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In Silico Primer Design for Accurate Detection of SARS-CoV-2 Delta Variants

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Abstract

Designing primer tools targeting conserved regions of the SARS-CoV-2 (Severe acute respiratory syndrome coronavirus-2) - Delta variants genome is an ideal approach to avoid false-negative results and reduce the requirement of standardization for various PCR protocols. For this work, we designed universal primers and probes targeting the conserved regions identified through multiple sequence alignment of 183 of SARS-CoV-2- delta variant genomes retrieved from Global Initiative on Sharing All Influenza Data (GISAID). We obtained 27 conserved regions and selected 10 systems (forward primer + reverse primer +probe) suitable for the qRT-PCR. In silico tools were used to design primers designed to anneal to the conserved regions of all the delta variants. We also conducted metadata analysis through the data available from GISAID. We found amino acid mutations most common among the delta variants. The metadata analysis gave us an overview of the phylogenetic lineage, sub-lineage, and common mutations in the delta variants. Through this study, we designed universal primers and probes that can detect conserved sites of all delta variants and can be used for evolutionary study and development of new drugs.

Keywords: COVID-19, In Silico Primers and Probes, qRT-PCR, SARS-COV-2 Delta Variant

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a novel coronavirus, was first identified in Wuhan, China late December 2019 [1]. SARS-CoV-2 is a highly infectious pathogen leading to unusual mortality; thus, the coronavirus disease 2019 (COVID-19) was then declared a pandemic by WHO in early March 2020 [2]. To date (September 7, 2021, 02:36 pm GMT+6), global confirmed COVID-19 cases are 2,20,904,838, out of which 4,570,946 were confirmed deaths reported

(https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Daily new COVID-19 cases were 404,360 (September 6, 2021), and Daily deaths were 6,704 (worldometer.info/coronavirus). In Asia, the total cases are 71,704,512 and counting (as of September 7, 2021). COVID-19 cases are increasing at an alarming rate; hence, it is fundamental to develop assays for timely and accurate detection of the virus.

Most diagnostic tools are available to detect SARS-CoV-2 based on the reverse transcription-polymerase chain reaction (RT-PCR) [3]. RT-PCR is the standard method for identifying acute respiratory infection, as it allows specific and sensitive virus detection from human samples [4]. Despite possible false positives (due to off-target reactivity) and false-negative results (due to lower viral load during the early onset of COVID), RT-PCR is being used widely in COVID diagnosis [5]. Few possible ways to overcome the limitations include testing at the correct time frame of viral replication and designing primers and probes targeting conserved sequences of the genome [3,6]. Until now, ten variants found that arose due to the continuous evolution of the virus (GenBank NC_045512) (Coronavirus Disease (COVID-19) Weekly Epidemiological Update, 2021) [7]. Among them, four are the Variants of Concern (VOC) because of their virulence. WHO labeled these VOCs as the Alpha, Beta, Gamma, and Delta. The Delta variant belongs to Global Initiative on Sharing Avian Influenza Data (GISAID- https://www.epicov.org) clade GK, Pangolin lineage B.1.617.2, and Next strain Clade 21A/S: 478K. Recent studies showed that patients infected with Delta variants have a higher risk of hospitalization. In addition, increased secondary attack rates among Delta cases manifest the severity of this variant. It is imperative to have rapid and specific testing of infections with Delta variants to contain the spread of this high-risk variant [8].

The SARS-CoV-2 proteome consists of four structural proteins including, Spike (S) glycoprotein, Envelope (E) glycoprotein, Nucleocapsid (N) protein, and Membrane (M) glycoprotein [9]. It further consists of 16 non-structural proteins (NSP1-NSP16) and six accessory proteins (NS3, NS6, NS7a, NS7b, NS8, and ORF10) [10]. To date, eleven spike mutations and many more other mutations from the remaining part of the genomes of Delta variants were of concern due to high diversity across different nations throughout the globe. The emergence of the variants due to mutations in the SARS-CoV-2 genome arguably restricts the efficiency of available PCR kits [8]. Mutations in the annealing sites will hinder the attachment of the primers lowering the specificity of the PCR tests and leading to false-negative results [11,12]. In a previous study, Nayer et. al. (2021) [8], highlighted how the primers do anneal to the target sequence due to the apparent mutations. Also, primers worked better against specific variants within a country than global sequences [8]. Since December 2019, the virus has mutated to produce virulent variants, such as Delta, rapid testing using primers that are sensitive and specific for qRT-PCR tests is required for their reliable and confirmatory diagnosis.

In this study, we identified 27 conserved sequences (CS) in the SARS-CoV-2 Delta variants based on multiplesequence alignment of 183 full genome sequences submitted at GISAID from Asian countries and then designedprimers and probes in silico using these targeted regions. Our work aims to prevent false-negative results bdesigning universal primers that will not need standardization for PCR protocols as these primers will target conserved regions. These primers and probes will help identify SARS-CoV-2 Delta variants via early rapid testing and will remove the necessity for costly and time-consuming sequencing of the amplified gene fragments for this purpose.

Material and Methods

SARS-CoV-2 Dataset

We retrieved the high coverage complete (>29kb) genomic sequences of SARS-CoV-2 submitted to the GISAID website (https://www.epicov.org) between May and July 2021. To be more specific, we selected the VOC Delta GK/478K.V1 (B.1.617.2+AY.x) variant first detected in India. We also activated filters "complete ", "high coverage only", "w/Patient status", and "collection date complete" at sequence retrieval in the GISAID database. We found 183 sequences of Delta variants that

led to outbreaks in 11 Asian countries (India, Indonesia, Hong Kong, Kuwait, Cambodia, Singapore, Malaysia, Bangladesh, Taiwan, Nepal, and Vietnam). All whole-genome FASTA format sequences were aligned by using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) software. The multiple sequence alignment (MSA) data of 183 genomes of Delta variants allowed us to identify the highly conserved regions for further analyses. We used the Hong Kong sequence (GISAID Accession code EPI_ISL_2787015) as a reference for all alignments to identify site and region positions. We successfully detected 27 highly similar regions and conserved domains to look for potential primer or probe targets.

Primer and probe design

We used the Open access Tool Primer quest for COVID-19 PCR primer and probe design. We identified 27 conserved regions from 183 sequences submitted from patients of 11 different Asian countries. Running the sequences from these conserved regions in an online tool named Primer quest (https://sg.idtdna.com/pages/tools/primerquest) generated a list of 121 potential qRT-PCR primers and probes based on by default parameters.

Quality control for primers

Primer specificity and sensitivity evaluation are crucial for a successful PCR reaction. For checking the in silico specificity of PCR primers and probes we used MFEprimer (https://mfeprimer3.igenetech.com/spec) Bioinformatics tool. We optimized the binding capacity of Primers (ΔG) based on SYBR green chemistry ranging from −20.55 to −23.25 (Kcal/mole), and TaqMan probes having the binding capacity (ΔG) ranged from −26.35 to −29.49 (kcal/mole). Few other parameters that we considered when choosing the primers were: Primer length= 18-30 bP, Primer GC content (%) =40.91-55.00, and Probe GC content (%) =41.38-50.00, Primer melting temperature (Tm)(°G) = 56.84-59.05, and Probe Tm(°G) = 64.14-65.25. We submitted a total of 121 primers and probes into the MFEprimer tool to check specificities such as size, sequence, binding capacity (ΔG), GC content, and calculating melting temperature (Tm) against the Primer specificity and sensitivity evaluation are crucial for a successful PCR reaction. For checking the in silico specificity of PCR primers and probes, we used MFEprimer (https://mfeprimer3.igenetech.com/spec) Bioinformatics tool. We optimized the binding capacity of Primers (ΔG) based on SYBR green chemistry ranging from −20.55 to −23.25 (Kcal/mole), and TaqMan probes having the binding capacity (ΔG) ranged from −26.35 to −29.49 (kcal/mole). Few other parameters that we considered when choosing the primers were: Primer length= 18-30 bP, Primer GC content (%) =40.91-55.00, and Probe GC content (%) =41.38-50.00, Primer melting temperature (Tm) (°G) = 56.84-59.05, and Probe Tm(°G) = 64.14-65.25. We submitted 121 primers and probes into the MFEprimer tool to check specificities such as size, sequence, binding capacity (ΔG), GC content, and calculating melting temperature (Tm) against the SARS-CoV-2 genomic database. Later, the ten best Primers were selected based on SYBR Green Chemistry and TaqMan methods

PCR suitability test

To evaluate potential primers and probes for the SARS-CoV-2 Delta variant, and to check their efficiency, we used an online tool named (Sequence Manipulation Suite; https://www.bioinformatics.org/sms2/) PCR primer stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). We screened the selected best ten candidates (Forward primers, reverse primers, and probes) based on properties such as self-annealing bases and Hairpin formation bases for PCR suitability tests for the SARS-CoV-2 Delta variant.

Phylogenetic tree construction

To understand the genetic distance and the number of mutations or evolutionary changes among SARS-CoV-2 (Delta variant) genome data from 11 different Asian countries, we calculated and counted the differences among these sequences. Firstly, we made a phylogenetic tree for sequences from each country using multiple sequences alignment tools (Clustal Omega) software (http://www.clustal.org/omega/). We constructed a countrywide tree to choose samples fully compatible with our set parameters. From these data, we selected 31 sequences of 11 Asian countries. Multiple sequences alignment (MSA) and the phylogenetic tree were constructed by MEGA X (Molecular Evolutionary Genetic analysis) software MEGAX (https://www.megasoftware.net / version10.1). Selected sequences were in-line with using the MUSCLE software tool (http://www.drive5.com/muscle/). We set some parameters to build up a Neighbor-joining (NJ) phylogenetic tree. The Tree Topology was assessed using a fast-bootstrapping function with 1000 replicates. The Tamura_Nei parameter was used for calculating the distance and constructing the tree.

Evolutionary relationships of taxa

The evolutionary history was hypothesized using the Neighbor-Joining method. The optimal tree is shown in Figure 1. The analysis involved 31 nucleotide sequences of 11 Asian countries. All unclear sites were extracted for each sequence pair (pairwise deletion option). There were a total of 29950 positions in the final dataset. Evolutionary analyses were directed in MEGA X.

Metadata Analysis

Amino acid Substitutions

We have conducted a thorough analysis of amino acid (AA) substitutions among 183 genomes of SARS-CoV-2 (delta variants) (Figure.2). Mutations were observed and recorded in the N-terminal Domain and RBD (Receptor–binding domain), furin-cleavage site, and S2 region, respectively.

SARS-CoV-2 (delta variant)-Lineage

We used metadata from GISAID (01/05/2021) to (01/07/2021) date frame. Based on characteristics of the splitting out sub-lineage of Delta cases, we also analyzed the Lineage details of 183 samples (Table.2).

Age-specific viral loads in SARS-COV-2 (delta variant) infection

In the SARS-CoV-2 (delta) variant outbreaks of Asian nations (From May to July 2021), we analyzed the transmission patterns by looking at age-specific viral loads among all the clinically infected patients (Figure.3).

Results

RT-PCR primers and probes Analysis and properties of SARS-COV-2 Delta variant

We attempted to design new Primers and probes for the SARS-CoV-2 Delta variants that were highly contagious virus strains in Asian countries and still circulating globally. A total of 121 potential qRT-PCR primers were designed from 27 conserved regions (acquired from 183 SARS-CoV-2 Delta sequences) based on in silico analysis. We evaluated the specificity of these 121 primers and probes and selected the ten best candidate primers (Forward and Reverse) and probes. These primers and probes can be used for successful and quick identification of the specific strains of SARS-CoV-2 Delta (B.1.617.2) variants as revealed by their physicochemical parameters. We also tested the self-complementarity of the selected primers using the PCR suitability test. All the primer sets showed higher specificity against the SARS-CoV-2 (B.1.617.2) virus (Table 1). Furthermore, we checked the efficiency of these primers and probe sets and they passed PCR suitability tests in silico.

Table1: Tabulated qRT-PCR primers and probes synthesized in the study of SARS-COV-2 Delta variants in 11 Asian countries. SN=Serial Number

SN	Primer ID	Sequence (5' → 3')	Size (bp)	GC (%)	T_m (°G)	ΔG (kcal/mol)	Conserved domains (Targets)
1	P1-F	TCCGTGGAGGAGGTCTTATC	20	55.00	58.44	-21.04	Nsp1
	P1-R	ACACATAGGGCTGTTCAAGTT	21	42.86	58.00	-21.21	
	P1-Probe	TGGCACTTGTGGCTTAGTAGAAGTT-GA	27	44.44	64.94	-28.35	
2	P2-F	GAGGCTGTGTGTTCTCTTATGT	22	45.45	58.58	-22.17	Nsp2
	P2-R	CCTTCTCCAACAACACCTGTAT	22	45.45	58.50	-21.99	
	P2-probe	AAGTGTGCCTATTGGGTTCCACGT	24	50.00	65.20	-26.62	
3	P3-F	AAACTCAAACCCGTCCTTGAT	21	42.86	58.28	-21.33	Nsp2
	P3-R	ACAATTTCCCAACCGTCTCTAA	22	40.91	58.15	-21.74	
	P3-Probe	TGGCTTGAAGAGAAGTTTAAG-GAAGGTGT	29	41.38	65.11	-29.49	

4	P4-F	TTCCCATCTGGTAAAGTTGAGG	22	45.45	58.21	-21.76	Nsp5
	P4-R	CAGATCACATGTCTTGGACAGTA	23	43.48	58.45	-22.57	
	P4-probe	ACGGTCTTTGGCTTGATGACGTAGT	25	48.00	65.03	-27.39	
5	P5-F	GCTATGAGGCCCAATTTCACTA	22	45.45	58.70	-22.10	Nsp5
	P5-R	CTAAGTCTGTGCCAGCATGAA	21	47.62	58.80	-21.84	
	P5--probe	AGGGTTCATTCCTTAATGGTTCAT-GTGGT	29	41.38	65.24	-29.45	
6	Env-F	GCACAAGCTGATGAGTACGA	20	50.00	58.50	-21.39	ORF3b and Cov_E
	Env-R	GCAAGAATACCACGAAAGCAAG	22	45.45	59.00	-22.64	
	Env-Probe	TCGTTTCGGAAGAGACAGGTACGT	24	50.00	64.14	-26.35	
7	RDRP-F	GCTGCCGGTACTACACAAA	19	52.63	58.27	-20.72	Nsp8
	RDRP-R	TCGGATAACAGTGCAAGTACAA	22	40.91	58.25	-21.98	
	RDRP-probe	CTGCTTGCACTGATGACAATGCGT	24	50.00	65.19	-27.19	
8	P8-F	ACTGACATAGCCAAGAAACCA	21	42.86	57.72	-21.07	Nsp15-NTD and Nsp11
	P8-R	GAACACCATTACGGGCATTTC	21	47.62	58.35	-21.73	
	P8-probe	CTGAAACGATTTGTGCACCATCACT-GT	27	44.44	65.14	-28.89	
9	P9-F	GGTGTTATCACGCATGATGTTTC	23	43.48	59.16	-23.25	Nsp13-helicase
	P9-R	TTTCTCCAAGCAGGGTTACG	20	50.00	58.31	-21.07	
	P9-probe	ACAGGCCACAAATAGGCGTGGTAA	24	50.00	65.25	-26.68	
10	P10-F	GCACAAGCTTTAAACACGCT	20	45.00	58.36	-21.32	Spike_S1-S2_S2
	P10-R	GTCTGCCTGTGATCAACCTATC	22	50.00	59.05	-22.51	
	P10-Probe	TCACGTCTTGACAAAGTTGAGGCT-GA	26	46.15	65.24	-28.11	

Phylogenetic Analysis of SARS-COV-2 Delta (B.1.617.2) in Asian countries:

We explored the high confidence gene sets indicating evolutionary history and other insights on mutations of the SARS-CoV-2 (B.1.617.2) variant. It was revealed that the distance between each pair of SARS-CoV-2 sequences was closely related. Similarity analyses of SARS-CoV-2 genomes submitted from 11 Asian countries have suggested that most of the genomes are highly homologous (98% -100 %) (Figure.1). The two unique genomes (EPI ISL 2502615 and EPI ISL 2534079) that shared 99% similarity with all the other isolates belonged to Indonesia (Figure 1). These findings suggest that the Asian Delta variant mutations were not unique for the region and are similarly associated with higher pathogenicity of the SARS-CoV-2 Delta variant, as expected (19).

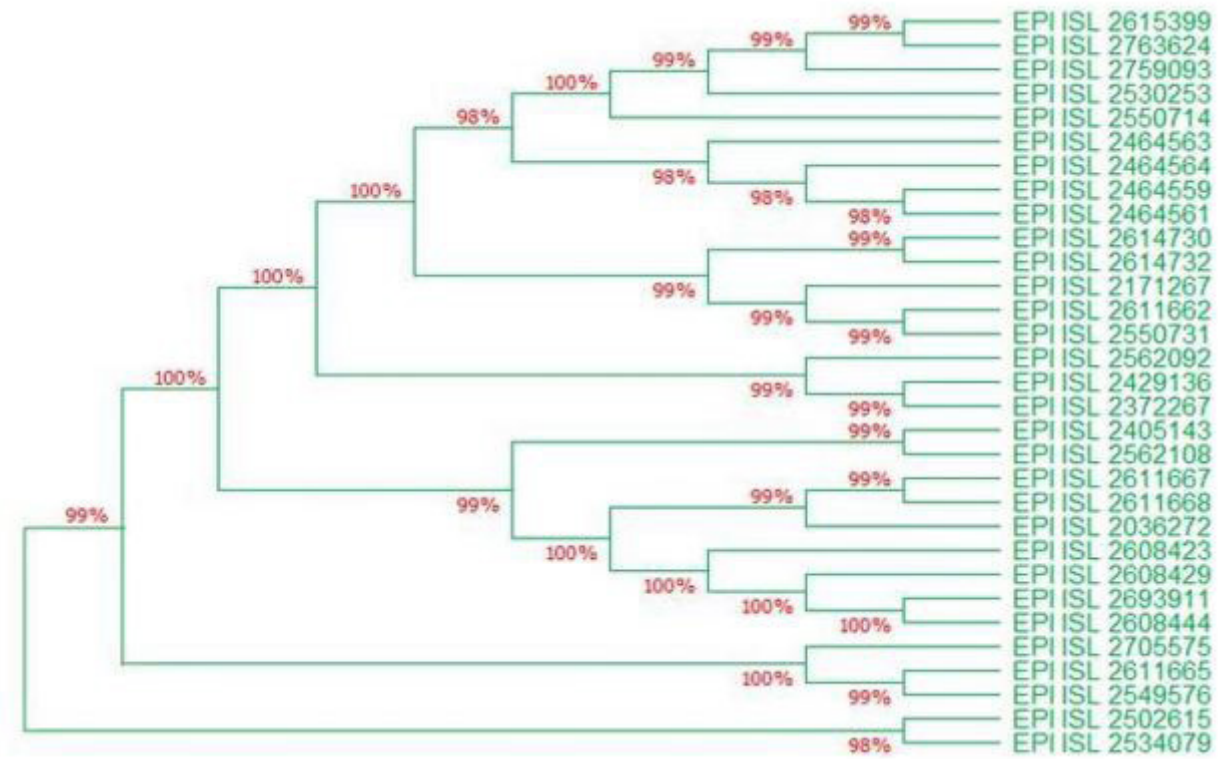

Figure 1: Evolutionary histories and relationships among a taxonomic group of SARS-CoV-2 (Delta variant)

Metadata analysis findings

AA substitutions and Mutation analysis

Another interesting observation of our systematic analysis of 183 SARS-CoV-2 (B.1.617.2) sequences was a high number of 329 Amino acid (AA) Substitutions (Fig.2). Our analysis also revealed 11 Amino acid Deletions. The most common substitutions were: Spike D614G (99.5%), Spike L452R (99.5%), Spike P681R (99.5%), N D377Y (99.5%), N R203M (99.5%), NS3 S26L (99.5%), NS7a T120I (99.5%), Spike T19R (99%), NSP12 P323L (99%) and M182T (98%).

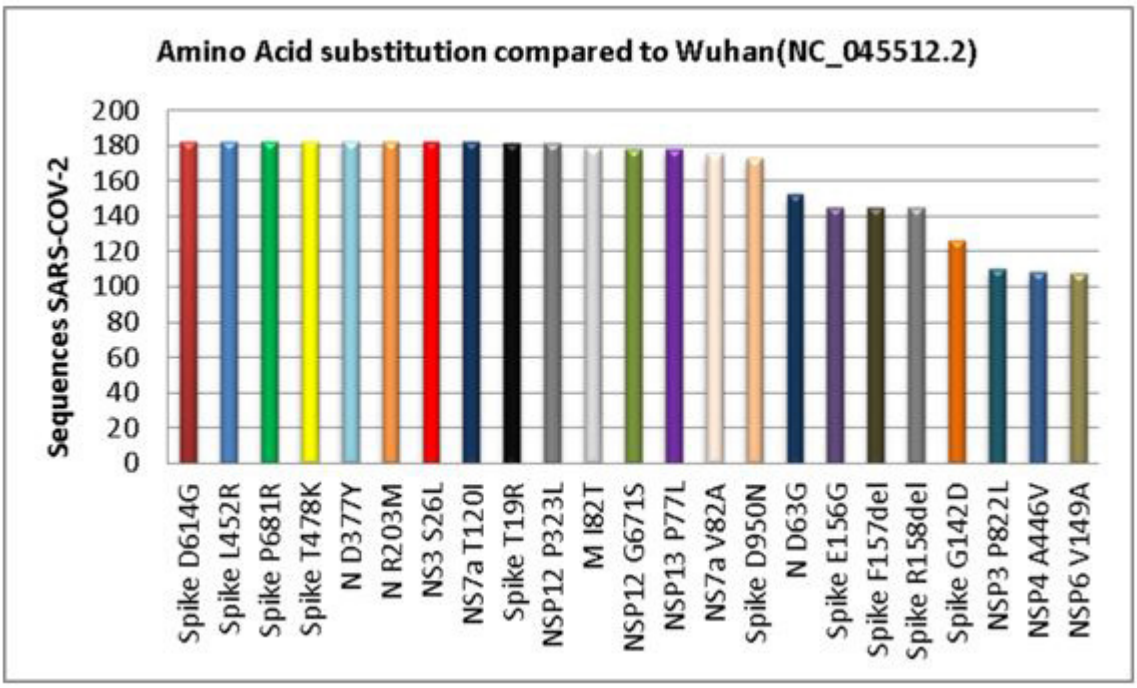

Figure 2: Dominant missense mutations in the SARS-COV-2 Delta sequences

Lineage analysis of SARS-COV-2 (B.1.617.2)

We observed most of the people in Asia belonged to B.1.617.2 (Pango v.3.1.10), Delta (B.1.617.2-like) (Scorpio) Pango lineage. However, a few exceptions such as the new Delta sub-lineage AY.3 (Pango v.3.1.9) were seen in all sequences from Cambodia and a few from Singapore. Indonesian sequences and a few Indian genomes had shown AY.23 and AY.4 respectively (https://github.com/cov-lineages/pango-designation/blob/master/lineage_notes.txt) (Pango v.3.1.11). (Table 2).

Table2: Lineage analysis of 183 SARS-COV-2 Delta sequences. B.1617.2 was the most prevalent lineage. Other sub-lineages AY.3, AY.23 AY.4 were also found in our data. This data indicates that there are sublineages in the delta variant

Pango	Sample
B.1.617.2 (Pango v.3.1.10), Delta (B.1.617.2-like) (Scorpio)	150
AY.3 (Pango v.3.1.9), Delta (B.1.617.2-like) (Scorpio)	29
AY.23 (Pango v.3.1.11), Delta (B.1.617.2-like) (Scorpio)	2
AY.4 (Pango v.3.1.11 2021-08-09), Delta (B.1.617.2-like) (Scorpio)	2

Age-specific viral loads in SARS-CoV-2 (B.1.617.2) Delta variants

Analyzing the age-specific viral loads among 183 identified patients revealed that the adults (19-59 years) were most vulnerable (75%) compared to others. Only 6% of the patients were seniors (60≥ years), while 3% were adolescents (13-18 years) and 2% were children (0-12 years) (Figure 3). These findings suggest that the Delta variants are highly transmissible in adults and require special attention in elderly patient care.

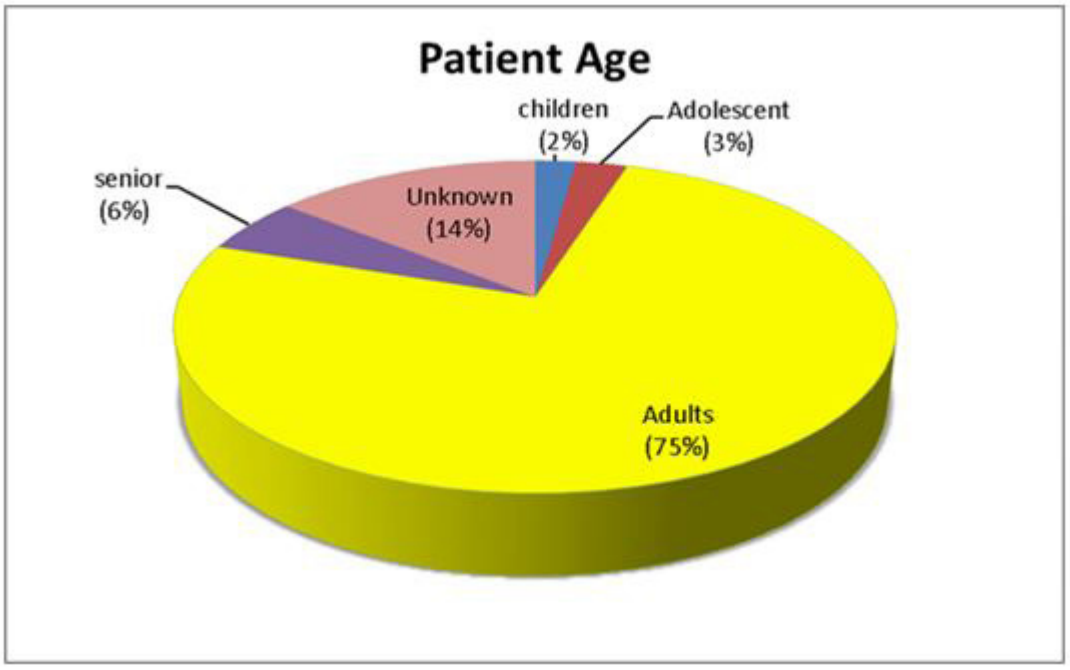

Figure 3: Viral load distribution among children, adolescents, adults, and seniors. The SARS-CoV-2 variant mostly infected adults

Discussion

The Delta variant (B.1.617.2) is classified as a Variant of Concern VOC due to its higher transmissibility, pathogenicity, and infectivity and its emergence as a dominant variant compared to other variants during July 2021 [13]. The transmission between fully vaccinated individuals raised concerns regarding the severity of this unique variant. Our metadata analysis confirms that vaccines are not 100% effective in preventing COVID-19 since ten of the subject patients were vaccinated yet tested positive for COVID-19 (as revealed from clinical history). Despite resulting in clinical infections, vaccinated individuals were effective against hospitalization and transmitted the virus for a shorter time [14,15]. We can contain the virus and subsequently control the pandemic via early detection of the Delta virus using qRT-PCR as this will not require any post PCR sequencing to confirm the variant identification. The qRT- PCR is a specific and sensitive method allowing faster detection of pathogens from patient samples [16]. The efficiency of PCR assays relies on the specificity of primers; thus, we decided to use the conserved regions of the Delta variant for universal primer designing to avoid false negatives. Multiple sequence alignment of the 183 whole-genome sequences helped identify these conserved regions using various bioinformatics tools [17]. The mutations and conserved sequences among the sequences were identified manually. We selected ten candidates (qRT-PCR primer and probe) at ten separate conserved regions from the whole genome (Table 1 and Supplementary Table 1). The primers and probes were selected based on the Tm values (°G), GC content, ΔG (Kcal/mole), and self-complementarity. The conserved regions cover- Nsp1, ORF1a, Nsp2, Nsp5, Nsp11, Nsp13, Nsp8, Nsp9, Coronavirus envelope (E), ORF3b, RNA-dependent RNA polymerase, porcine Delta Coronavirus (PDCoV), Nsp15, S1/S2 cleavage region, and the S2 fusion. The specificity and sensitivity of the proposed primers and probes should be highly suitable for rapid and reliable SARS-COV-2 Delta variant detection and clinical applications.

We collected sequences from patients enrolled in Asian countries to find the most dominant mutations and similarities in the region. Several genomic changes compared to the other variants make the Delta variant the most infectious VOC globally. We detected a total of ten missense mutations in the following Delta variant proteins. Among them the D614G mutation in the spike protein increases the virus transmissibility, infectivity, and fitness and is present among all the VOCs, including the Alpha, Beta, and Gamma, becoming a globally dominant form in the pandemic [18,19]. The L452R mutation in the receptor-binding domain (RBD) of the spike protein allows the virus to strongly interact with the ACE2 (Angiotensin-converting enzyme 2) host receptor, increasing the protein stability and promoting virus fusion facilitating the entry of the virus [20,21] (Figure.2). This mutation in Delta, Kappa, and Epsilon inhibits antibody recognition and neutralizing activity via abolishing intermolecular interactions and increasing infectivity [22,23]. Again, the P681R mutation is highly conserved in Delta variants and is present within the spike protein in the furin S1-S2 cleavage site (Saito et al., 2021). The cleavage of the spike protein to the S1 and S2 subunit by furin increases cell-cell fusion, and internalization of the virus, and thus enhances transmissibility. A separate study reported the prevalent mutations in several sequences, including the Alpha, Gamma, Delta, and Beta [24]. The authors further stated that except for ND377Y and NSP12, P323L (RNA-dependent RNA polymerase), all the other mutations mentioned above are exclusive to the Delta variant [24]. We found them as the most dominant missense mutations among our sequences, suggesting they as a crucial factor in making the virus more infectious than the other VOCs.

We also attempted to identify sequences from different Pango Lineages using data retrieved from the GISAID. The designation stated by the WHO does not show functional and biological differences between the lineages but clusters significant country-wise mutations and epidemiological differences into specific AY lineages (i.e. Cov-Lineages; https://cov-lineages.org/). Most isolates studied here belonged to the B.1.617.2 lineage, and sequences retrieved from Cambodia (n=25) and Singapore (n=4) belonged to sub-lineage AY.3. Sequences retrieved from Indonesia (n=2) belonged to AY.23, and isolates from India (n=2) belonged to AY.4 (Table.2). Though all our sequences belong to the GK clade, we constructed a phylogenetic tree to identify the most closely related isolates. We used the neighbor-joining method to produce a minimum-evolution tree using all of our similar clade sequences [25] (Figure.1). It was revealed that a high sequence similarity (98-100%) existed among the Asian isolates. There was 98% similarity among the sequences submitted from patients from Singapore (EPI_ISL_2464563, EPI_ISL_2464564, EPI_ISL_2464559, EPI_ISL_2464561), and these sequences also shared 98% similarity with sequences from Kuwait (EPI_ISL_2615399, EPI_ISL_2763624, EPI_ISL_2759093), India (EPI_ISL_2530253) and Malaysia (EPI_ISL_2550714). These sequences arose from the different Pango lineages as two

of the Singapore sequences belonged to AY.3, while all other sequences belonged to B.1.617.2. Exceptionally, all the selected candidates from Cambodia shared 100% sequence similarity and belonged to AY.3 lineage. Bangladeshi sequences shared 99% sequence similarity to Hong kong sequences (EPI_ISL_ 2611667, EPI_ISL_2611668). Indonesian sequences (EPI_ISL_2502615, EPI_ISL_2534079) shared 98% sequence similarity between them, while 99% with the other 29 sequences from Asian nations. The analyses revealed that mutations among sequences collected from the same country varied greatly for most and might not necessarily be fully similar. Additionally, despite belonging to the same clade, most sequences belonged to different sub-lineages (Table.2). The present study shows that adults (19-59 years) are more vulnerable to SARs-CoV-2 infection than children. Our data aligned with previous reports who found the viral load increases with age [26]. There are many possibilities for lower viral load in children as they are usually dependent on their parents for seeking medical care and might not express their discomfort, which might cause a delay in testing when the viral load is already low (Figure 3). Another study showed low expression of ACE2 in the early years, hindering the virus entry which explains the lower viral load in the children [27, 28]. Other membrane proteins including TMPRSS2 (Transmembrane protease, serine 2) and CD147, which facilitate the virus entry were also seen to have lower expression in children [29].

Conclusion

We have demonstrated that the designed primer sets and SYBR green-based or Taqman probe-based qRT-PCR can be considered for a further suitable commercial kit to continue rapid cost-effective molecular diagnosis of SARS-CoV-2 Delta variants (B.1.617.2). To our understanding, this is the first report on designing primer and probe sets of the SARS-CoV-2 Delta variants. Viruses constantly change through mutation, and the insertion of viral sequences from infections appearing in 11 Asian countries helped to demonstrate the dynamics of virus circulation in Asia. The universal primers and probes that we have proposed here are crucial at this time and will assist in controlling the pandemic.

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Supplementary data

Supplement Table: Showing conserved domains and targets of 10 primers and probes

No	Conserved region	Conserved domains(Targets)
1	AAGGTAAGATGGAGAGCCTTGTCCCTGGTTTCAACGAGAAAACACACGTCCAACTCAGTTTGCCTGTTTTACAGGTTCGCGACGTGCTCGTACGTGGCTTTGGAGACTCCGTGGAGGAGGTCTTATCAGAGGCACGTCAACATCTTAAAGATGGCACTTGTGGCTTAGTAGAAGTTGAAAAAGGCGTTTTGCCTCAACTTGAACAGCCCTATGTGTTCATCAAACGTT	Nsp1 is a cleavage product of ORF1a.
2	CCTTTGGAGGCTGTGTGTTCTCTTATGTTGGTTGCCATAACAAGTGTGCCTATTGGGTTCCACGTGCTAGCGCTAACATAGGTTGTAACCATACAGGTGTTGTTGGAGAAGGTTCCGAAGGTCTTAATGACAACCTTCTTGAAATACTCCAAAAAGAGAAAGTCAACATCAATATTGTTGGTGACTTTAAACTTAATGAAGAATCGCCATTATTTTGGCAT	Nsp2 N-Terminal
3	TATGAAAAACTCAAACCCGTCCTTGATTGGCTTGAAGAGAAGTTTAAGGAAGGTGTAGAGTTTCTTAGAGACGGTTGGGAAATTGTTAAATTTATCTCAACCTGTGCTTGTGAAATTGTCGGTGGACAAATTGTCACCTGTGCAAAGGAAATTAAGGAGAGTGTTCAGACATTCTTTAA	Nsp2- which is 100% preserved and fit for more genetic studies.
4	CTCTATCACCTCAGCTGTTTTGCAGAGTGGTTTTAGAAAAATGGCATTCCCATCTGGTAAAGTTGAGGGTTGTATGGTACAAGTAACTTGTGGTACAACTACACTTAACGGTCTTTGGCTTGATGACGTAGTTTACTGTCCAAGACATGTGATCTGCACCTCTGAAGACATGCTTAACCCTAATTATGAAGATTTACTCATTCGTAAGTCTAATCATAATTT	Nsp5- called the prime protease (Mpro or 3C-like protease 3CL-pro) dominant enzyme.
5	AGGACAGACTTTTTCAGTGTTAGCTTGTTACAATGGTTCACCATCTGGTGTTTACCAATGTGCTATGAGGCCCAATTTCACTATTAAGGGTTCATTCCTTAATGGTTCATGTGGTAGTGTTGGTTTTAACATAGATTATGACTGTGTCTCTTTTTTGTTACATGCACCATATGGAATTACCAACTGGAGTTCATGCTGGCACAGACTTAGAAGGTAACTTTTATGGACCTTTTGTTGACAGGCAAACAGCACAAGCAGCTGGTACGGACACAACTATTACAGTTAATGTTTTAGCTTGGTTGTACGCTGCTGTTATAAATGGAGACAGGTGGTTTCTCAATCGATTTACCACAACTCTTAATGACTTTAACCTTGTGG	Nsp5- in this procedure also building it a peak value target for the circumstances of anti-coronavirus therapeutics.
6	CCTTTGTAAGCACAAGCTGATGAGTACGAACTTATGTACTCATTCGTTTCGGAAGAGACAGGTACGTTAATAGTTAATAGCGTACTTCTTTTTCTTGCTTTCGTGGTATTCTTGCTAGTTACACTAGCCATCCTTACTGCGCTTCGATTGTGTGCGTACTGCTGCAATATTGTTAACGTGAGTCTTGTAAAACCTTCTTTTTACGTTTACTCTC	Coronavirus envelope (E) proteins -that are highly pathogenic SARS-CoV-2. Also accessory protein ORF3b-that blocks IFN synthesis, signaling and stops spreading to the nucleus in transfected cells.

7	AACAACATTATCAACAATGCAAGAGATGGTTGTGTTCCCTTGAACATAATACCTCTTACAACAGCAGCCAAACTAATGGTTGTCATACCAGACTATAACACATATAAAAATACGTGTGATGGTACAACATTTACTTATGCATCAGCATTGTGGGAAATCCAACAGGTTGTAGATGCAGATAGTAAAATTGTTCAACTTAGTGAAATTAGTATGGACAATTCACCTAATTTAGCATGGCCTCTTATTGTAACAGCTTTAAGGGCCAATTCTGCTGTCAAATTACAGAATAATGAGCTTAGTCCTGTTGCACTACGACAGATGTCTTGTGCTGCCGGTACTACACAAACTGCTTGCACTGATGACAATGCGTTAGCTTACTACAACACAACAAAGGGAGGTAGGTTTGTACTTGCACTGTTATCCGATTTACAGGATTTGAAATGGGCTAGATTCCCTAAGAGTGATGGAACTGGTACTATCTATA	Nsp8 grants processivity on RNA-dependent RNA polymerase. Key component Nsp9 constantly seizes other proteins in the replicase complex to moderate efficient virus transcription and replication. Oligomerization of Porcine Delta Coronavirus (PDCoV).
8	ATCTGGGACTACAAAAGAGATGCTCCAGCACATATATCTACTATTGGTGTTTGTTCTATGACTGACATAGCCAAGAAACCAACTGAAACGATTTGTGCACCATCACTGTCTTTTTTGATGGTAGAGTTGATGGTCAAGTAGACTTATTTAGAAATGCCCGTAATGGTGTTCTTATTACAGAAGGTAGTGTTAAAGGTTTACAACCATCTGTAGGTCCCAAACAAGCTAGTCTTAATGGAGTCACATTAATTGGAGAAGCCGTAAAAACACAGTTCAATTATTATAAGAAAGTTGATGGTGTTGT	Nsp15-NTD Nsp15 diverges from that of further coronavirus members and has been shown to exist as a dimer and a monomer in solution. Nsp11 from the product of enveloped positive-strand RNA virus PRRSV performs as a dimer.
9	GGCGTTGTCCTGCTGAAATTGTTGACACTGTGAGTGCTTTGGTTTATGATAATAAGCTTAAAGCACATAAAGACAAATCAGCTCAATGCTTTAAAATGTTTTATAAGGGTGTTATCACGCATGATGTTTCATCTGCAATTAACAGGCCACAAATAGGCGTGGTAAGAGAATTCCTTACACGTAACCCTGCTTGGAGAAA	The helicase domain Nsp13 from delta coronavirus, along with pathogenic human viruses (SARS-CoV-2).
10	ATGTGGTCAACCAAAATGCACAAGCTTTAAACACGCTTGTTAAACAACTTAGCTCCAATTTTGGTGCAATTTCAAGTGTTTTAAATGATATCCTTTCACGTCTTGACAAAGTTGAGGCTGAAGTGCAAATTGATAGGTTGATCACAGGCAGACTTCAAAGTTTGCAGACATATGTGACTCAACAATTAATTAGAGCTGCAGAAATCAGAGCTTCTGCTAATCTTGCTGCTA	S1/S2 cleavage region and the S2 fusion subunit of the spike (S) glycoprotein SARS-CoV-2 depict a central role in viral entry by inaugurating fusion of the viral and cellular membranes.