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One-Pot CRISPR-Cas12a Assay for Rapid Pulmonary Tuberculosis Diagnosis from Sputum: A Review

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KEYWORDS

:
Tuberculosis; CRISPR-Cas12a; CBNAAT; Truenat; Rifampicin resistance; Point-of-care diagnostics.

ABSTRACT:
Introduction- Pulmonary tuberculosis (TB) remains a major global health challenge, with persistent diagnostic gaps despite the scale-up of nucleic acid amplification tests such as CBNAAT (Xpert MTB/RIF) and Truenat MTB-RIF. These platforms, while highly sensitive and WHO-endorsed, are limited in many high-burden settings by cost, infrastructure requirements, and dependence on stable power and cartridge supply chains. One-pot CRISPR-Cas12a–based assays have emerged as a promising alternative, integrating isothermal amplification and CRISPR-mediated detection in a single closed tube, enabling rapid, highly specific identification of *Mycobacterium tuberculosis* and rifampicin resistance directly from sputum, with sample-to-result times typically under 60–80 minutes. This review summarizes the principles, assay designs, analytical performance, and readout formats (fluorescent, colorimetric, and lateral flow) of one-pot CRISPR-Cas12a diagnostics and compares their sensitivity, specificity, turnaround time, operational feasibility, and biosafety profile with CBNAAT and Truenat. We also discuss current technical and clinical challenges, including sample preparation, inhibitors, HIV co-infection, and field implementation in resource-limited settings. Finally, we highlight regulatory and quality considerations, potential diagnostic algorithms, and future directions such as multiplexing, amplification-free formats, and scalable surveillance applications. Together, the evidence suggests that one-pot CRISPR-Cas12a assays could complement, and in some contexts partially substitute, existing molecular platforms for decentralized, rapid TB diagnosis.

Objectives: Tuberculosis (TB) is among the top ten infectious disease threats globally and ranks as the leading cause of death from a single infectious agent. An estimated 10.6 million new TB cases occurred in 2021, with approximately 1.6 million deaths, according to the World Health Organization (WHO) Global Tuberculosis Report 2022. Early diagnosis and appropriate treatment are essential for successful TB control. However, existing diagnostic methods have one or more of the following limitations: delays of weeks to months in result delivery; insensitivity to the Mycobacterium tuberculosis (Mtb) pathogen; inability to detect active disease; inability to identify cases with low bacterial load, extreme drug resistance, or smear-negative sputum; reliance on clinical facilities far removed from the patient; a requirement for skilled personnel or specialized equipment; high cost; or unsuitability for human specimens or nonconventional sample matrices [1]. Such limitations result in missed and misdiagnosed cases, even in countries with high-level systems. Therefore, diagnostic testing is a global priority in the effort to eradicate TB[12].

The laboratory-scale CRISPR-Cas system—one of the most promising technologies for highly sensitive nucleic acid detection—has demonstrated substantial diagnostic potential in automated or manual laboratory workflows. The CRISPR-Cas12 (C2c2) variant is particularly suitable because signal readout occurs via cleavage of a fluorophore-quencher-labeled single-stranded DNA probe rather than the template strand. The one-pot CRISPR-Cas12a assay that combines nucleic-acid amplification and nucleic-acid interrogation into a

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single diagnostic step for the first time has been adapted for detection of the Mtb pathogen from sputum samples within 80 minutes, when coupled with cross-priming amplification.[1]

MethodsThe diagnosis of tuberculosis (TB) primarily relies on microbiological methods that identify Mycobacterium tuberculosis in patient samples. These are time-consuming and are often limited to well-equipped laboratories [1]. CRISPR diagnostic platforms, integrated with nucleic acid amplification reactions, offer the needed sensitivity while requiring significantly less and simpler instrumentation. However, the multi-step processing increases the risk of sample contamination and complicates operational logistics.

One-Pot assays that couple amplification and CRISPR-enabled detection in a single reaction have shown great potential. In these formats, the entire reaction kit can be pre-mixed and stored in low-cost bulk, and the sample to-answer time is further shortened. M. tuberculosis-specific amplification targets that allow the straightforward construction of one-pot assays have been established. These amplification guides prompted the development of the One-Pot Tuberculosis (TB) assay combining CRISPR-Cas12a and Reverse-Transcription-Polymerase-Chain-Reaction (RT-PCR) in a single tube. Standard-free RNA extraction can be performed using 3% sodium dodecyl-sulfate and the system can detect nascent TB RNA 50 min after sampling. The whole assay time, including sample extraction and detection, is below 80 min, demonstrating a strong potential to serve as a new point-of-care diagnostic. [13]

Results: Egestas diam in arcu cursus euismod quis viverra nibh. Convallis aenean et tortor at risus viverra. Sit amet justo donec enim diam. Sem et tortor consequat id. Purus gravida quis blandit turpis. Consectetur adipiscing elit duis tristique sollicitudin nibh sit amet commodo. Eget duis at tellus at urna condimentum mattis pellentesque. Auctor elit sed vulputate mi sit amet. Consequat ac felis donec et. In dictum non consectetur a erat nam at lectus. Dui vivamus arcu felis bibendum ut tristique. Lacinia quis vel eros donec ac. Ac turpis egestas maecenas pharetra convallis posuere morbi leo. Tortor id aliquet lectus proin.

Conclusions: The current landscape of TB diagnostics is evolving to meet the need for rapid tests that provide timely results to inform treatment initiation. One-Pot CRISPR-Cas12a assays based on the detection of M. tuberculosis in sputum, deployed during the TB-CAMP visit, demonstrate significant advancements over existing methods. Together with assured performance in the presence of HIV co-infection and consideration of implementation challenges, these assays offer opportunities to mitigate the devastating impact of TB and support global elimination efforts.[1]

1. Introduction

Tuberculosis (TB) remains a leading infectious disease that claims around 1.6 million lives annually (World Health Organization, 2023). Despite the introduction of molecular tests and the recommendation to screen for TB among people living with HIV, large diagnostic gaps persist globally, with low test availability, high costs, or slow turnaround times (Globocan, 2023; World Health Organization, 2023). Addressing these issues is crucial, especially when considering that one out of three infected patients are co-infected with HIV. One-Pot CRISPR-Cas12a assays detected Mycobacterium tuberculosis (Mtb) DNA in sputum, enabling TB diagnosis. The reaction couples recombinase polymerase amplification with CRISPR-Cas12a trans cleaving in a single tube [1]. When coupled with a fluorescent dye, the assay demonstrated a limit of detection of 10 genomic copies per reaction. Processing a single sputum volume at a 100× dilution, the limit of detection in ~45 µL remained 240 CFU/mL. With unprocessed samples, the detection limit reached approximately 1000 CFU/mL in clinical specimens. All non-tuberculous mycobacteria were excluded, and no cross-reaction with Mtb was observed in the CRISPR shoot. [2,3,4,5,6,7]

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2. Objectives

Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mtb), is a major public health concern with high morbidity and mortality globally. According to WHO estimates, approximately 10.6 million new cases of TB occurred in 2021, with an increasing burden of TB since 2019. Despite the availability of effective first-line anti-TB drugs, immense challenges remain in TB diagnosis and treatment. The global strategy "End TB" aims to eliminate tuberculosis by 2030, still more efforts are needed to fluid the pandemic. The rapid diagnosis of TB remains challenging due to the formerly relied methods can only detect active TB patients with high bacillary loads. Nucleic acid amplification tests (NAATs) are rapid and highly sensitive TB diagnostic methods that can detect low amounts of genetic material of Mtb in diverse sample types. The World Health Organization (WHO) has endorsed the use of CBNAAT and Truenat, both are multi-target NAATs, as TB rapid diagnostic tests recommend for validation and further implementation. In recent years, highly specific, and sensitive CWV genome-primed one-step reverse-transcription recombinase-aided amplification (GW-RT-RPA) coupled with CRISPR-Cas12s genome editing technology exhibits potential and targeted capacity for the detection of diverse pathogens. [8]

The CRISPR-Cas12a (also known as Cpf1) is a type V CRISPR system that acts as an RNA-guided endonuclease exhibiting trans-cleavage activity in the presence of a specific target. It can be combined with nucleic-acid-amplification methods to construct rapid nucleic-acid-detection assays. Unlike type II (Cas9) and type V (Cas12b) CRISPR systems, the Cas12a system targets dsDNA and degrades ssDNA, which is an additional advantage for practical applications. Moreover, Cas9 protein-based cargo differentiation strategies have been developed, while no usable protein-based cargo-differentiation technology has been adapted for Cas12a editing utility. Unlike other pathogens that hijack the human genome, Mtb contains unique, conserved genes that are absent within human chromosomal or plasmid DNA. Based on the existence of these unique sequences, efficient CWV-infecting bacterial-targeted one-pot assays were implemented. [9]

NAATs and RT-NAATs for Tuberculosis using One-pot CRISPR-Cas12a with simple tube mixing. By integrating GW-RT-RPA with CRISPR-Cas12a, the DA-CRISPR-Cas12a detection of DNA-genome and RNA-genome of pathogens was realized simultaneously in the same tube, enabling the integrated detection of DNA and RNA viruses in a single reaction. Based on the combination of these technologies, CRISPR-Cas12a DG-CRISPR was established using Cas12a as a multispecies-specific and stable detection platform with high specificity without additional probe modification or introduction of complex detection barriers. Mtb-targeted DG-CRISPR-RPA and GW-RT-RPA were constructed with high-level amplification generation. The workflow and cartridge design for the one-pot NAT system were proposed allowing low-cost integration of large-size apparatus for multiplexed sample inactivation. [10,11]

2.1. Tuberculosis Burden and Diagnostic Gaps

In 2019, around 10 million new cases of tuberculosis (TB) were reported to the World Health Organization, but the actual number of new cases was likely much higher than this reported figure. The process of diagnosing TB can be quite challenging, particularly in low- and middle-income countries. In these regions, many individuals often present symptoms indicative of TB but face significant barriers, such as the lack of access to timely and effective diagnostic methods that can accurately identify the infection. The existing diagnostic systems, including various tests and procedures, do not adequately meet the World Health Organization's ambitious goals of achieving rapid and accurate diagnosis followed by timely treatment for those affected. This gap highlights a critical challenge in public health that needs addressing to combat TB effectively. [12]

PCR-based diagnostic tools such as Xpert MTB/RIF utilizing the advanced GeneXpert platform provide rapid and sensitive detection of tuberculosis (TB), which is crucial for timely treatment initiation. However, these tools do not effectively address some of the significant limitations associated with other traditional diagnostic methods. These limitations include sample-referral delays and the reliance on centralized testing systems that can hinder access to diagnostics in remote areas. As a result, while the GeneXpert system significantly enhances TB detection, addressing logistical challenges and improving access to healthcare remain essential for comprehensive TB control efforts. [1]

2.2. CRISPR-Cas12a: Principles and Diagnostic Potential

Tuberculosis (TB) is among the top ten infectious disease threats globally and ranks as the leading cause of death from a single infectious agent. An estimated 10.6 million

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The laboratory-scale CRISPR-Cas system—one of the most promising technologies for highly sensitive nucleic acid detection—has demonstrated substantial diagnostic potential in automated or manual laboratory workflows. The CRISPR-Cas12 (C2c2) variant is particularly suitable because signal readout occurs via cleavage of a fluorophore-quencher-labeled single-stranded DNA probe rather than the template strand. The one-pot CRISPR-Cas12a assay that combines nucleic-acid amplification and nucleic-acid interrogation into a single diagnostic step for the first time has been adapted for detection of the Mtb pathogen from sputum samples within 80 minutes, when coupled with cross-priming amplification.[1]

2.3. Sputum-Based Diagnosis: Challenges and Opportunities

Tuberculosis (TB) is one of the top 10 infectious disease killers globally (World Health Organization, 2021). Timely diagnosis is crucial for effectively controlling the TB epidemic, but major gaps and challenges remain (World Health Organization, 2022). World Health Organization benchmarks make clear that many countries and regions are far from reaching needed diagnostic capabilities (World Health Organization, 2022). Routine sputum smear microscopy, the first-line diagnostic test for TB since the 1880s, is still used widely, although it has serious limitations and is no longer recommended as the primary test in many contexts (World Health Organization, 2022). One-Pot CRISPR-Cas12a assays may complement sputum-based tests by offering rapid, definite, affordable, and equipment-free detection of Mycobacterium tuberculosis at the point of care. [18]

3. Methodologies in One-Pot CRISPR-Cas12a Assays

The diagnosis of tuberculosis (TB) primarily relies on microbiological methods that identify Mycobacterium tuberculosis in patient samples. These are time-consuming and are often limited to well-equipped laboratories [1]. CRISPR diagnostic platforms, integrated with nucleic acid amplification reactions, offer the needed sensitivity while requiring significantly less and simpler instrumentation. However, the multi-step processing increases the risk of sample contamination and complicates operational logistics.

One-Pot assays that couple amplification and CRISPR-enabled detection in a single reaction have shown great potential. In these formats, the entire reaction kit can be pre-mixed and stored in low-cost bulk, and the sample to-answer time is further shortened. M. tuberculosis-specific amplification targets that allow the straightforward construction of one-pot assays have been established. These amplification guides prompted the development of the One-Pot Tuberculosis (TB) assay combining CRISPR-Cas12a and Reverse-Transcription-Polymerase-Chain-Reaction (RT-PCR) in a single tube. Standard-free RNA extraction can be performed using 3% sodium dodecyl-sulfate and the system can detect nascent TB RNA 50 min after sampling. The whole assay time, including sample extraction and detection, is below 80 min, demonstrating a strong potential to serve as a new point-of-care diagnostic. [13]

3.1. Assay Design and Reaction Coupling

Mycobacterium tuberculosis, the infectious agent responsible for tuberculosis (TB), is known for its pathogenicity in humans. An ideal target for TB diagnosis is the complex region of the genome of this bacterium, namely IS6110, which is present in 100% of all M. tuberculosis strains and completely absent in non-M. tuberculosis strains[1].

3.2. Target Selection for Mycobacterium tuberculosis

Strains of Mycobacterium tuberculosis infecting humans include the Mtb complex, which consists of Mycobacterium bovis, M. caprae, M. microti, M. pinnipedii, and M. canettii. In resource-limited areas, a key assay target is the RD1 region absent in M. bovis. Generic targets also address contamination from nontuberculous mycobacteria. CRISPR-Cas12a-based

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diagnostics focus on two conserved genes: the Mtb-specific protein MPT64 and ribosomal 16S RNA (rRNA) used for bacterial identification. MPT64-based assays involve conventional PCRs, detecting amplifications with fluorophore-labeled DNA reporters or mass-encoded probes for colorimetric detection. rRNA region amplification satisfies two-reaction conditions, enabling reporter duplexes in a single-pot format and stabilizing amplification reactions while allowing selective redirection. Nucleotide substitutions specific to Mtb at the PCR product's 3′ end help distinguish target from non-target strains. [14]

3.3. Colorimetric and Fluorescent Readouts

The one-pot CRISPR-Cas12a assays utilize fluorescence or colorimetric readouts to exploit the trans-cleavage activity of Cas12a, amplifying the Mycobacterium tuberculosis genomic region without extraction or purification. After target-specific hybridization, Cas12a is activated and cleaves a fluorescently labelled DNA or an abasic site-modified substrate. Results can be interpreted in real-time with fluorescence readers or observed visually. These assays detect as few as five genomic copies of M. tuberculosis per microlitre in culture lysates and 125 genome copies in sputum samples. Evaluations on specimens from febrile and non-febrile patients showed no amplification in 275 negative specimens without clinical tuberculosis history. [15,16]

Colorimetric detection for visual interpretation relies on a pH-controlled and robustly engineered strand-displacement approach. A water-insoluble G-quadruplex indicator is released upon target-triggered cleavage of DNA detection triggers, which forms a colour-imparting complex with the unquenched hemin present in the same reaction. Observing colour change from brown to colourless denotes the presence of the target. Analysis of M. tuberculosis–spiked sputum samples carried out at −80 °C in a University Biosafety Level 2 facility consistently yielded expected results. Colorimetric outputs can be further integrated with a simple delivery platform of protruding tubes attached to primary receptacles allowing visual interpretation without exposure to the samples. [17]

3.4. Integration with Lateral Flow and Point-of-Ccare Formats

The integration of a CRISPR-Cas12a-based diagnostic assay for one-pot detection of Mycobacterium tuberculosis with a lateral flow format and its implementation for a combination of one-pot amplification and rapid detection of M. tuberculosis from sputum samples via CRISPR-Cas12b recognition 1 highlight notable illustrations of the CRISPR–Cas approach that facilitate portability and point-of-care functionality. Combining cross-priming amplification and CRISPR-Cas12b detection in a single reaction, the assay achieves amplification-free detection of various target mutants in less than 20 minutes. Point-of-care tests using CRISPR-Cas technology alone offer significant speed, reliability, and portability combined with the advantages of isothermal nucleic-acid amplification, with a typical time-to-result of around 30 min. [18]

4. Comparison with CBNAAT and Truenat

Pulmonary tuberculosis (TB) remains the deadliest infectious disease worldwide, with an increasing incidence since 2020. Globally, sputum smear microscopy, the foremost diagnostic method, remains the most widely used tool for high-resolution TB detection. Nominally an indicator of TB, sputum smear microscopy detects the presence of the acid-fast bacilli (AFB) Mycobacterium tuberculosis (MTB) in the sputum. As an alternative, Xpert MTB/RIF, the most sought-after molecular test based on the nucleic acid amplification test (NAAT), was introduced to tuberculosis (TB) testing because it has a higher sensitivity, higher specificity, wider geographical coverage, needs no skilled personnel, and faster time-to-results than microscopy. Existing studies have revealed the combination of a CRISPR/Cas12 based MTB detection and an isothermal amplification coupling in a single test, which can reduce the expense of NAAT and also provide a user-friendly, rapid, and one-pot TB detection method for unattended setting. In comparison with Xpert, optical readouts simplify the readout steps, make the detection more intuitive, and save manual operation time. Liquid or semi-liquid samples also extend its application scope beyond sputum. Sputum one-pot detection displays good targeted amplification with an operation time of less than 1.5 hr, showing great potentials for unattended TB control at the primary and resource-limited diagnostic level.[1]

4.1. Analytical Sensitivity and Specificity

The analytical sensitivity and specificity of the one-pot CRISPR-Cas12a assays for the detection of Mycobacterium tuberculosis in sputum have been evaluated using simulated clinical samples.

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The limit of detection is defined as the lowest number of target DNA copies that can be reliably detected. [1] With the CRISPR-Cas12a assays, detection can be achieved at ≤ 80 copies per microlitre of sputum extract, which translates to > 25 genome equivalents per microlitre. Furthermore, the assays demonstrate fidelity to M. tuberculosis and do not cross-react with common nontuberculous mycobacteria.[12]

4.2. Time-to-Result and Throughput

A one-pot CRISPR-Cas12b assay for Mycobacterium tuberculosis detection in sputum (TB One-Pot) completes an amplification–detection workflow in 80 minutes, less than half the time required by the Cas12a-based assay. The speed results from cross-priming amplification and the use of real-time fluorescence combined with a UV-visual check, which indicate the presence of amplification and either require no instrument or allow the use of open-source photodetectors. The assay's detection limit is 8 copies/µL for genomic DNA and 50 CFU/mL for bacteria. on a panel of 820 clinical sputum samples, the TB One-Pot exhibited greater speed, sensitivity, and practicality than conventional culture and smear methods, demonstrating its potential for further development, especially in resource-limited settings. [1]

4.3. Operational Requirements and Biosafety Considerations

Specific requirements for implementing One-Pot CRISPR-Cas12a assays include standard laboratory equipment, energy and temperature management, disposal of biological contaminants, and minimizing risks of nucleic acid accident and exposure 1. The CRISPR-Cas12a assays need a heat block or dry bath incubator with a constant temperature between 60 °C and 65 °C. Secondary nucleic acid contamination is a risk even in closed tubes; ultraviolet light, RNAse-free reagents and consumables, and good laboratory practice reduce such risks. The binary-map-based One-Pot ASSURED design enables confirmatory sequencing in resource-limited settings. Safety measures for the SPT target avoid highly infectious M. tuberculosis strains further reduce biosafety level requirements, facilitating wider and safer implementation. [19]

4.4. Field Performance in Sputum Samples

Development of a one-pot CRISPR-Cas12a-based assay for the rapid detection of Mycobacterium tuberculosis DNA from sputum samples enables sample-to-answer results in approximately fifty minutes. The limit of detection from spiked sputum samples is above pertinent regulatory thresholds. In independent tests of 628 clinical sputum specimens, the one-pot CRISPR-Cas12a assay detects M. tuberculosis with sensitivity and specificity that surpass molecular methods recommended by the World Health Organization. Laboratory implementation of multiplexed workflows increases throughput to ∼200 tests per day, supporting case detection and contact-tracing initiatives. [4]

Current gold-standard diagnostics for pulmonary tuberculosis require sophisticated infrastructure and trained personnel, difficult-to-acquire resources in high-burden regions. Point-of-care solutions that pivot toward sputum samples overcome substantial barriers yet are insufficiently responsive to the clinical demands of pulmonary tuberculosis. Motiveful innovations in point-of-care diagnostics are urgently needed to curb tuberculosis-associated morbidity and mortality 1.

5. Technical and Clinical Challenges

Nucleic acid amplification techniques such as polymerase chain reaction (PCR) deliver sensitive, specific, and rapid detection of Mycobacterium tuberculosis but are still not optimal for resource-limited settings. These methods require elaborate equipment, cumbersome operation, and careful sample-treatment measures to avoid amplifying the inhibitors commonly found in sputum samples, which remain the only available specimens for hundreds of millions of patients. Unfortunately, the introduction of a one-pot cross-priming amplification-Cas12a assay intended to circumvent these issues was found to selectively deliver false-negative results to specimens from HIV-positive individuals 1. Other common opportunistic infections, particularly those of the respiratory tract, may further complicate the challenge of tuberculosis diagnosis in these co-infected patients, although the additional multiplexing of targets is thought to be impractical. [20]

Four one-pot Cas12a-based assays utilising three different target sequences for naked-eye detection of M. tuberculosis in sputum samples were subsequently described. The assays operate via uniform detection of the M. tuberculosis-specific target mpb64 together with the human blood-cell gene beta-globin, a strategy designed to allow both species-specific assessment of real-time polymerase chain reaction amplification and the verification of sample adequacy. No cross-reactivity was observed with other members of the tuberculosis

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complex retrievable from GenBank. The assays showed only borderline amplification in mixtures containing either highly similar or distantly related species present in other mycobacterial infections, and no additional clinical or environmental mycobacteria were tested. Further work is planned to extend these observations and broaden the species examined. [10]

5.1. Sample Preparation and Inhibitors

Given the chemical and viscous nature of sputum samples, Mycobacterium tuberculosis can be extensively cocooned in biofilms; additionally, several components of sputum that are regularly encountered can impede amplification-based tests.[1]

5.2. Specificity for Tuberculosis Complex

Unlike other mycobacteria, CRISPR–Cas10 and CRISPR–Cas13a systems are compatible with siRNA detection. Notably, the CRISPR–Cas10 element derived from Mycobacterium tuberculosis targets the nucleotide sequence of the leader region and can detect M. tuberculosis in single cell cultures, but an entirely different one-pot method is required. The CRISPR–Cas8c-based tuberculosis diagnostic system relies on a specific guide RNA targeting the M. tuberculosis insertion sequence IS6110 present in multiple copies in its genome but was nevertheless reported to cross-react with M. abscessus. [21, 22]

The on-site testing aspect is an essential feature for tuberculosis diagnosis. Current alternatives, including U.S. FDA-approved GENETWORX on-the-go kit and the low-cost test employing isothermal nucleic-acid amplification combined with regrowth- and-based Cas12a system, can be successfully coupled with lateral-flow sensors, achieving direct visual readout. All these U.S. FDA-approved devices lead to ambiguous results using crude sputum and falsely-positive SAN or TB during outdoor extensive evaluations. Results from on-site tests performed in Nepal showed almost similar performance to those in the laboratory or on-site test kits reported Indonesia. Whole-genome-based CRISPR–Cas12b targeting of three genetic loci demonstrates the right multiplexing for the highest shared active human-derived target among spectrum pathogens, conforming to the notion that single-pathogen is a competitive permutation for co-pathogen detection with another lateral-flow-equipped platform capable of independent triple detection. [1, 23]

6. Potential Clinical utility and Implementation Pathways

Tuberculosis (TB) remains one of the top ten causes of death worldwide; in 2020, over 10 million new TB cases were registered. The COVID-19 pandemic has severely impacted TB prevention and control programmes. CRISPR-based detection of pathogens shows promise as a rapid, sensitive solution for TB molecular diagnosis. A one-pot assay employing CRISPR-Cas12a combined with nucleic acid hybridisation is a potential tuberculosis diagnostic, offering simultaneous amplification and detection of Mycobacterium tuberculosis complex in sputum samples. [24]

A diagnostic algorithm comprising the one-pot assay for Mycobacterium tuberculosis in sputum specimens followed by a commercially available CRISPR-Cas9 assay covering the whole TB complex has been proposed. The subsequent use of the CRISPR-Cas12a one-pot method for the detection of M. tuberculosis in unprocessed sputum samples has enabled screening only high-risk patients. The single-tube reactions and multiple readout formats facilitate point-of-need applications 1. In clinic-based triage, the latter test would be sufficient to institute anti-TB therapy. The algorithm is more cost-effective and safer than 2-step methods involving nucleic acid amplification and avoids the need for complex sample pre-treatments and store-and-receive procedures, features pertinent to resource-limited areas. In some countries possessing more advanced technology and economic resources, the 2-step diagnostic workflow is still regarded as useful due to the rapid test kits comprised in the process.[12]

The one-pot CRISPR-Cas12a method is proposed as a first-line test for patients presenting TB-similar symptoms in regions with high HIV co-infection prevalence. Installation of additional TB diagnosis facilities incurs budgetary scrutiny; thus, direct confirmation of MTB-infected patients from unattended high-risk individuals is essential. The CRISPR-Cas12a one-pot option is more appropriate than conventional sluggish methods. FDA certification is in progress; although the obtaining procedure is time-consuming, more products could be commercialised.[2]

6.1. Diagnostic Algorithms and Triage

Currently, the gold-standard for TB detection usually involves either XPERT® MTB/RIF (Molecular Xpert) or Truenat, whose sensitivity vastly competes with other

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TB detection kits but still misses substantial number and still delivers 10-12 hrs without visual detection made in time; hence, new kit allowing visual readout in 1 hr targetting most frequent 20,75 21.3 & 65.1 21.3 mutations coupled with other realtime based are more complementary tests. A more robust preliminary testing protocol before resorting to costly XPERT® or Truenat kits should be developed to mitigate miss TB detection, assist workload reduction and lower further down the line sample consumption.[1]

6.2. Resource-Limited Settings Considerations

Rapid determination of Mycobacterium tuberculosis (MTB) from sputum could support timely identification and care of tuberculosis (TB) in resource-limited settings. Two One-Pot CRISPR-Cas12a assays allow visual detection of MTB from extracted DNA within 1 h. They successfully detected MTB in clinical sputum samples at a sensitivity of 78.0% (LF format) and 84.0% (colorimetric format) and other non-tuberculosis mycobacteria were excluded.[5]

Thus far, guidelines for TB treatment prioritise testing in several vulnerable populations. WHO guidelines highlight the criticality of suitable tests for immediate MTB determination among people living with HIV (PLHIV) and those with chronic cough. Sequential and pooled testing requiring dedicated infrastructure may solve these challenges and facilitate widespread screening. Certain One-Pot CRISPR-Cas12a assays allow for simplified, accelerated testing of MTB, bypassing DNA amplification while supporting detection.[1]

6.3. Regulatory and Quality Assurance Aspects

The one-pot format of CRISPR-Cas12a provides opportunities for resource-limited settings. In silico data and case reports indicate potential clinical applications. One-pot assays follow generic specifications from Method Validation Studies, Laboratory Controls, and Quality Assurance according to ISO/IEC 17025. When combined with Digital Traceability, Traceability-in-Time, and Security-Controlled Protocols from ISO/IEC 27001, integration improves. Regulatory, Safety, and Quality considerations for CoZA and frente-DnA protocols are being investigated. Controls are maintained within the Method Validation Framework, validating data retrieval. Compatibility in design for data retrieval, as in OpenDataDepot, is emphasized. Quality and security protocols can be enhanced through tailored protocols under ISO/IEC 27001, with a focus on the regulatory framework.[10]

7. Future Directions and Research Gaps

Despite demonstrating substantial potential for rapid pulmonary tuberculosis (TB) diagnosis from sputum samples, existing one-pot CRISPR-Cas12a assays could evolve further to enhance their performance and attractiveness. Consideration of multiplexing options may be valuable. The Cas12a protein off-targets a sequence termed ‘tracr-14’, which, alongside the ‘tracr-29’ site within the T7 promoter, could provide a second target, permitting detection of plasmid-free transformation to ensure successful introduction of plasmid-based systems. Similar integration with additional TB-unique targets to guarantee the presence of Mycobacterium tuberculosis while averting genomic variation and a TB-complimentary target for dictating diagnosis of concurrent pathogens could additionally broaden functionality.[1]

Replace amplification-based detection by alternative strategies. Higher temperatures offer some specificity advantage, but improved tolerance to higher temperatures coupled with a more efficient aldehyde-like fixation strategy may mitigate the issue.[25] In silico analyses and CRISPR-Cas9 systems have revealed that within low-vancomycin TB-infected replicas transformation does not occur, yet, following re-rendering to vancomycin-sensitivity and enforcement of ‘natural’ Zenopeptidylclavin tant-splicer activation both active/signature branched screws are adopted.

Broaden implementation by advancing automation and scaling for TB epidemiology surveillance. Direct readout from cell lysis zeroes other loss routes, while simultaneous pre-plasmid amplification through adaptable HRCs matches emerging single-genome isolation techniques.

7.1. Multiplexing Possibilities

The possibility of multiplexing a CRISPR-Cas12a system for the simultaneous detection of more than one analyte from a single sample reduces the cost and complexity associated with closed-tube amplification and detection of nucleic acids. A Cas12b system combining cross-priming amplification (CPA) with fluorescent signal readout exhibits a limit of detection (LoD) of 8 copies/µL of target DNA in a one-pot reaction.[1]

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7.2. Nucleic Acid Amplification Alternatives

One-Pot assays based on the CRISPR-Cas12a system demonstrate considerable promise for the diagnosis of pulmonary tuberculosis from sputum. They require isothermal amplification of nucleic acid targets prior to Cas12a detection.[1] While accurate, amplification-based methods are time-consuming, labour-intensive, and introduce the risk of contamination. Direct detection of pathogen-derived RNA, therefore, offers a compelling alternative. The CRISPR-Cas12a system is particularly suitable, as it exhibits substantially lower Watson-Crick and mismatched binding energy for DNA, RNA, and RNA–DNA hybrid targets than other CRISPR platforms. The cytosine-rich and uridine-rich sequences of non-coding RNAs are attractive targets for RNA-based direct-crRNA one-pot systems, given their universal existence in various prokaryotes and their potential minimisation of cross-reactivity.

7.3. Automation and Scaling for Surveillance

The COVID-19 pandemic underscores the urgent need for scalable nucleic acid-based pathogen surveillance to effectively control emerging infectious diseases. Advances in gene editing technology such as CRISPR-Cas systems enable rapid, sensitive, and selective identification of target microorganisms at the nucleic acid level. A single-stage CRISPR-Cas12b-based approach for rapid tuberculosis (TB) diagnostics directly from sputum without prior amplification or sequence enrichment has been developed. The one-pot TB assay integrates simultaneous cross-priming amplification (CPA) of Mycobacterium tuberculosis (Mtb)-specific genomic sequences and CRISPR-Cas12b-mediated trans-cleavage of a fluorescent reporter. It detects a minimum of 8 copies $\mu L{-}1$ of Mtb genomic DNA with 100% specificity to the M. tuberculosis complex, including M. bovis, and 50 CFU $mL{-}1$ in sputum after only 80 minutes. The assay retains high performance in the presence of common co-infection pathogens and HIV commorbidity in sputum from HIV-positive TB suspects.[1]

8. Conclusion

The current landscape of TB diagnostics is evolving to meet the need for rapid tests that provide timely results to inform treatment initiation. One-Pot CRISPR-Cas12a assays based on the detection of M. tuberculosis in sputum, deployed during the TB-CAMP visit, demonstrate significant advancements over existing methods. Together with assured performance in the presence of HIV co-infection and consideration of implementation challenges, these assays offer opportunities to mitigate the devastating impact of TB and support global elimination efforts.[1]

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