Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i16.2415

CRISPR-BASED MOLECULAR DETECTION METHODS FOR RNA VIRUSES: APPLICATIONS IN THE DIAGNOSIS AND TREATMENT OF COVID-19 VARIANTS

Myle Akshay Kiran[1], Muhammed A. Bakhrebah[2], Muhammad A. Halwani[3], Bashayer M. AlShehail[4], Abdulrazzag Abdulaziz Othman[5], Abdullah Jaber AlShahrani[6], Fahd A. Alshehri[7], Ahmad A. Alshehri[8], Mohammed Alissa[9], Ali A. Rabaan[10]*

[1]Department of Clinical Research, Clinical practise and Pharmacology, General and Integrative Medicine, Clinical Research, Health care and Hospital Administration Department.. National Institute of Medicine Sciences, Andhra Pradesh State, India. Email: myleakshaykiran@gmail.com
[2]Advanced Diagnostics and Therapeutics Institutes, Health sector, King Abdulaziz City for Science and Technology (KACST) Riyadh 11442, Saudi Arabia. Email: Mbakhrbh@kacst.edu.sa
[3]Department of Medical Microbiology, Faculty of Medicine, Al Baha University. Al Baha 4781, Saudi Arabia. Email: mhalwani@bu.edu.sa
[4]Pharmacy Practice Department, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam 31441, Saudi Arabia. Email: Bmalshehail@iau.edu.sa
[5]Department of clinical laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Shaqra 11911, Saudi Arabia. Email: aothman@su.edu.sa
[6]Public Health Department, Health affairs, Asir region, Abha 62523, Saudi Arabia. Email: ajalshahrani@moh.gov.sa
[7]Nursing administration, East Jeddah Hospital, Jeddah 22253, Saudi Arabia. Email: FALSHEHRI12@MOH.GOV.SA
[8]Department of Clinical Laboratory Sciences, Faculty of Applied Medical Sciences, Najran University, Najran 61441, Saudi Arabia. Email: aaalshehri@nu.edu.sa
[9]Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Prince Sattam bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia. Email: m.alissa@psau.edu.sa
[10]*Molecular Diagnostic Laboratory, Johns Hopkins Aramco Healthcare, Dhahran 31311, Saudi Arabia
[10]*College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia
[10]*Department of Public Health and Nutrition, The University of Haripur, Haripur 22610, Pakistan
Email: arabaan@gmail.com

***Corresponding Author:** Ali A. Rabaan
*Molecular Diagnostic Laboratory, Johns Hopkins Aramco Healthcare, Dhahran 31311, Saudi Arabia. Email: arabaan@gmail.com
*College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia
*Department of Public Health and Nutrition, The University of Haripur, Haripur 22610, Pakistan

Abstract:
Introduction: The COVID-19 pandemic caused by the SARS-CoV-2 virus has affected millions worldwide. In this study, we investigate the potential of CRISPR systems Cas12 and Cas13 for diagnosing and treating RNA viruses, particularly SARS-CoV-2.

Aim: This study aims to explore the role and application of CRISPR-system-based methods in detecting and treating RNA viruses, especially SARS-CoV-2, using Cas12 and Cas13 proteins.
Materials and Methodology: CRISPR enzymes, types V and VI, can target RNA or DNA through processes known as RNA and DNA targeting. Cas12 and Cas13 enzymes are specific for single stranded RNA viruses and play a crucial role in diagnosing and treating these viruses. We used the DETECTR assay to detect mutations in SARS-CoV-2, including circulating and rare or new variants, in 304 respiratory swab samples collected from patients.
Results: Our results showed that Cas12 and Cas13 enzymes could be used for diagnosing and treating SARS-CoV-2. The DETECTR assay was able to detect circulating and rare or new variants, including mutations in the 452, 484, and 501 sites of SARS-CoV-2 associated with different COVID-19 variants
Conclusion: Future studies should focus on using the DETECTR assay to screen for circulating and rare or new variants by targeting the crucial sites mentioned above, and these approaches could be applied for other RNA viruses' detection and treatment.

Acknowledgments
We would like to express our gratitude to the faculty members at the University and funding support from Prince Sattam bin Abdulaziz University project number (PSAU/2023/R/1444) and King Abdulaziz City for Science and Technology (KACST). We also acknowledge the support of our research team and the participants who provided the respiratory swab samples for this study.

Keywords: CRISPR-Cas system, DETECTR assay, SARS-CoV-2, RNA viruses, molecular detection, COVID-19 variants.

Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences are an adaptive immune system present in archaea and bacteria, where they provide antiviral and antiphage defenses. The CRISPR-Cas system consists of three major distinguishable classes with types with further ten subtypes each encoded by specific genes. Type I systems are produced by an operon containing cas1, 2, 5, 6 and 7 genes while the signature gene for type II systems is the cas9 gene. The cas9 gene is responsible for the formation of multidomain protein key in crRNA maturation. Type III systems typically express cas10 gene which also encodes a multidomain protein [118]. Type V systems is majorly characterized by a group of RNA-guided ds DNA targeting effector proteins called Cas 12. Additionally, Cas13 has been shown to be defining the group of RNA-led nucleases that target single-stranded RNA in type VI CRISPR-Cas system [119]. Of these, CRISPR-Cas9 has been implicated as the most essential leading to development of technology based on the CRISPR-Cas9 system due to its potential to edit the genes of living organisms.

In bacterial and archaeal species, CRISPR sequences are responsible for providing support with antiviral and antiphase defenses – a defense mechanism against the infection caused by phages. As such, technologies based on this system have been developed to explore avenues for antiviral therapy as well as biosensing through nucleic acid detection by quantitative PCR [119]. Owing to the noteworthy specificity as well as the versatility of the various CRISPR-related proteins especially Cas9, 12 and 13, CRISPR-Cas system has become a great tool for genetic engineering. Cas9 is one of the proteins involved in the CRISPR system and is used to find complementary DNA strands and break them apart after they have been located [12-13, 111, 116].

CRISPR-Cas9 is a powerful tool that can be used for genetic modification in living organisms. Its potential applications include creating disease-resistant crops, developing new gene therapies, and advancing our understanding of genetic diseases [14-15, 110, 117]. Thanks to CRISPR-Cas9 enzymes and CRISPR sequences, researchers now have a promising tool for advancing the field of genetic engineering.

Research Methodology

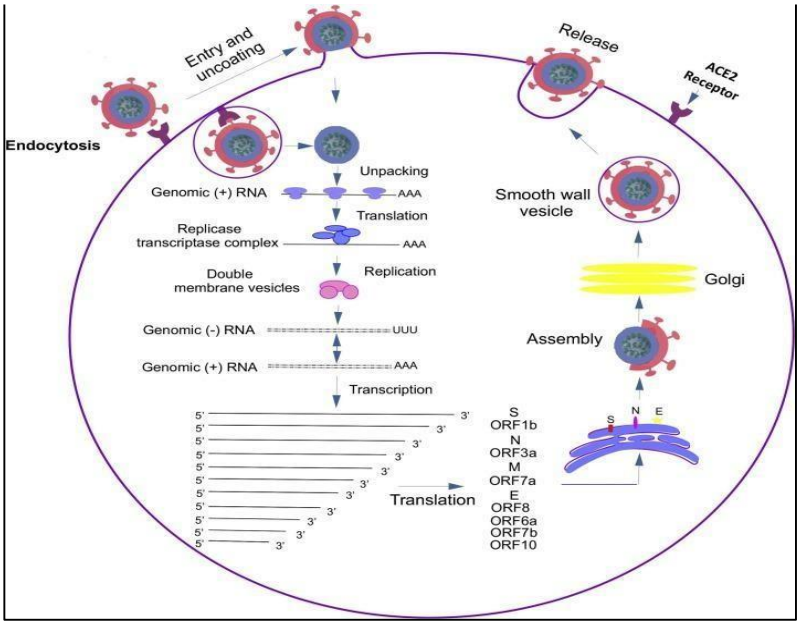
CRISPR-Based Methods for Diagnosing COVID-19 Variants: In this study, we aimed to diagnose COVID-19 variants using CRISPR-based methods. To achieve this, we utilized the DETECTR assay to detect SARS-CoV-2 mutations in respiratory swab samples collected from 304 patients. This technique involves modifying the Cas12 and Cas13 proteins present in the human body to bind complementary RNAs to their CRISPR RNAs, enabling the identification and targeting of RNA viruses, specifically ssRNA viruses such as SARS-CoV-2. By analyzing the samples, we identified mutations associated with each COVID-19 variant.

Detection of Alpha, Beta, Delta, and Omicron Variants using CRISPR: We also investigated the use of CRISPR-based methods to detect specific COVID-19 variants, including the Alpha, Beta, Delta, and Omicron variants. For each variant, we employed the DETECTR assay and modified the Cas12 and Cas13 proteins to bind complementary RNAs to their CRISPR RNAs, enabling the identification and targeting of ssRNA viruses. This technique enabled the identification of mutations associated with each variant in each sample.

Overall, our study demonstrates the effectiveness of CRISPR-based methods in diagnosing COVID-19 variants and highlights the potential for future development and improvement in this field.

3.Results and Analysis

3.1. Structure And Detection of Sars-Cov-2



When it infects host cells, the SARS-CoV-2 virus goes through various stages shown in Figure 1. During the second phase of the life cycle of the Coronavirus, the protein (S) creates a connection with the ACE2 receptor. Once the connection has been established, the endosomal pathway offers support allowing fusion of the viral envelope with the cell membrane [16-17, 109] enabling the virus to proliferate inside the cell. This process is then repeated upon release of the SARS-CoV-2 RNA into the environment as the virus is no longer hindered in its capacity to enter the host cell and to begin replicating [18-25].

Another essential step in the process is the translation of the genomic RNA which is key in the formation of viral replicase polyproteins required for viral replication. This was an unavoidable outcome because the procedure in question had already been completed (pp1a and 1ab). In addition, the polymerase enzyme, being an active participant in the transcription process, eventually synthesizes a wide variety of sub-genomic mRNAs thus facilitating gene copying from one form to another.

Protein synthesis then follows as the second stage in the manufacture of essential viral proteins, which occurs after translation of these mRNAs into their corresponding proteins [26-30, 108].
Upon completion of the aforementioned procedure, virions are then created by mixing the viral RNA extracted from the viral genome with the viral proteins producing the virus. The extraction process comes to a close with this stage. After exiting the Golgi complex, these viral particles enter vesicles and continue their journey through the cytoplasm until they are expelled from the cell. This process continues until the cell's contents can no longer be contained within it [31-32, 107].

Fig. 2 The SHERLOCK system entails combining RPA pre-amplification, Cas13 detection, and a colorimetric reporter for a visual readout.

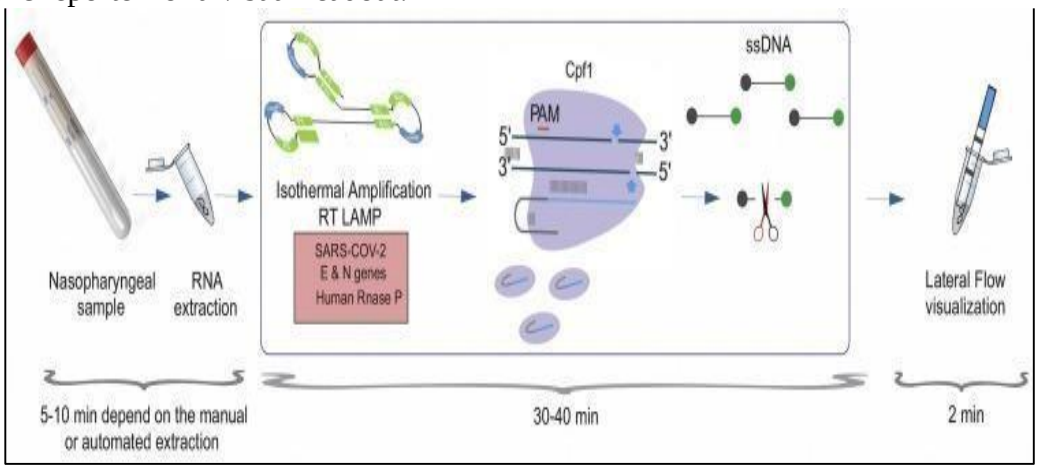


Fig. 3 The visual readout is produced by the DETECTR system by combining the LAMP pre-amplification with the Cas12a detection as well as the colorimetric reporter.

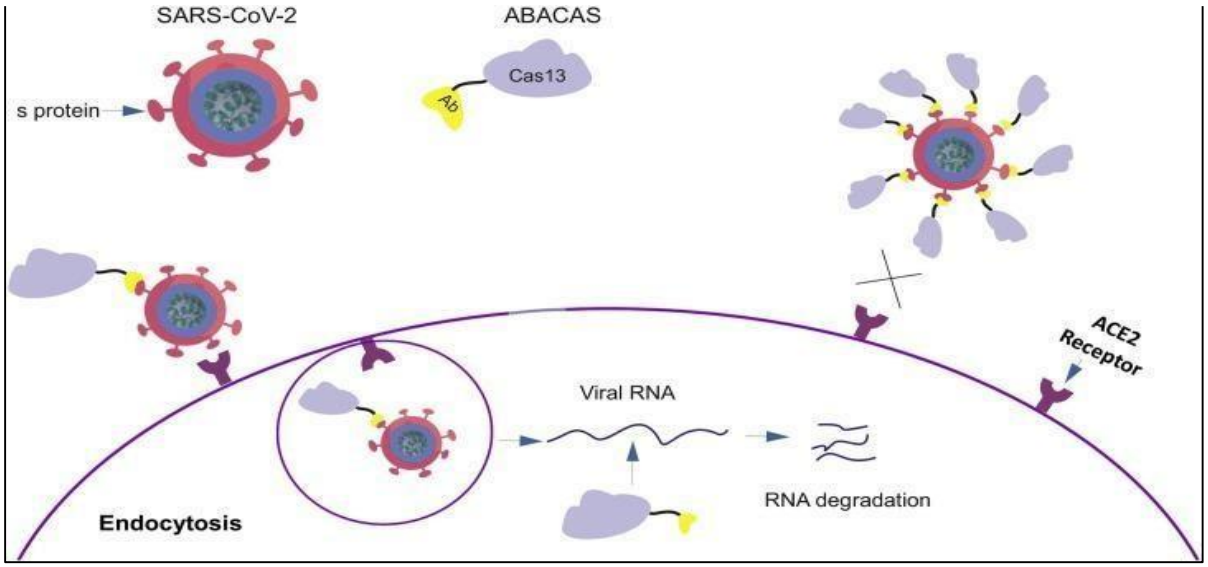


Figure 4: The diagram illustrates how the ABACAS virus and SARS-CoV-2 are connected.

Endocytosis is the mechanism by which viruses infiltrate cellular structures. At that exact moment, the ABACAS protein and the virus are absorbed into the cell. The release of ABACAS activates Cas13, which in turn destroys viral RNA. Additionally, there is the possibility that ABACAS might stop viral proteins from accessing target cells [34-40].

As such, laboratory tests that reveal SARS-CoV-2 mutations in a timely and accurate way could assist in the treatment of COVID-19 and in the management of infections. The authors developed and validated a quick COVID-19 variant DETECTR test. This test was used to detect variants. LAMP and

CRISPR-Cas12 are technologies used to search for SNP alterations in the spike (S) gene associated with SARS-CoV-2. This test aims explicitly to detect the L452R, E484K/Q/A, and N501Y mutations [41-45]. The vast majority of severe cases of COVI-19 infections exhibit the presence of at least one mutation.

CasDx1, an enzyme that belongs to the Cas12 family and just discovered relatively recently, was the only one that could recognize all of the targeted SNP changes. Using an analytical technique for CRISPR-based SNP identification from 261 clinical samples, the SNP concordance was confirmed to be 97.3% while the lineage classification agreement was determined to be 98.9% (258 of 261) [46-48,106].

The SARS-CoV-2 whole-genome sequencing and/or real-time (RT) PCR were used as comparative methods in this research endeavor. The reality of this situation was shown when the findings of pipeline testing were compared to those of earlier research. In addition, we found that a single mutation in the E484A gene was sufficient to differentiate Omicron from other large circulating abnormalities in patient samples. Another item that we uncovered was the fact that finding the mutation was required and could be sufficient for differentiation which denotes that patient samples might be used to differentiate between the two possibilities [49-55].

These findings suggest that the DETECTR approach, which is based on CRISPR, might be a more time and labor-efficient alternative to sequencing when it comes to detecting SARS-CoV2 mutations in clinical and public health laboratories. The method of sequencing has evolved into a regular procedure. Further mutations in SARS-CoV-2 may make COVID-19 more persistent. Even in regions with high COVID-19 immunization rates, SARS-CoV-2 variants, some particularly worrisome, have been linked to the outbreak of comeback outbreaks (VOCs). SARSCoV-2 variants have a higher risk of initiating viral mutations, which might ultimately result in sickness hence the need to detect such variants [1,2,5,6,7,8-11].

As a consequence of alterations in the spike protein, which interacts with the human ACE2 receptor, there is a chance that the virus might become more infectious in the future [12]. It makes no difference whether one acquires immunity via vaccination or natural means [13,-14]. In addition, variations may affect the treatment, which is particularly important considering that some mutations may reduce the efficacy of monoclonal antibody therapy for the disorder [15]. Monitoring SARS-CoV-2 variations in the community can potentially affect public policy about testing and immunization, in addition to assisting with tracing contacts. Despite the long turnaround times and the need for expensive laboratory equipment, viral whole-genome sequencing (WGS) and single nucleotide polymorphism genotyping (SNP genotyping) are often utilized to find changes [16-18]. Several diagnostic techniques are based on CRISPR [19], and a significant number of them can promptly identify SARS-CoV-2 in clinical samples [13, 20-23]. The FDA has authorized an emergency use authorization (EUA) for specific exams. Because of this clearance, it is permissible to carry out several emergency tests [24-26]. These assays, which can be used in labs as well as at the point of care, are not only inexpensive, but they also require very little in terms of equipment, and has a turn-around-time of less than two hours. Thus, they can be used in laboratories and at the point of care i.e., both clinical laboratories and field settings may bank on the reliability of these diagnostic tools [20, 23, 27-29].

3.2. Infection and sample collection of SARS-CoV-2

We created a CRISPR-based COVID-19 variant DETECTR tool to find SARS-CoV-2 mutations in 304 patient respiratory swab samples and compared its performance to that of WGS (hence abbreviated as DETECTR assay). Through this technique, mutations in SARS-CoV-2 were identified in each sample (Fig. 1A). This experiment used both the RT-LAMP pre-amplification approach and the CRISPR-Cas12 enzyme fluorescence detection method. Both methods are described below.

According to our analysis of a wide variety of potential Cas12 enzymes, high test performance necessitates the determination of specificities possessed by the recently discovered CRISPR-Cas12 enzyme CasDx1 in recognizing substantial SNP mutations of functional importance in the spike

protein at amino acid positions. This is the case because CasDx1 is the only enzyme capable of doing so. The demonstration that CasDx1 is responsible for regulating specificity allowed for the achievement of this outcome.

3.3. Multi-Channel Detection of Sars-Cov-2 And Other Respiratory Infectious Diseases

The DETECTR test was prepared for administration as shown. (A) A comparison of the DETECTR test to whole-genome sequencing (WGS) of SARS-CoV-2 using either a benchtop sequencer from Illumina or a nanopore sequencer from Oxford Nanopore Technologies (ONT).

(B) This is a depiction in the form of a schematic of a gRNA that was created to target SARS-CoV-2 S gene changes using the CRISPR-Cas system.

(C) A schematic demonstrating the positions of the SARS-CoV-2 S gene mutations (shown by arrows), primers (shown in black and black-gray), and gRNAs (shown in blue for wild-type and green for mutant) positioned within the S gene target region.

(D) A heat map analysis of the data obtained from the testing of three Cas12 enzymes employing 10 nM PCR-amplified synthetic gene segments over a period of thirty minutes [56-60]. (E) A dot plot with a total of n = 6 positive RT-LAMP duplicates derived from heat-inactivated viral cultures. These duplicates are identical to known variations within a dynamic range of 4 logs.

(F) A heat map that compares the fluorescence of viral cells that have been rendered nonviable by heat and the endpoint fluorescence of three Cas12enzymes recorded after 30 minutes [61-62,105].

The most efficient form of the CRISPR-Cas12 enzyme was used to detect SNPs. In order to discover which of the three distinct trans-cutting CRISPR-Cas effectors is the most efficient at locating SNPs, we put each of them through their paces in a series of tests. CasDx1, an uncharacterized Cas12 enzyme, was one of the effectors. The other effectors were LbCas12a and AsCas12a. The first thing that we did was investigate the impact of guide RNAs (gRNAs) made using CasDx1 and LbCas12a on synthetic gene segments that generated SARS-CoV-2 S-gene sections with either WT or MUT sequences at amino acid positions 452, 484, and 501 (Fig. 1B, C) (Fig. 1B, C) [63-65].

Using this initial activity screen, we could determine which gRNAs are the most effective for each S-gene mutation expressing L452R, E484K, or N501Y. (Fig. 1D). CasDx1 revealed the largest SNP difference between wild-type and mutant sequences for all targeted S-gene alterations when tested with their corresponding gRNAs on synthetic gene segments (Fig. 1D; Fig. S1A) (Fig. 1D; Fig. S1A). The LbCas12a gene could distinguish between SNPs at sites 452 and 484 but not at position 501, while the AsCas12a gene could only differentiate between SNP 452. In order to determine the identities of the multiple heat-inactivated viral cultures, the comprehensive DETECTR test was utilized to search for SNPs and seek for differences. For RNA extraction, multiplexed RT-LAMP amplification (Fig. 1C), and CRISPR-Cas12 detection, the spike receptor-binding domain (RBD)targeting guide RNAs were used (Fig. 1B) [66-70,104]. During the synthesis procedure, two sets of six LAMP primers were used to produce spike RBD amplicons that included all three mutations (L452R, E484K, and N501Y). There was evidence that the spike RBD amplicons of both groups overlapped. During the course of this pandemic, multiple escape mutations in the spike RBD were discovered, and as a result, we set out to improve the assay's resistance to being disrupted. Based on the results of the first tests, it seemed that a redundant LAMP design seemed to boost the detection sensitivity.

Consequently, we concluded that the best course of action would be to adopt a LAMP architecture with built-in redundancy [13]. We know that 91.7% of viruses will have one LAMP primer nucleotide mismatch, and 99.9% will have two mismatches based on inclusiveness research that used sample sequences from the Alpha, Beta, Gamma, and Delta subtypes [70-72].

This information was obtained via the use of sample sequences and through the analysis of test sequences. This suggests incorrect matches in the virus's genome causing the illness are prevalent (Data set S1). The research was carried out on the WA-1 strain of SARS-CoV-2 because it has the

wild-type (WT) version of the spike protein on the surface of its particles. In order to generate vaccines against this protein, licensed mRNA (either Pfizer's BNT162b2 or Moderna's mRNA-1273; 37, 38) was combined with a DNA adenovirus vector (J&J's Ad26.COV2. S; 39) [74-76]. In addition, VBMs acquired from previous viral cultures were also investigated (40). Using digital droplet PCR, the pre-amplification technique's analytical sensitivity was evaluated using heat-inactivated viral culture materials to quantify SARS-CoV-2 lineages within a dynamic range of 4 logs. This was done in order to determine whether or not the pre-amplification technique was effective. In order to assess whether or not the pre-amplification procedure was successful, this step was taken. There were seven distinct strains of the SARS-CoV-2 virus included among these samples. We used six sets of viral culture duplicates to test the likelihood of RT-LAMP amplification. When the virus was present at a concentration of 2 105 copies/mL (10,000 copies/reaction), all seven lineages of SARS-CoV-2 could successfully undergo amplification (Fig. 1E). This number is crucial to the process used to identify the SARS-CoV-2 variant (a value of 30 Ct, which is more than 2 105 copies/mL) [41-42].

CasDx1, LbCa12a, and AsCa12 were used to locate the SNPs responsible for the L452R, E484K, and N501Y alterations. Research should be done on the specificity of the Cas12 enzyme. CasDx1 was found in each LAMP-amplified, heat-inactivated viral culture at 452, 484, and 501, and it correctly identified wild-type (WT) and mutant (MUT) targets, as was expected based on gene fragment studies (Fig. 1F; Fig. S1B). LbCas12a could discriminate between WT and MUT targets at position 501 in LAMP-amplified viral cultures; however, it had a substantially higher background for the WT target at position 452 and for both WT and MUT targets at position 484. (Figure 1F; Figure S1B) [77,78].

Although there was a considerable lot of background noise, AsCas12a could discern between WT and MUT targets at position 452; however, it could not make this difference at positions 484 and 501. 1F; S1B). CasDx1 could recognize mutations such as L452R, E484K, and N501Y with a better degree of certainty if it had access to and used this information. Throughout the experiment, we used the high-fidelity CasDx1 enzyme, which increased the experiment's overall efficacy [79-80,103].

3.4. Difference Between Covid Alpha, Delta, Belta, Omicron

parameter	omicron	Delta	Beta	alpha
Transmission rate	More than delta	2x than alpha and beta	10%-20%more transmissible than delta	50% more transmissible than alpha
Detection by RT-PCR	Yes, detectable.2/3 genes detected	Yes	yes	yes
Infection severity	Unclear	More severe infections for unvaccinated people	More severe than other variants and can lead to hospitalization and death.	More severe than the SARS-COV-2 strain, it leads to hospitalization and death.
Prevention through vaccination	People vaccinated may contract and spread the virus	Effective against illness, hospitalizations, and death. Easily spread by vaccinated people.	Vaccinations are effective	Effective against diseases that are severe and can lead to hospitalization.

3.5. Statistics Showing Countries Affected by The Types Of Variants

Name of country	Type of variant	No of the people affected	No recovered people	diabetes	Pulmonary patient
Britain	Alpha	100000	47890	no	Yes
India	Delta	50000	40000	yes	Yes
South Africa	Beta	46679	43000	yes	Yes
Brazil	Gamma	200000	70000	yes	Yes
South Africa	Omicron	125790	25000	yes	Yes

4. Discussion

As a direct result of this research, we developed a CRISPR-based DETECTR test capable of detecting SARS-CoV-2 mutations. We investigated three unique CRISPR-Cas12 enzymes: LbCas12a from NEB, AsCas12a from IDT, and a customized enzyme from Mammoth Biosciences (CasDx1) [80-82]. CasDx1 correctly identified each of the SNPs that were being targeted, outperforming the other two enzymes in terms of activity levels.

The DETECTR test can differentiate between WT and MUT findings, depending on the analysis processes used. Our pipeline achieved an SNP concordance of 97.3% (1,326 out of 1,335 calls), and a lineage classification agreement of 98.9% with viral WGS samples (258 out of 261) [8486, 102]. We used the Dia Sorin variation test as a third comparator throughout the assessment of discrepancies. The DETECTR test and viral WGS yielded identical results for the categorization of variants and the identification of SNP mutations. Compared to using the DETECTR test, discovering new COVID-19 variants and keeping track of existing ones via sequencing is significantly more difficult and time-consuming [118-120]. Our research findings indicate that CRISPR-based diagnostics are most reliable when the Cas enzyme is produced in a manner that is suitable for the area being examined. The COVID-19 variation DETECTR test can identify the Alpha, Delta, Kappa, and Omicron variants when the L452R, E484K/Q/A, and N501Y SNP targets are present. However, it cannot resolve any of the other VBMs or VOIs. Monitoring significant changes, the majority of which are anticipated to be the result of convergent evolution, will become an increasingly crucial component of pandemic surveillance as the pandemic continues to spread [55]. In this work, we developed a CRISPR-based SNP calling data analysis pipeline that can automate the examination of the fluorescence signal pattern [8890, 101].

These investigations are limited in either the circulating lineage coverage they provide, the clinical sample assessment scope they provide, or both. Additionally, the tests themselves are somewhat complicated. Despite their effectiveness, CRISPR-based approaches have several significant shortcomings that must be overcome to detect SARS-CoV-2 mutations. To find N501Y, E484K, and Y144Del in eight unique lineages (WA-1, Alpha, Beta, Gamma, Eta, Iota, and Mu and Zeta), the mi SHER LOCK variant test uses LbCas12a (NEB) in conjunction with RPA pre-amplification [57]. To detect N501Y, E484K/Q/A, and L452R, including Omicron, in all circulating lineages, the DETECTR test uses CasDx1 with LAMP pre-amplification, combining two amplification methods. We analysed a total of 261 clinical samples from eight different lineages, which were WA-1, Alpha, Gamma, Delta, Epsilon, Iota, Mu, and Omicron. Through this research, we showed that Omicron can be correctly detected by using the E484 WT and A484 MUT values as our only criterion [93, 98-100].

5. Conclusion

In conclusion, this research showcases the potential of CRISPR-based diagnostic tests for identifying SARS-CoV-2 mutations. We developed a DETECTR test capable of detecting key SNPs associated with different SARS-CoV-2 variants, including the highly transmissible Omicron variant. Our results demonstrate that CRISPR-based approaches can provide reliable and accurate results when the Cas enzyme is produced in a suitable manner for the target area.

However, there are limitations to the current CRISPR-based tests, such as the inability to detect all mutations and the need for further improvement in differentiation between SNPs. Future studies should focus on developing more comprehensive CRISPR-based assays that can detect a wider range of mutations and improve the specificity of SNP identification.

Overall, CRISPR-based approaches have the potential to greatly enhance pandemic surveillance and response efforts. By providing rapid and accurate detection of SARS-CoV-2 mutations, CRISPR-based tests can help public health officials make informed decisions and take appropriate action to

control the spread of the virus. With continued advancements in CRISPR technology and the development of more comprehensive diagnostic assays, we can be better prepared to respond to future pandemics and emerging infectious diseases.

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