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# Variant-specific RT-qPCR for rapid screening of B.1.617 mutations in SARS-CoV-2

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**ABSTRACT**

The continuous emergence of new SARS-CoV-2 variants required rapid and reliable diagnostic methods for early detection and monitoring of the spread of the virus, especially in low-resource countries where whole genome sequencing is not available. We aimed to evaluate and compare the performance of two different RT-qPCR screening assays for the detection of B.1.617 lineage mutations. A total of 85 SARS-CoV-2 positive samples were collected between 9$^{th}$ August and 10 September 2021 and screened by two mutation-specific RT-qPCR assays for simultaneous detection of B.1.617.1 and B.1.617.2 lineage mutations. VIASURE Variant II PCR assay identified 2 Delta variant-specific mutations (L452R, and P681 R) in 80% of tested samples, while the PKamp™ Variant Detect™ assay was only able to detect one Delta variant specific mutation (L452R) in 75% of tested samples. This is the first report to show the Delta variant as the cause of the third wave in Libya. The use of multiplex RT-qPCR assays has allowed the identification of new variants for rapid screening. However, RT-qPCR results should be confirmed by whole genome sequencing of SARS-COV-2.

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## 1. Introduction

Since the outbreak of COVID-19 in Wuhan, China, in 2019, several mutations have been shown in SARS CoV-2 [1,2]. From September 2020, new variants of concern (VOC) emerged in Europe [3], started from the UK, B.1.1.7 (alpha variant) [4], followed by two other variants: B.1.351 (beta variant) [5], and P.1 (gamma) [6]. In late 2020/early 2021 B.1.617 lineage was first identified in Maharashtra in India, and rapidly spreads around the globe [7]. The first sub-lineage to be detected was B.1.617.1 [7], followed by B.1.617.2, both carrying L452R mutation in the receptor binding domain of spike protein, which is also observed in Epsilon variant B.1.427/B.1.429 [7,8]. This mutation showed increased infectivity with little loss of susceptibility to neutralizing antibodies [9,10]. The mutations L452R, P681R, and E484Q within the receptor binding domain (RBD) are specific to the Kappa variant (lineage B.1.617.1) while L452R and P681R are specific to the Delta variant (lineage B.1.617.2), where P681R mutation is located in the S1-S2 furin cleavage site [11]. B.1.617.2, termed as Delta variant by WHO, has since dominated over B.1.617.1 (Kappa variant) and other lineages[12]. The symptoms of delta infection differ from pre-existing strains, usually starting with flu-like symptoms such as sore throat, headaches, and runny nose before progressing to pneumonia [13]. Because of the higher transmissibility and mortality rate of the Delta variant, WHO alerted that this variant is rapidly becoming the main dominant strain of SARS-CoV-2 worldwide [14,15].

Since June 2021, the propagation of these variants in Libya required rapid screening based on RT-qPCR typing tests; because of their clinical implications of high transmissibility, and escaping from the immune system [16]. RT-qPCR is the gold standard for detection of SARS-CoV-2 in clinical samples [17], and has been used for the detection of many spike gene mutations focusing on deletion and SNPs in previous studies, using different techniques such as specific primers, and probes, and high-resolution melting curve analyses [18–24]

Here, we aimed to evaluate two mutation-specific RT-qPCR assays for screening of B.1.617 lineage mutations.

## 2. Material and methods

### 2.1. Samples collection

This study was conducted at the Molecular laboratory of the Libyan Biotechnology Research Center. A total of 85 clinical samples for SARS-CoV-2 positive patients were selected with a cycle threshold (Ct value) less than 35. All samples were previously tested N-gene positive by the Da An® detection kit for 2019-Novel Coronavirus (2019-nCOV) RNA (PCR-Fluorescence

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Probing), V4, Da An® Gene Co., Ltd. of SunYat-sen University, which targeted the *ORF1ab* and *N* genes. The samples were collected from the Tripoli region in the period between 9th August and 10th September 2021.

Ethical approval was obtained from the Bioethics Committee of the Libyan Biotechnology Research Centre.

### 2.2. RNA extraction

RNA was extracted from the clinical samples using the Automated (Kingfisher flex Instrument, Thermo Fisher Scientific, Waltham, MA, USA), the kit used was viral Nucleic Acid (DNA/RNA) Extraction kit, Techstar, (WUXI TECH-STAR TECHNOLOGY CO., LTD) with 96 well pre-filled plates, following the manufacturer's instructions.

### 2.3. RT-qPCR and SARS-COV-2 variants detection

Positive samples were screened; on the same day, for the main mutations in lineage B.1.617, using two RT-qPCR variant-specific screening tests (VIASURE SARS-CoV-2 Variant II, Certest Biot, Zaragoza, Spain, and Pkamp™ VariantDetect™ SARS-CoV-2 RT-PCR assay, Combo F, PerkinElmer. Boston). The VIASURE SARS-CoV-2 Variant II assay screen for the presence of the *S* gene- E484Q, L452R, and P681R, whereas Pkamp™ VariantDetect™ screens for *S* gene- E484Q, L452R, E484K mutations, in addition to *N/ORF1ab*. Simultaneous detection of the L452R, E484Q, and P681R SNPs further allows differentiation between the B.1.617.1 and B.1.617.2 lineages in a single reaction.

The kits contained positive and negative control and Master-Mix reagents, including primers and probes for the detection of SARS CoV-2 targets. Briefly, VIASURE SARS-CoV-2 Variant II used 20 µl volume of reaction (15 µl M.M, and 5 µl sample), amplification was carried out with the following protocol: 45°C for 15 min, followed by 95°C for 2 min, and 45 cycles of 95°C for 10 sec, and 63°C for 50 sec. Another screening kit (Pkamp™ VariantDetect™ SARS-CoV-2 RT-PCR Kit, Combo F) used 30 µl volume of reaction (20 µl M.M, and 10 µl sample), with amplification protocol: 37°C for 2 min, followed by 50°C for 15 min, and 94°C for 10 min, and 45 cycles of 94°C for 10 sec, 62°C for 15 sec, and 65°C for 45 sec. following the manufacturer's recommendations. Amplification and detection were carried out on the Bio-Rad CFX96 Touch™ Real-Time PCR detection system, Bio-Rad (Bio-Rad, California, USA).

## 3. Results

The following RT-qPCR assays were primarily evaluated for their ability to detect the following mutations: E484K, E484Q, P6 81R, and L452R.

### 3.1. Pkamp™ Variant Detect™ SARS-CoV-2 RT-qPCR Kit

From the positive samples set, 75% of samples (n = 64) showed an L452R+/E484Q–/E484K – profile. Six samples were only positive for N and ORF 1ab gene, i.e. mutations in the *S* gene were not detected (L452R–/E484Q–/E484K–). The remaining 15 samples did not feature any of the targeted mutations.

### 3.2. VIASURE SARS-CoV-2 Variant II RT-PCR detection kit

Unlike Pkamp™ VariantDetect™, VIASURE SARS-CoV-2 Variant II assay additionally detects the presence of P681R mutation characteristic of B.1.617.1 and B.1.617.2 lineages. Sixty-eight (80%) samples had a P681R+/L452R+/E484Q– profile, which is consistent with the Delta variant. Only three cases (3.5%) showed a P681R+/L452R+/E484Q+ profile which is consistent with Kappa. No mutation specific to the Epsilon variant was detected. Furthermore, 14 samples showed no amplification curves for any targeted mutations.

### 3.3. Comparison of the two assays

Of note, three samples that were consistent with kappa variant (P681R+/L452R+/E484Q+) by VIASURE SARS-CoV-2 Variant II were only positive for L452R by PKamp™ VariantDetect™ RT-qPCR detection kit. Pkamp™ VariantDetect™ kit does not target P681R mutation; hence the variant type cannot be determined based on E484K and E484Q alone, this decreases the kit specificity in comparison to VIASURE SARS-CoV-2 Variant II. A Comparison of results obtained by the two assays is summarized in Table 1. The difference between the two means of Ct value of L452R was statistically significant ($P < 0.0001$).

Collectively, Lineages B.1.617.2 (Delta variant) appeared to be predominant in positive samples. Interestingly, lineages B.1.427/B.1.429 (Epsilon variant) was not detected. VIASURE SARS CoV-2 Variant II was able to detect E484Q in three samples but Pkamp™ VariantDetect™ didn't. CT values of all mutations

**Table 1.** Comparison of results obtained by PKamp™ VariantDetect™ (Combo F) and VIASURE SARS-CoV-2 Variant II assays for the detection of B.1.617 mutationanalyzedlysed samples (n = 85).

| | PKamp™ VariantDetect™ (Combo F) | | VIASURE SARS CoV-2 Variant II | |
|---|---|---|---|---|
| Type of Mutation | # Of positive cases | $C_{T\ (Mean)}$ | # Of positive cases | $C_{T\ (Mean)}$ |
| L452R | **64** | **33.23** | **68** | **25.7** |
| E484Q | **ND** | | **3** | **21** |
| E484K | **ND** | | **NA** | |
| P681R | **NA** | | **68** | **26.5** |

ND – Not Detected, NA- Not Applicable.

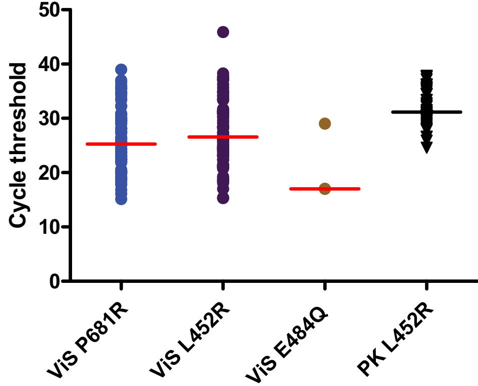


**Figure 1.** Dot plot of cycle thresholds in the two assays.

obtained by both assays are represented as dot plots (Figure 1).

## 4. Discussion

Rapid screening of SARS CoV-2 mutations can early identify circulating variants, may alert for early infections, rapid infection control, and public health interventions [25]. This study has evaluated two multiplex RT-qPCR-assays for rapid screening of newly circulating SARS-CoV-2 Variants. We identified 75% of cases with an L452R+/E484Q–/E484K– profile using Pkamp™ VariantDetect™ assay which detects three targets (N and ORF1ab genes, and E484K, E484Q, L452R in S-gene). Importantly, the variant couldn't be specified as Delta, since L452R mutation is also present in other variants (e.g. B.1.427&B.1.429) [26]. It is noteworthy, that six samples were only positive for *N* and *ORF1ab* but not for S gene mutations, four of these samples showed Ct above 35 for L452R mutation with VIASURE SARS-CoV-2Variant II assay. This may be due to the relatively higher Limit of detection of Pkamp™ VariantDetect™ (200 copies per PCR reaction) in comparison to 160 genome copies/reaction of VIASURE SARS CoV-2Variant II assay.

All samples used in this study were selected with Ct value ≤ 35 for the N gene using Da An® detection kit for 2019-Novel Coronavirus (2019-nCOV) RNA (PCR-Fluorescence Probing), V4, Da An® Gene Co., Ltd. of SunYat-sen University and the majority had Ct values <30. Although N gene has greater sensitivity for detection of SARS-CoV-2 since it is abundant among other gene targets [27,28], some samples showed negative results for N gene using Pkamp™ VariantDetect™; this may suggest lower sensitivity of this kit. Furthermore, multiplex PCR assays using many target genes although have many advantages, they can cause self-inhibition among different sets of primers; low amplification efficiency; and variable efficiency in different templates [29].

After the screening for S gene mutations many samples showed a relatively low viral load (Ct values ≥ 31) using both kits, this may be due to low sensitivity of both assays. Since the Ct values for all samples were taken to be less than 35 then the issue of lower DNA concentration could be excluded.

Samples that showed no amplification with any target genes (15 samples with Pkamp™ VariantDetect™, 14 samples with VIASURE SARS CoV-2 Variant II) need sequencing since they might belong to other variants. Pkamp™ VariantDetect™ has lower efficiency to amplify S gene, since the Ct value of L452R mutation was higher than VIASURE SARS-CoV -2Variant II. Notably, these samples tested positive for the *N*-gene and *ORF1ab* with Da An gene Kit which is routinely used in our laboratories for SARS-COV-2 diagnosis and has a high sensitivity for N gene [30] and then screened for using Pkamp™ VariantDetect™, and VIASURE SARS CoV-2 Variant II on the same day of detection.

The detection of L452R mutation in the samples without any combination with other S gene mutations is meaningless for variant specification; because L452R is found in many circulating variants and its use for screening cannot determine which variant has infected the patient [26]; therefore it is crucial to differentiate between all samples that contain L452R, either through multiplex PCR assays targeting specific mutations commonly found along with L452R or by sequencing of S gene.

It is noteworthy, that T478K mutation or its combination with other mutations in spike gene showed a greater fitness to Delta variant [11], therefore its detection could discriminate between B.1.617 sublineages. Both RT-PCR kits used in this study lack T478K detection and since delta variant was the most dominant variant at the time of the study the detection of T478K in combination with other mutations is very important to avoid misdiagnosis.

As in many other countries, the delta variant wave began to spread around to many countries [13], Libya was no exception, in late June a sharp increase in the number of positive samples was attributed to Delta, this was in agreement with our results because the frequency of Delta cases was dominant (using VIASURE SARS CoV-2Variant II assay).

The unique combination of spike mutations L452R, E484Q, P681R has been observed in B.1.617.1 and B.1.617.3 in Maharashtra, India [11], this was consistent with our findings. Cherian and colleagues showed that the combination of two RBD mutations, L452R and E484Q, could affect the neutralization by specific monoclonal Antibodies [11]; therefore, the rapid detection of L452R and E484K/Q mutations is necessary; because this confers clinical implications on the vaccine and COVID-19 treatment; since these mutations associated with reduced efficacy of the vaccine, or confers resistance to neutralizing antibodies [31].

Several publications have evaluated the potential performance of different variant-specific PCR screening kits in comparison to whole genome sequencing using next-generation sequencing (NGS) technology. The authors confirmed a high concordance between the two methods, and demonstrate their varying degrees of suitability for practical use in diagnostics and screening methods [19,24,32–35]. However, the misidentification of variants by RT-qPCR which was then confirmed to belong to other variants by sequencing was reported by Boudet and colleagues [36].

The difficulty for accessing sequencing reagents and their costs are the main challenges for performing whole genome sequencing in Libya, in addition to the need for capacity building on bioinformatics and genomic data analysis.

Furthermore, continuous development of these PCR assays is important since SARS CoV-2 is continuously mutating resulting in the emergence of new variants for example Omicron which could be quickly detected by identifying its new spike mutations (i.e. ins214EPE, S371L, and S373P) [37].

Multiplex RT-qPCR assays demonstrate practical results to specifically screen for B.1.617 lineage variants (delta/kappa) [38], this was consistent with our results since VIASURE SARS- CoV-2Variant II assay was able to detect E484Q mutation, and hence variant classification (according to manufacturer instructions).

## 5. Conclusion

To summarize, the results obtained from RT-qPCR screening kits represent an ideal balance of costs, turnaround time, and preliminary call for variants, and it is simple for implementation in the routine clinical laboratory. Ultimately, the definitive answer should only be made based on whole genome sequencing.

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## Disclosure statement

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