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M.Zh. Amanzholova[1], A.M. Shaizadinova[1,2], S.K. Abeldenov[1,3*]

[1]National Center for Biotechnology, Astana, Kazakhstan;
[2]al-Farabi Kazakh National University, Almaty, Kazakhstan;
[3]S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan
**Corresponding author: abeldenov@gmail.com*

Enhancing Pathogen Detection Methods through a Novel Molecular Diagnostic Approach with CRISPR/Cas Technology

Molecular diagnostics is widely recognized as one of the most efficient approaches for detecting and characterizing microorganisms. This method relies on the identification of specific nucleic acid sequences and enables the quick and precise determination of pathogen presence in various samples. Conventional methods for pathogen detection rely on the culture and identification of the pathogen in a laboratory setting, which can be time-consuming and expensive. Molecular diagnostic methods, such as PCR and DNA sequencing, have emerged as powerful alternatives to culture-based methods, offering greater sensitivity and specificity. However, these methods are still limited by their reliance on costly equipment and specialized expertise. In recent years, the CRISPR/Cas technology has emerged as a powerful tool for genome editing and manipulation, as well as for molecular diagnostics. This review presents a novel approach for improving pathogen detection methods through the utilization of CRISPR/Cas technology. The proposed method offers several distinct advantages over existing molecular diagnostic techniques. Notably, it provides enhanced specificity and accuracy, thereby minimizing the occurrence of false-positive results. Additionally, this method can rapidly and effectively detect pathogens, making it particularly attractive for use in clinical and laboratory settings. Therefore, molecular diagnostics using CRISPR diagnostics based on Cas12a is a powerful tool for pathogen detection and improving the accuracy and speed of diagnosis. Its prospects for future use are wide-ranging and may encompass many areas of life sciences.

Keywords: CRISPR, Cas12a, diagnostics, bacteria, viruses.

Introduction

CRISPR-based diagnostics: CRISPR (short palindromic repeats regularly spaced in clusters) is a revolutionary technology that allows precise editing of DNA sequences. In recent times, scientists have made significant progress in the development of CRISPR-based diagnostic methods that enable rapid and specific detection of particular nucleic acid sequences within a sample. These methods are very accurate and can potentially be very cost effective.

CRISPR/Cas

CRISPR/Cas is an innate adaptive immune system employed by prokaryotes, including bacteria and archaea, as a defense mechanism against viral and plasmid invasions. Upon initial infection by a virus, a small segment of the viral genetic material is integrated into the genome of the bacterium. Subsequently, if the same bacterium is exposed to the same virus again, it transcribes these integrated viral sequences into CRISPR RNA. The CRISPR RNA then combines with tracrRNA and a CRISPR-associated (Cas) protein, forming a complex. This complex recognizes and binds to complementary sequences present in the invading viral DNA or RNA. By doing so, it initiates the cleavage of the viral DNA or RNA, creating double-strand breaks (DSBs). The introduction of DSBs ultimately leads to the destruction of the virus, effectively neutralizing the viral infection

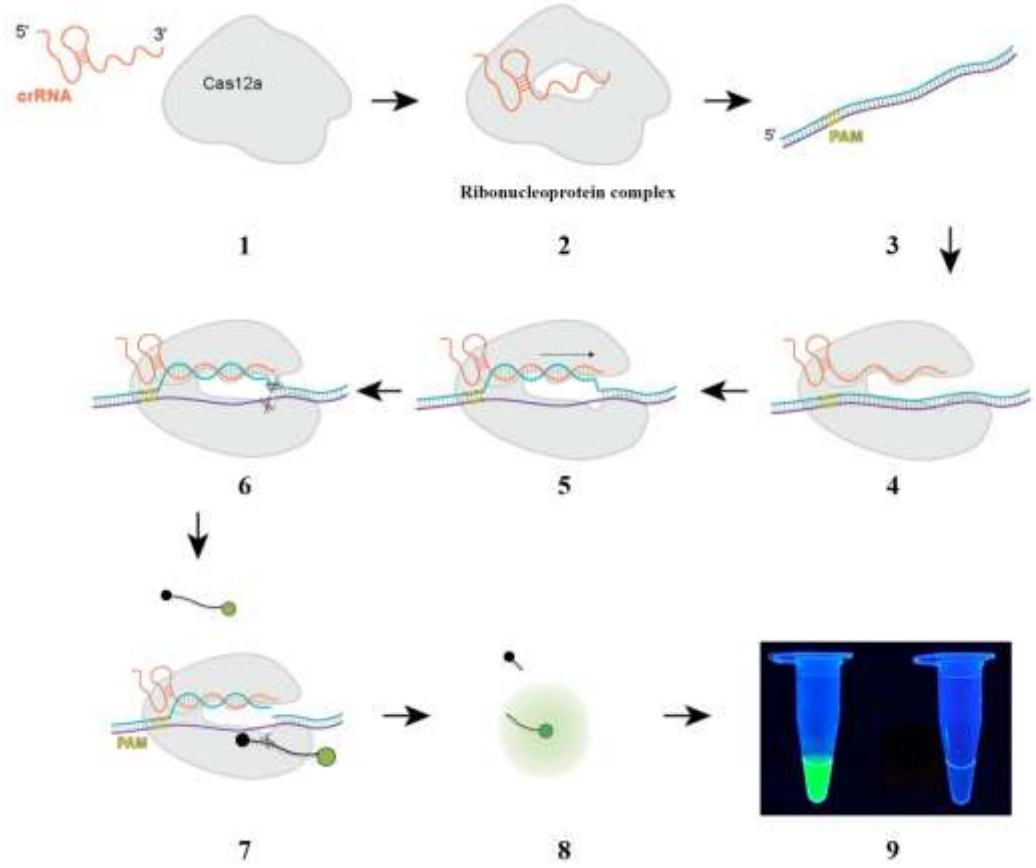
CRISPR-Cas systems function through a series of three main steps: adaptation, expression, and interference. Adjacent to the CRISPR array are genes that encode Cas proteins responsible for governing these phases of immunity: adaptation, CRISPR RNA (crRNA) biogenesis, and interference. During adaptation, foreign genetic material is selected, processed, and integrated into the CRISPR array, creating a memory of the infection. This memory is retrieved when the CRISPR array is transcribed, resulting in the production of crRNA which is further processed within the repetitive sequences to generate mature crRNA molecules. In the event of subsequent infection, the interference mechanism is activated, wherein the crRNA guides Cas

proteins to cleave complementary sequences known as protospacers within the foreign genetic material, effectively neutralizing the invader.

With the help of modern bioinformatics algorithms, in a fairly short time, it was possible to describe and classify the CRISPR-Cas system. The most studied Cas effector is Cas9. Quite quickly, the use of this enzyme led to preclinical studies in genome editing. Doudna and Charpentier received the 2020 Nobel Prize in Chemistry for their significant contributions to the advancement of CRISPR-Cas9 genome editing technology.

In 2015, a new endonuclease Cas12a was discovered [1]. This enzyme has a number of differences in the mechanism of action. First, Cas12a uses only one guide RNA. Second, Cas12a utilizes a specific region known as the protospacer adjacent motif (PAM), which is a TTTN sequence, to bind the target sequence to the guide RNA. The PAM motif is necessary for the CRISPR-Cas system to be able to distinguish its own nucleotide sequence from a foreign one. Third, Cas12a forms sticky-end double-strand hydrolysis of DNA, while Cas9 forms blunt ends. All of the above features of the enzyme's mechanism of action make it an alternative to the well-studied Cas9 in certain cases.

In 2018, it was discovered that Cas12a possesses an additional function that, after binding to the target sequence, the enzyme undergoes conformational changes and begins to cleave any single-stranded DNA [2]. Furthermore, this additional activity exhibited by Cas12a is commonly referred to as collateral or trans-cleavage activity, which will be further used for diagnostic purposes by adding fluorescently labeled single-stranded DNA (reporter with a quencher) to the reaction, the cleavage of which leads to fluorescence. Most homologues exhibit this activity (Fig. 1). Swarts described the mechanism of cis- and trans-cleaving DNase activity [3].



1 – crRNA design; 2 – Cas12a forms a ribonucleoprotein complex with crRNA; 3 – adding a substrate; 4 – Cas12a-crRNA searches for sequences of the PAM region; 5 – Upon recognition of the PAM site, a complex is formed with the target; 6 – Cas12a cleaves the substrate, creating a double-strand break; 7 – Target recognition/cleavage is followed by non-specific reporter cleavage; 8 – Cleavage of the reporter releases the fluorophore; 9 – Fluorescence is detectable with the naked eye

Figure 1. CRISPR/Cas12a detection mechanism

Cas effectors from types V (Cas12a) and VI (Cas13a) can be effectively utilized in the development of biosensor systems. Since their discovery, these enzymes have received immediate attention due to their differences from Cas9. These Cas effectors have the side activity of non-selectively cleaving single-stranded oligonucleotide sequences upon recognition of a specific target. If the activity of the first enzyme is a single turnover, then the trigger activity exhibits a multi-turn mechanism, which is very useful in the development of biosensor systems. Indeed, the Cas enzyme can recognize its target nucleic acid, and then its trigger activity can be used to report this event in an enhanced way.

To enhance the sensitivity of CRISPR diagnostics, pre-amplification methods such as (LAMP) loop-mediated isothermal amplification and (RPA) recombinase polymerase amplification are utilized.

Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) represents a straightforward and rapid DNA amplification technique, suitable for deployment as a preliminary amplification approach in CRISPR/Cas-centered diagnostic procedures. This approach employs a group of four to six primers designed to precisely focus on numerous segments of the intended DNA sequence, ultimately leading to the precise and heightened amplification of the desired sequence [4].

Loop-mediated isothermal amplification (LAMP) is based on the activity of the Bst DNA polymerase enzyme from *Bacillus stearothermophilus* at 60-65 °C. The formation of specific structures occurs through the transformation into a “stem-loop” structure, facilitated by the use of four pairs of primers (forward outer primer (F3), reverse outer primer (B3), forward inner primer (FIP), and reverse inner primer (BIP)). Subsequently, the “stem-loop” structure, containing multiple initiation sites, serves as the reaction matrix for cyclic amplification in the LAMP process, ultimately generating multiple nucleotide chains of varying lengths.

Utilizing LAMP as a preliminary amplification technique in Cas12a-based diagnostics offers numerous benefits. LAMP stands out as a rapid and uncomplicated procedure, devoid of the necessity for specialized apparatus or specialized knowledge. Furthermore, LAMP exhibits the capability to magnify extremely minute amounts of the intended DNA, underscoring its exceptional sensitivity. Integrating LAMP with Cas12a-based diagnostics has the potential to heighten the assay's sensitivity, thereby diminishing the potential for incorrect negative results [5-9].

Recombinase polymerase amplification (RPA)

RPA, or recombinase polymerase amplification, is an innovative DNA amplification technique used to amplify targeted regions of genetic material. This method has been developed to provide high sensitivity and specificity, as well as a fast amplification process, making it suitable for various applications such as infection diagnosis, genetic research and biological research.

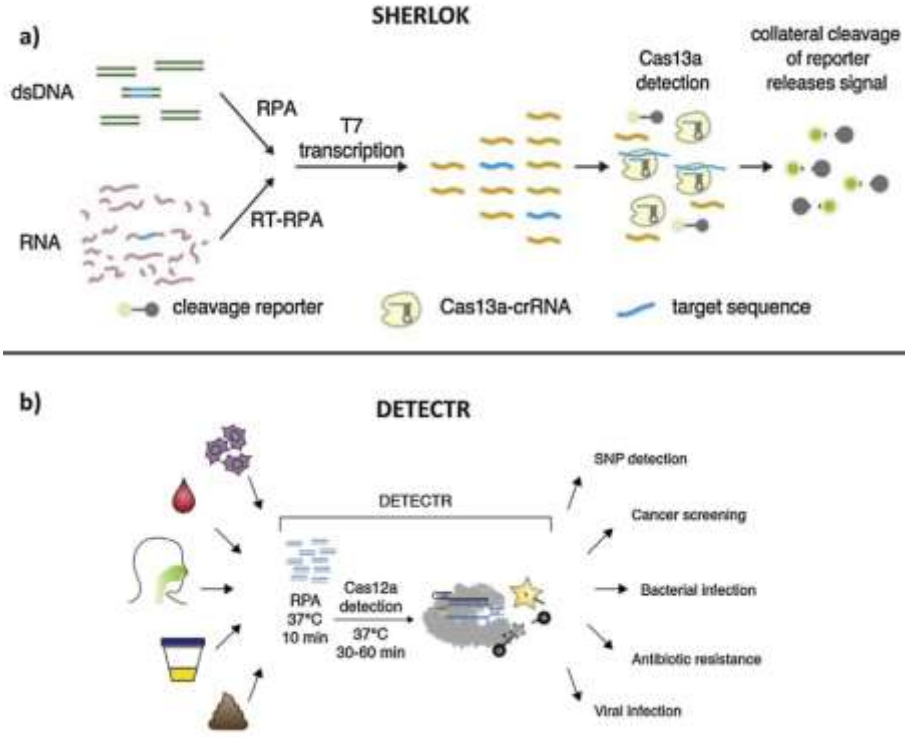
The basic idea of RPA is that specific recombinases, such as the UvsX protein from the T4 virus, are used to separate two complementary target DNA sequences. After that, short single-stranded starting sequences, known as primers, are attached to them. Primers have the property of directing recombinases to the target sequence, and then the polymerase enzymes begin to synthesize a new DNA strand using one of the separated strands as a template.

RPA occurs under isothermal conditions, that is, at a constant temperature, and this provides a fast amplification reaction without the need for thermal cycling, which is required for PCR. This method is specific and sensitive, since the use of specific primers allows you to select only those DNA regions that correspond to the desired target sequence. The result is an amplification of only the DNA that is really of interest to the researcher, which significantly increases the efficiency of diagnostics and analysis [10-12].

CRISPR-based SHERLOK and DETECTR platforms

Several publications have reported the use of Cas enzymes in combination with various reading strategies such as fluorescent, colorimetric and electrochemical methods. In 2017, Zhang and his collaborators first discovered the side activity of Cas13a and demonstrated its possible use for biosensing through a highly sensitive enzymatic reporter (SHERLOK) system. In this system, a target RNA or DNA gene was amplified and transcribed with RT-RPA + T7 (or RPA-T7) and then recognized with a specific Cas13a/crRNA complex. This triggered side activity and cleavage of the quenched reporter RNA resulting in an increase in fluorescence that was recorded in real time. System sensitivity was similar to digital drop polymerase chain reaction (ddPCR) and quantitative PCR (qPCR). The Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOK) platform is a molecular diagnostics system that leverages the enzymology of CRISPR-Cas to selectively identify specific DNA or RNA targets [13]. Viruses such as Zika, Dengue Fever and African Classical Swine Fever have been detected using SHERLOK.

In 2018, Doudna and her colleagues made a significant discovery regarding the side activity of Cas12a, which they subsequently harnessed for biosensor research. They pioneered the development of the DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) system, utilizing the CRISPR-Cas12a technology (Fig. 2). The Cas12a/crRNA complex was able to detect the target DNA amplified by RPA and the side activity was used to cleave the single-stranded fluorescence quencher DNA reporter [14]. The reaction proceeds quickly (~30 min), the method is inexpensive and accurate for the detection of viral infections as well [2].



a)SHERLOK; b) DETECTR

Figure 2. Diagnostic platforms: a) SHERLOK; b) DETECTR [15]

After the discovery of trans-cleavage activity, the scope of Cas12a for diagnostic purposes began to expand rapidly.

Virus diagnostics based on CRISPR-Cas12a

The global COVID-19 pandemic has prompted the rapid advancement of cost-effective and efficient diagnostic solutions. A huge number of Cas12a-based methods with different isothermal amplification methods, different targets, different reaction time, detection limit and method of reading the result have been proposed in publications, for example, methods iSCAN, DETECTR, AIOD-CRISPR, CASdetec, CRISPR-FDS, ITP-CRISPR, CRISPR/Cas12a-NER, Lyo-CRISPR, dWS-CRISPR, PGMs-CRISPR, symRNA-Cas12a, VaNGuard, deCOViD, CASCADE, opvCRISPR and CRISPR-ENHANCE.

The attractiveness of methods using isothermal amplification (isothermal loop amplification, recombinase polymerase amplification) is that a portable thermoblock or water bath is enough to perform them.

Bacterial diagnostics based on CRISPR-Cas12a

In addition to the detection of viruses, a significant number of publications are devoted to the detection of bacterial pathogens. Publications began to appear on the diagnosis of such bacterial infections as tuberculosis [16] and *Yersinia pestis* [17]. There are also articles on the detection of such important pathogens as *Salmonella typhimurium* (salmonellosis) [18], *Bacillus anthracis* (anthrax) [19], *Francisella tularensis* (tularemia) [10], *Escherichia coli* O157: H7 (acute diarrhea) [8], *Helicobacter pylori* (gastritis, peptic ulcer, stomach cancer) [11].

In addition to determining the pathogen, using CRISPR-Cas12a it is possible to determine the antibiotic resistance of pathogens, which will allow for the correct antibiotic therapy. Articles on the diagnosis of antibiotic resistant *Staphylococcus aureus* (MRSA strain) [20] and *Klebsiella pneumoniae* [9] have been published.

The implementation of the CRISPR-Cas12a technique in the agriculture

Researchers and analysts pin hopes that field diagnostics in agriculture will serve as a revolutionary moment in the use of the Cas12a enzyme.

Jiao et al. published results on diagnosing apple tree viruses [21]. Luo et al. described the RPA-Cas12a system for the identification of *Xanthomonas arboricola*, a bacterial pathogen of peach [22]. Fungal diseases of citrus fruits lead to significant yield losses. In a study by Shin et al, an RPA-Cas12a diagnostic for citrus scab was established. A feature of the work is the use of immunochromatographic test strips and the possibility of analysis within 1 hour [12].

In addition to the ability to determine the presence of a pathogen, work is underway to determine GMOs [23]. A highly sensitive fluorescent analysis has been successfully developed for the detection of organophosphate pesticides [24]. The latest research focuses on the development of the platform, which aims to enable early detection of phytopathogens.

Lin et al. have presented a novel method for the detection of pathogenic *Aeromonas hydrophila* using CRISPR technology. In this study, the researchers devised a detection method that is rapid, reliable, sensitive, and could be applied without any specialized equipment. The method includes recombinase amplification and Cas12a technology to identify pathogen. Method exhibits high sensitivity, enabling the rapid detection in less than 1 hour with a LoD of 2 copies of target. Moreover, the method demonstrates excellent specificity [25].

Wang et al used a similar method to accurately identify and detect *Staphylococcus aureus* in clinical specimens [26]. *Staphylococcus aureus* is a significant contributor to hospital-acquired infections. Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) result in higher mortality rates compared to those caused by methicillin-susceptible *Staphylococcus aureus*, posing a serious global concern. Consequently, there is a critical need for the prompt and highly sensitive identification of patients with clinical staphylococcal infections, as well as timely implementation of infection control measures. One promising approach lies in utilizing CRISPR and CRISPR-associated proteins (Cas) for nucleic acid detection methods, known for their exceptional diagnostic features. In this regard, a powerful method has been introduced, which combines CRISPR with RPA and employs a fluorescent detection for precise clinical identification of *Staphylococcus aureus* in samples. The results have demonstrated that technology can detect as low as ten copies within a 1-hour time. Furthermore, specificity analyses have confirmed the technology's ability to differentiate *Staphylococcus aureus* from other relevant pathogens in clinical settings. Notably, the results have exhibited strong agreement with antimicrobial susceptibility testing and PCR testing. These findings highlight the technology's exceptional diagnostic parameters, making it an indispensable tool for fast identification of *Staphylococcus aureus*.

Chen et al. conducted a comprehensive review of the current techniques employed for different viruses [27]. The prompt and accurate diagnosis of these viruses is crucial for implementing preventive measures to contain the spread of these diseases. While reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR are established and robust methods, there’s a need in specific equipment. In recent years, LAMP and RPA, have emerged as rapid, and equipment-free alternatives for POC diagnostics.

Detection of foodborne pathogens

Based on statistics provided by the World Health Organization (WHO), it is estimated that approximately 600 million individuals globally become ill due to the consumption of contaminated food each year. This leads to approximately 420,000 deaths annually, in addition to significant economic losses. These figures highlight the significant impact of foodborne illnesses on public health, underscoring the importance of ensuring food safety and implementing effective preventive measures throughout the food supply chain. Bacterial food and environmental contamination, *Escherichia coli* (*E.coli*), *Listeria monocytogens* (*L. monocytogens*), *Staphylococcus aureus* (*S. aureus*), *Salmonella* species (spp.) [28], poses a constant threat to food safety products, which is a global public health problem. The application of fluorescent sensing technology, utilizing the CRISPR-Cas12a system, has extended beyond disease diagnosis and ventured into the realm of food safety monitoring. This advancement has introduced a novel approach to food safety testing, offering a new strategy in ensuring the quality and safety of food products. By leveraging the CRISPR-Cas12a system

in combination with fluorescent sensing, rapid and accurate detection of potential contaminants or pathogens in food samples can be achieved, thereby enhancing food safety standards and safeguarding public health.

Zhang et al. constructed the corresponding cRNA by selecting the *Vibrio parahaemolyticus* specific heat-labile hemolysin gene as the target sequence, pre-installed the CRISPR-Cas12a system in the cap of the tube, mounted it on a microthermocycler, and then performed PCR amplification. After mixing the reagents, they were subjected to centrifugation and incubation. The minimum concentration achieved by this method was determined to be several copies [29].

Chen et al. developed the CRISPR-Cas12 system for rapid identification of bacterial genotypes in urinary tract infections, which detected concentrations of ampicillin-resistant (AmpR) *E. coli* in urine samples up to 10^3 CFU/mL within 1 hour, allowing accurate decisions on antibiotic treatment for 1 hour [30].

Conclusion

This review provides an up-to-date assessment of the recent advancements in the field of molecular diagnostics for pathogen detection. Infectious diseases are highly prevalent and often result in severe conditions that pose significant risks to human life and well-being. Effective detection methods are critical for accurate diagnosis and timely treatment. Therefore, the development of detection technologies plays a crucial role in achieving rapid and precise pathogen detection. Conventional methods employed for pathogen detection typically involve microbiology, microscopy, enzyme immunoassays, PCR-based detection methods, and others. However, these methods suffer from limitations such as lengthy reaction times and weak sensitivity. Hence, there is a growing necessity to explore and develop new approaches for pathogen detection.

CRISPR diagnostics utilize isothermal amplification methods, such as LAMP and RPA, as initial-amplification techniques for the targets. Compared with conventional PCR, LAMP has the advantages of higher sensitivity, shorter reaction time and easy operation. The RPA reaction temperature ranges from 37°C to 42°C for 5–60 min, depending on the initial concentration of nucleotides. The LAMP reaction temperature is 65°C, the reaction time is 15–60 min. The combination of Cas12a, LAMP and RPA can provide ultrasensitive nucleic acid detection. Pathogenic microorganisms, mycotoxins and genetically modified crops are key food safety concerns. The development of new detection technologies is essential to achieve and respond to potential food safety. CRISPR-Cas12a, a biosensor analysis technology, has a huge advantage in combating food biosafety agents and needs to be further developed.

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М.Ж. Аманжолова, А.М. Шайзадинова, С.К. Абельденов

CRISPR/Cas технологиясын қолдану арқылы молекулярлық диагностикаға жаңа көзқараспен патогенді анықтау әдістерін жетілдіру

Молекулярлық диагностика микроорганизмдерді анықтау мен сипаттаудың ең тиімді әдістерінің бірі ретінде кеңінен танылды. Бұл әдіс нуклеин қышқылының белгілі бір реттілігін анықтауға негізделген және әртүрлі үлгілерде патогеннің болуын тез және дәл анықтауға мүмкіндік береді. Патогенді анықтаудың дәстүрлі әдістері зертханада қоздырғышты өсіруге және анықтауға негізделген, бұл уақыт пен шығынды қажет етеді. ПТР және ДНҚ секвенциясы сияқты молекулярлық диагностикалық әдістер жоғары сезімталдық пен ерекшелікті ұсына отырып, тарату әдістеріне қуатты балама болады. Дегенмен, бұл әдістер қымбат жабдыққа және арнайы білімге тәуелді болғандықтан әлі де шектеулі. Соңғы

жылдары CRISPR/Cas технологиясы геномды өңдеу мен манипуляциялаудың, сондай-ақ молекулалық диагностиканың қуатты құралына айналды. Шолуда CRISPR/Cas технологиясын қолдану арқылы патогенді анықтау әдістерін жақсартудың жаңа тәсілі ұсынылған, бұл әдістің қолданыстағы молекулалық диагностика әдістеріне қарағанда бірнеше айқын артықшылықтары бар. Атап айтқанда, ол жоғары нақтылық пен дәлдікті қамтамасыз етеді, осылайша жалған оң нәтижелердің пайда болуын азайтады. Сонымен қатар, бұл әдіс патогендерді тез және тиімді анықтай алады, бұл оны клиникалық және зертханалық жағдайларда қолдануға ерекше тартымды етеді. Осылайша, Cas12a негізіндегі CRISPR диагностикасын қолданатын молекулалық диагностика патогендерді анықтауға және диагностиканың дәлдігі мен жылдамдығын арттыруға арналған қуатты құрал болып саналады. Оның болашақта пайдалану перспективалары өте кең және өмір туралы ғылымның көптеген салаларын қамтуы мүмкін.

Кілт сөздер: CRISPR, Cas12a, диагностика, бактериялар, вирустар, молекулярлы диагностика, қоздырғышты анықтау.

М.Ж. Аманжолова, А.М. Шайзадинова, С.К. Абельденов

Совершенствование методов обнаружения патогенов с помощью нового подхода к молекулярной диагностике с использованием технологии CRISPR/Cas

Молекулярная диагностика широко признана одним из наиболее эффективных подходов к обнаружению и характеристике микроорганизмов. Данный метод основан на идентификации специфических последовательностей нуклеиновых кислот и позволяет быстро и точно определить присутствие патогена в различных образцах. Традиционные методы обнаружения возбудителя основаны на культивировании и идентификации возбудителя в лабораторных условиях, что может потребовать много времени и средств. Методы молекулярной диагностики, такие как ПЦР и секвенирование ДНК, стали мощной альтернативой культуральным методам, предлагая более высокую чувствительность и специфичность. Однако они по-прежнему ограничены из-за их зависимости от дорогостоящего оборудования и специальных знаний. В последние годы технология CRISPR/Cas стала мощным инструментом для редактирования и манипулирования геномом, а также молекулярной диагностики. В настоящем обзоре представлен новый подход к совершенствованию методов обнаружения патогенов за счет использования технологии CRISPR/Cas, который имеет ряд явных преимуществ по сравнению с существующими методами молекулярной диагностики. В частности, он обеспечивает повышенную специфичность и точность, тем самым сводя к минимуму возникновение ложноположительных результатов. Кроме того, этот метод может быстро и эффективно выявлять патогены, что делает его особенно привлекательным для использования в клинических и лабораторных условиях. Таким образом, молекулярная диагностика с применением CRISPR-диагностики на основе Cas12a является мощным инструментом для выявления возбудителей и повышения точности и скорости диагностики. Его перспективы для использования весьма обширны и могут охватывать многие области наук о жизни.

Ключевые слова: CRISPR, Cas12a, диагностика, бактерии, вирусы, молекулярная диагностика, выявление возбудителей.