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Introduction of the STexS PCR Technique and the Advantages of Simultaneous Multi-target PCR Application

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Abstract

Detecting and diagnosing small changes occurred by gene alteration *via* PCR is globally considered as an effective and precise method for handling various diseases. From the internal factors which change the normal body to develop disorders such as cancer to mutated external sources of infection that alters the intact human body, mainly bacteria and viruses. Among these, SARS-CoV-2 has emerged to be one of the most concerning threats to public health due to its rapid mutation. From the initial outbreak in 2019, research groups continuously identified various subtypes, some of which branched out as a novel variant of the original form that caused global health crisis among the population. While the standard PCR method of real-time quantitative reverse transcription is considered effective in detection and prevention, as more SARS-CoV-2 variants emerged, the overall process of identifying each variant one by one has deteriorated the standard procedure to be tiresome and time-consuming. To overcome the inefficiency, a novel PCR method using oligonucleotides (STexS) was introduced to significantly increase the specificity in detecting single nucleotide polymorphisms. Furthermore, the implementation of simultaneous multi-target PCR successfully isolated both Delta and Omicron variants within a single PCR trial, condensing the excessively long PCR procedure to be overall efficient and precise at the same time. The improvements will provide crucial insight in SARS-CoV-2 detection and diagnosis.

Keywords: Single nucleotide polymorphisms • Polymerase Chain Reaction (PCR) • SARS-CoV-2• Molecular diagnosis • Variant detection

Introduction

With the presentation of DNA polymerase and the discovery of Taq DNA polymerase, the understanding of DNA structure was able to be practiced with precise accuracy [1]. The dominant method to this day is the Polymerase Chain Reaction (PCR) which allowed detection of gene sequences start from a small fraction to produce large volumes of targeted DNA [2]. The simply yet effective strategy branched to contribute invaluable research projects such as the human genome project [3]. While the completion of the genome was a great achievement, it was not the end to DNA insight as variations of polymorphisms were found among the population [4]. Series of alterations were easily detected worldwide ranging from subtle variations occurred in the noncoding regions to crucial mutations leading to genetic malfunctions [5,6]. In search for solutions, PCR was also implemented to detect DNA polymorphisms such as Single Nucleotide Polymorphisms (SNPs) as in theory would amplify mutated DNA and rule out non targeted sequences [7]. However, in practice this wasn't the case. Constant nonspecific binds between primers and mismatched strands were seen in various PCR trials. This phenomenon occurs when the DNA polymerase tolerates the mismatched base pair between the primer and wild type strand and further continues its elongation process [8]. Research groups across the world continuously presented improvements in order to successfully reduce unwanted amplification. Almost all the improvements were adjusting or adding supplements around the three fundamental steps of PCR: Denaturation of the template, primer annealing for new strand synthesis, and extension of new DNA strands primer synthesis [9].

Alongside the practical issues occurring within the PCR, various researches soon realize the human DNA was not the only concern for disease and genetic treatment. Various strands of bacteria and viruses infecting unsuspecting personnel were also needed to be identified by its DNA structure [10]. To make things matters worse, somewhat dormant strands had the potential to break havoc once certain genes mutate and create novel variants [11,12]. The current spread of SARS-CoV-2 is the most major example as it continuously mutates its original strand, creating various mutations that impacted human population worldwide [13]. As the nature of the virus is based on single-strand RNA, nucleotide mutation occurs at a higher rate during reverse transcription. Furthermore, viral RNA polymerases lack the ability to proofread between transcription, resulting in a series of simultaneous SNPs that led to infamous variants such as the Alpha, Beta, Gamma, Delta, and Omicron variant [14]. Such hazardous variants were all identified and treated based on PCR detection. Despite most of the infection was detected, a series of false negative PCR results occurred which impacted an unexpected breakout in heavily populated regions [15,16]. When the Initial variants of Alpha and Beta were discovered, the response of treatment and prevention was quite fast, as PCR detection was limited to one or two trials. This process soon became troublesome once additional variants emerged. Not only several PCR tests were needed to identify the exact SARS-CoV-2 variant, but also similar mutations were overlapping among variants which needed to be excluded in the detection process [17]. As more and more variants were discovered due to the rapid mutation of reverse transcription, the overall global health efforts became tiresome and inefficient in both variant detection and patient treatment. However, current guidelines are limited to ruling out one variant per PCR trials. Furthermore, previously used PCR methods were not sufficient for precise SNP detection as abundant amounts of wild type interfered with target amplification as the overall background blocked successful variant selections. The review discusses previously used PCR methods for SNP detection and target strand amplification

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and the novel STexS method [18]. The implemented TaqMan PCR is further used as a successful method for simultaneous multi-target PCR that selects among several distinct SARS-CoV-2 variants such as the recently discovered Delta and Omicron variant.

Literature Review

Types of PCR assays for SARS-CoV-2 detection

TaqMan PCR: As a variation branched from real-time PCR, TaqMan PCR is known as using nucleic-acid probes which complements to an internal segment of the target DNA. Basically, probes are labeled with two fluorescent components. The emitted spectrum of a probe overlaps with the other produced spectrum, quenching the initial fluorophore. Probes are mainly present during the PCR process but once the target product is amplified, it degrades *via* the 5'-nuclease activity of the Taq DNA polymerases which specifically act as a DNA hybridizing method to the template also known as the TaqMan activity [19]. Probe degradation gradually separates the two fluorophores, reducing quenching and increase the intensity of light emission. As the assay is performed within a sealed PCR tube environment with minimal external exposures during fluorescence measurements, the risk of contamination is further decreased. Due to the advantages described above, TaqMan PCR provides sensitive, specific, and quantitative results which can be applied to various clinical and environmental practices. The implication stretches to virus detection and selection, as research reports the method to be effective in successfully evaluating different genogroups of Noroviruses [20]. Based on the hydrolysis technology of the TaqMan probe, low percentage of Norovirus have been detected in contaminated water which can easily develop to a serious health risk as the minimum infectious dosage needed for the infected patient can be little as 10 to 100 PCR units. Other previous reports regard the detection of Norwalk-like virus *via* real-time quantitative reverse transcription PCR [21]. 71 Norwalk-like virus positive stool specimens were used to isolate the virus which some of them contain low copy numbers of Norwalk-like RNA. Due to its high performance in detecting DNA and RNA based viruses, it was only a matter of time to be implemented in isolating SARS-CoV-2 as well. After the initial outbreak, TaqMan real-time reverse transcription PCR assays were used to target the nucleocapsid and the open reading frame 1b gene region of SARS-CoV-2 [22]. Further optimization revealed the overall detection range of each gene region to be 1-106 and 10-106 copies per reaction. As SARS-CoV-2 spread worldwide and simultaneously alters its original form, a novel approach for producing synthetic positive controls was introduced by TaqMan multiplex PCR. As a result, evaluation of the expression level of RdRP gene has been the standard for identifying positive detection of SARS-CoV-2 [22].

STexS PCR: Despite the TaqMan PCR's contribution in SARS-CoV-2 as a golden standard of real-time reverse transcription method, it was not the perfect method, as limitations were visible once single base SNPs developed distinct variants from the original form. Nonspecific strands attached to the primers were amplified resulting in false positive diagnosis. Furthermore, as important as treating the already severely infected patients, the emphasis of SARS-CoV-2 prevention were of equal concern.

Therefor the need for an effective alternative that can detect 1% or less included mutated targets among a large abundance of highly similar wild type. Alongside the required high specificity, the PCR method needed to diminish unnecessary primer dimers, reduce nonspecific mismatch binds and block efficiency drops occurred by high G/C rates [23,24]. "Hotstart PCR" was one of the suggestions for decreasing unwanted bindings, but this alone did not increase the CT value as the amplification of small percentage targets did also get hindered. To overcome the obstacle, double stranded oligonucleotides were implemented hence the novel STexS PCR platform was introduced [18]. A double helix forming oligonucleotide DNA (dbOligo) of at least 10 base pairs in design was used in the annealing process. Optimizing the melting temperature above the average annealing temperature was crucial as unwanted dimers were easily discarded. When compared to a typical TaqMan PCR in a normal matching situation, both methods did not present any difference in amplification rates. However, STexS shined its utility against the TaqMan PCR in mismatched cases. The attach and detach rate of DNA polymerases are altered, hindering the mismatch annealing which results in a decrease and a significant boost to PCR specificity (Figures 1a and 1b). Not only it was useful in isolating SNPs, but also significantly contributed to identifying early-stage cancer markers such as the EGRF mutation in non-small cell lung cancer (same). Further validation revealed the STexS method was viable to successfully discriminate SNP apart from the wild type template (Figures 1a and 1b).

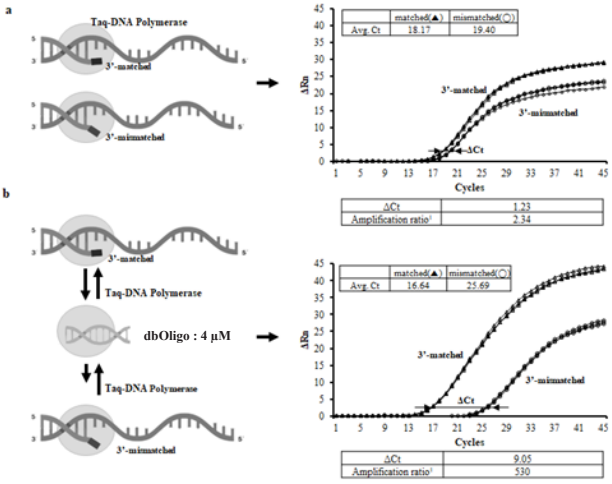

Figure 1. Schematics and CT value difference of the standard PCR model. A. SNP Typing –excellent specificity (STexS) PCR; B. within a mismatched amplification. Adapted from Inclusion of double helix structural oligonucleotide (STexS) results in an enhance of SNP specificity in PCR [18].

Note: (▲) Matched; (○) Mismatched.

Simultaneous multi-targeting of SARS-CoV-2 variants

Despite the solution to drastically decreasing nonspecific strands within abundant nonspecific strands was provided *via* STexS PCR, the platform alone could not fully function as a substitute method in viral detection. Limitations regarding the virus titers of each gene region to be 1~106 and 10~106 copies per reaction hindered the accuracy of amplification. Moreover, the current guideline for identifying SARS-CoV-2 variants was to validate the broad coronavirus RT-PCR against the published 2019-CoV sequence to sort out overlapping matches that can indicate a positive detection [25,26]. The more recent guidelines provide suggestions in SARS-CoV-2 detection yet needs further validation through sequencing to confirm the initial variant detection. This is mainly due to the concern of false-positives or false-negatives that are seldom detected when typical PCR methods are implemented. However, the lack of specificity had been solved by the mismatch signal diminished in STexS PCR, leading to possibilities of a condensed and efficient method of variant detection. As a result, a compact method based on TaqMan PCR was devised [27]. In order to detect specific SARS-CoV-2 variants, each primer was designed to match distinct variant mutations such as the T19R, L452, and P681R genomic regions in the Delta variant and T547K, D796Y, and Q954H genomic regions in the Omicron variant [28,29]. The percentage of each gene mutation locating specifically in its respective variant was estimated above 95% *via in silico* data (Table 1). These distinct features easily indicate if primers can match at least two mutations of any variant, the results would indicate a positive detection of either Delta or Omicron variant infection. Merging the TaqMan PCR and the concept of diagnosing multiple variants within a single reaction, each PCR tests were consisted of multi-targeting primers/probes of the Delta and Omicron variant, the positive control RdRp, and the negative wild type control [27]. Through optimization and validation to rule out possible interferences caused by the positive and negative control, the multi-target PCR procedure was able to successfully diagnose specific variant infected RNA samples (Figures 2a-2c). The combined signal of variant specific matched primers was easily visible and would provide a compact information to the patient of a reliable yet fast result (Table 1) (Figures 2a-2c).

Table 1. Mutation occurrence percentages of genes among SARS-CoV-2 variants.

VOC	Target Gene (%)					
	T19R	L452R	P681R	T547K	D796Y	Q954H
Alpha	ND	0.1	0.1	0.1	0.1	ND
Beta	0.1	0.1	0.1	0.1	0.1	ND
Gamma	0.1	0.1	0.4	0.1	0.1	ND
Delta	98.3	97.1	99.2	0.1	0.1	ND
Omicron	0.1	0.5	0.1	93.9	96.9	97.8

Note: ND: Not Determined

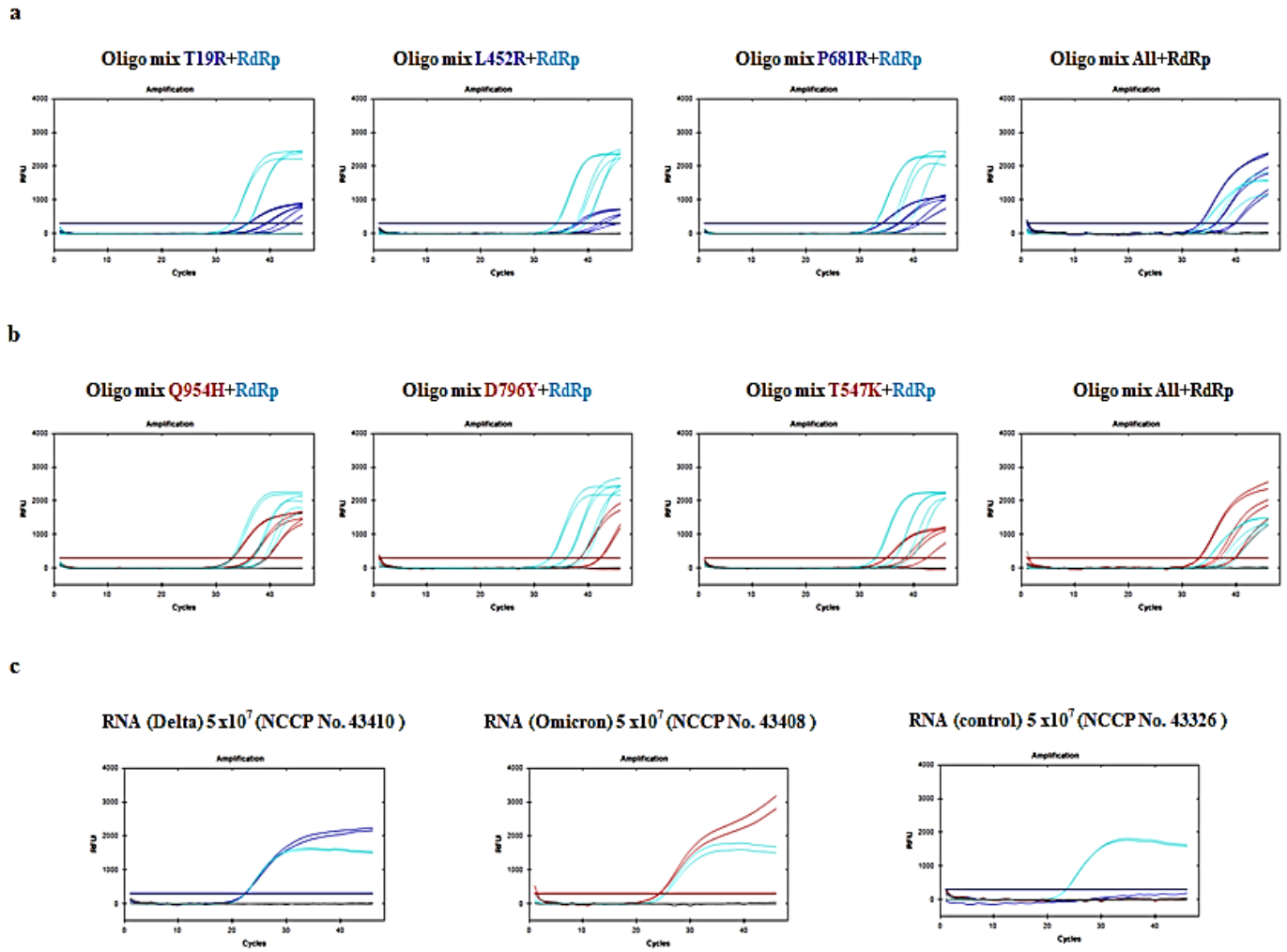

Figure 2. PCR test of standard RNA samples embedded with Delta. A. Omicron; B. variants and combined; C. Each validation was done with RNA copies from 5×10^2 to 5×10^4. Increased amounts of RNA were set to 5×10^7 for further amplifications. Adapted from Compact Method of SARS-CoV-2 PCR Detection Enhances Overall Process of Diagnosing Delta and Omicron Variants [27].

Discussion

The struggle to successfully detect, rehabilitate, prevent, and contain the global threat caused by the SARS-CoV-2 is a united attempt that constantly needs to improve through novel insight and feedback [30]. Despite the recent diminishing trend of the contagion, mass breakouts are still occurred within densely populated districts. Numerous samples of nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavages, tracheal aspirate or nasal wash, sputum, tissue from biopsy or autopsy derived from the lungs, serum or plasma containing whole blood vials, and urine are constantly collected and stored for laboratory testing [31-33]. Although PCR itself is a tight and rapid method to cultivate several trials, the ever-consistent demand of new samples waiting for detection and diagnosis can easily be a burden [34,35]. In a way to effectively prevent or response in the early-stage infection, the process of immediate testing is inevitably needed. The multi-targeting will shorten the process of variant detection within a single PCR test. The methods mentioned above will act as a time-efficient and cost-effective strategy in diagnosing the two most recent variants of SARS-CoV-2 [36].

The increased specificity contributed by STexS PCR is in itself a notable enhancement when detecting SNPs yet requires further augmentations to fully function in both DNA and RNA detection. As its intention was to work in 101~106, virus variants could be seen in far concentrated amounts that exceeds the current detection limit. To overcome this obstacle, enhancements could be found by altering the main conception of multiplex probes. While mainly using several probes within a single PCR reaction is based on the premise of no interference among probes, some probes could act to deliberately compete with other probes, decreasing the overall amplification. By improving the detection range of STexS PCR, it will serve as an enhancement in PCR methods diagnosing DNA based SNP derived diseases and RNA based virus infections.

Conclusion

As the ever-changing nature of the RNA virus, SARS-CoV-2 will likely exude novel variants that may develop to yet another major threat to global health. Some variants of interest described by the World Health Organization (WHO) contain Epsilon (B.1.427 and B.1.429); Zeta (P.2); Eta (B.1.525); Theta (P.3); Iota (B.1.526); Kappa (B.1.617.1); Lambda (C.37)and Mu (B.1.621). While most of the variants are not classified as a major concern or are safely contained due to neutralization efforts, it is without a doubt a novel variant capable enough to disseminate worldwide could appear in the foreseeable future. However, with each novel variant detected, simultaneous multi-targeting PCR methods will prove to be more valuable as it the method itself will update to target such variants. The suggested procedure will not be mentioned as a temporary replacement but noticed as a persistent method that may be used in various situations.

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