polymerase [10,27,28].

Mismatches between primers and target DNA lead to ineffective annealing [33]. The 3' end of the primer is crucial for initiating cDNA synthesis as it serves as the starting point for DNA polymerase to add nucleotides. During the extension phase, DNA polymerase begins adding nucleotides from the 3' end of the primer, a process that requires complete annealing between the primer and the template. This binding at the 3’ end is critical for successful amplification. Furthermore, mutations can compromise the reliability of RT-PCR testing, leading to false-negative outcomes and decreased fluorescence signals. Mismatches in the primer significantly lower fluorescence, while mismatches in the probe region tend to produce the lowest fluorescence signals [34,35]. The mismatch significantly compromises the efficiency of PCR amplification, threatening the sensitivity of qRT-PCR tests [36].

There is one interesting finding from this study that differs from previous studies, particularly regarding the G15451A mutation. Previous studies mentioned that Omicron did not carry the G15451A mutation [28]. However, our study found that the Omicron variant/subvariant circulating in East Java does carry the G15451A mutation. This mutation contributes to a high level of mismatch between the Charité primer sequence and the Omicron variant/subvariant (**Tables 1 - 3**).

The difference between previous studies and this study is related to the timing, which is closely linked to the variants emerging during those periods. Earlier studies focused on aligned variant sequences were limited to those circulating at the time, whereas this study analyzes a broader range of subvariants. This is supported by a study that mentions that SARS-CoV-2 tends to mutate, leading to the emergence of new mutations [37].

The findings of this study are also supported by laboratory studies that demonstrated the G29140U mutation at nucleotide 16 (which is five nucleotides from the 3' end) in the primer, which failed to detect the *N* gene in a qRT-PCR test [38]. Additionally, direct laboratory tests performed in other studies also indicated that mutations at the 3' end of the CN-CDC-*N* primer affected the test’s sensitivity. These mutations potentially caused shifts in the Ct value, leading to difficulties in detecting the virus and the potential for false-negative results [39,40].

This is also supported by earlier studies, which showed that false-negative results in the test were due to a mutation in the first five nucleotides from the 3' end of the primer [41]. Other studies showed that a single nucleotide mutation (C26340T) was associated with failure in detecting the *E* gene using Cobas RT-PCR. Additionally, a different single nucleotide mutation (C29200T) has been reported to hinder the detection of the *N* gene target in Xpert Xpress testing, while another mutation (G29196T) interfered with *N* gene detection in the Cepheid Xpert Xpress test [11,42-44].

The mutation may lead to a decrease in hydrogen bonds, possibly destabilizing primer and probe hybridization to their target sequence, which could decrease assay sensitivity [45,46]. Mutations located at the center of the probe target have been reported to cause greater destabilization compared to those at terminal regions, this is possibly due to more complex hydrogen bonding interactions that typically form in the central region of the probe [31,47]. This suggests that hydrogen bonding interaction may also be an important factor in the affinity of qRT-PCR primers/probes for target sequence.

Moreover, previous studies have demonstrated that hydrogen bonding has contributed significantly to crystal packing, supramolecular stability, and molecular recognition [48–51]. Molecular recognition is based on

molecular interactions, which are crucial because a molecule's biological activity is determined by its interaction with a specific target [52]. A relevant example is spiropyrrolidine-based compounds, which have been described to exhibit notable biological activity through selective interactions with protein binding sites [53]. Another previous study stated that one of the main causes of interactions in biological systems is hydrogen bond formation [54].

The limitation of this study is the restricted access to the primer and probe sequences from other widely used commercial qRT-PCR assays. If additional primer/probe sequences become publicly available in the future, further research can be conducted to continuously evaluate the alignment between the circulating variant sequences and the primer/probe targets of existing diagnostic tests. Additionally, the number of Alpha and Beta variant samples analyzed in this study is limited. For future research, a larger sample size would provide more representative results.

Conclusions

In conclusion, the mutations in SARS-CoV-2 are continually leading to the emergence of new variants or subvariants, resulting in ongoing changes in the distribution. A notable shift was observed from the dominance of the Delta variant in 2021 to the rise of Omicron and its subvariants from 2022 to 2024. These shifts may impact the performance of qRT-PCR assays, especially when mutations occur at critical primer or probe binding sites. Overall, the primer/probe from US-CDC, Pasteur, EasyDiagnosis, Sansure, Liveriver, and Daan kits showed high matching accuracy with almost all SARS-CoV-2 variants in East Java. However, the highest mismatches were observed with the Charité and CN-CDC for the Omicron variants/subvariants. Importantly, the G15451A mutation in the *RdRp* gene, previously unreported in Omicron variant, was detected across many Omicron subvariants in East Java. This study underscores the need to regularly update these assays based on the genetic evolution of SARS-CoV-2 and serves as a valuable guide for managing emerging and re-emerging variants in the future.

Acknowledgements

The authors would like to express our gratitude to all data contributors, including the Authors and their originating laboratories responsible for obtaining the specimens, and their submitting laboratories for generating the genetic sequences and sharing via the GISAID Initiative, on which this research is based.

Declaration of Generative AI in Scientific Writing

The authors declare that no generative AI tools were used in the writing or preparation of this manuscript.

CRediT author statement

ERVL: Investigation, Methodology, Data curation, Writing – original draft, Formal analysis, Visualization. J: Conceptualization, Supervision, Validation, Writing – review & editing. NLAM: Software, Writing – review & editing. AAF: Writing – review & editing. MIL: Supervision, Conceptualization, Validation, Writing – review & editing, Corresponding author.

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