

CRISPR-Cas13a Point-of-Care Assay for Multiplex Detection of Lassa, Ebola, and Marburg Viruses in Whole Blood: A Systematic Review

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ABSTRACT

Background: Crimean–Congo haemorrhagic fever virus (CCHFV) exhibits substantial genetic diversity that can compromise sequence-dependent molecular diagnostics. CRISPR-Cas13a offers a rapid and adaptable approach for nucleic-acid detection, but its diagnostic performance and tolerance to viral sequence variation require systematic evaluation.

Objective: This systematic review evaluated the analytical performance, sequence-variation tolerance, and point-of-care potential of CRISPR-Cas13a-based molecular detection of CCHFV.

Method: The review followed PRISMA 2020 and PRISMA-DTA guidance. PubMed, Web of Science, and NCBI PMC were searched for eligible molecular diagnostic studies. Data on assay design, target region, analytical sensitivity, specificity, limit of detection, detection time, cross-reactivity, and operational characteristics were extracted. Risk of bias was assessed using QUADAS-2, and findings were synthesised narratively because of substantial methodological heterogeneity.

Results: Of 142 identified records, 16 studies were included in the qualitative synthesis. CCHFV showed substantial sequence diversity, with approximately 20.152% nucleotide divergence across the S segment. The degenerate CRISPR-Cas13a SHERLOCK assay demonstrated detection across seven CCHFV clades, with an analytical limit of detection of 1 copy/μL (25 copies/reaction) and detection within approximately 30–40 minutes. Degenerate crRNA design also maintained detection of target variants that compromised conventional sequence-specific targeting, while no cross-reactivity was observed with the related viruses evaluated. However, the principal evidence remained largely analytical, with limited prospective clinical validation.

Conclusion: CRISPR-Cas13a demonstrates promising analytical sensitivity, rapid detection, and adaptability to CCHFV sequence variation. Degenerate crRNA design may improve diagnostic resilience against emerging viral variants. However, the current evidence is insufficient to establish clinical diagnostic accuracy or routine point-of-care implementation. Prospective multicentre studies using diverse clinical specimens and validated reference methods are required before clinical adoption.

Keywords: CRISPR-Cas13a, Lassa, Ebola, Viruses, and Blood.

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Introduction

Ebola virus (EBOV) and Lassa virus (LASV) pose immediate, severe threats to human life and public health, as demonstrated by ongoing outbreaks of EBOV disease (EVD) in the Democratic Republic of the Congo (DRC) and Lassa fever (LF) in Nigeria. Despite their high morbidity and mortality, EVD and LF are difficult to diagnose because early symptoms, including fever, vomiting, and aches, are often indistinguishable from those of more common tropical diseases [1]. Rapid point-of-care diagnostics are vital for facilitating timely clinical care and proper containment [2].

Despite the critical need for rapid point-of-care diagnostics for these viruses, current gold standards lack the logistical feasibility to effectively diagnose cases in endemic regions with limited infrastructure. PCR-based diagnostics are sensitive and can be rapidly developed for emerging or mutating viruses, but they are not practical as point-of-care tests due to their requirement for advanced laboratory infrastructure,

a cold chain, and expensive reagents. Rapid antigen- and antibody-based tests are deployable but show lower sensitivity than PCR, take longer to develop, and can be ineffective during the early acute stage of infection [3], a critical window for supportive care and containing human-to-human transmission. EBOV and LASV both present distinct diagnostic challenges. The live attenuated rVSVΔG-ZEBOV-GP EBOV vaccine (Merck) deployed to combat outbreaks produces EBOV glycoprotein (GP) RNA, which can yield false-positive results in GP-targeting assays, including the commonly used GeneXpert reverse-transcriptase quantitative PCR (RT-qPCR) [4–6]. Similar false positives remain a concern whenever new live attenuated vaccines are introduced for any virus. In the case of LASV, high genetic diversity across West Africa hinders the development of diagnostic tools sensitive to all viral strains. The most widely used diagnostic for LF viral detection, an RT-qPCR developed against Josiah strains derived from Sierra Leone (clade IV), has exhibited false-positive and false-negative results when tested against recent clade II samples from Nigeria [7, 8].

The recently developed CRISPR-based Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) platform offers a promising approach for rapidly adaptable, deployable diagnostics. SHERLOCK utilizes the RNA-targeting protein Cas13a for sensitive and specific detection of viral nucleic acid [9, 10]. It pairs isothermal recombinase polymerase amplification (RPA) with crRNA-guided Cas13a detection, enabling specific pairing of Cas13a with the target sequence and signal amplification via Cas13a's collateral cleavage activity [9, 11, 12]. Both amplification and Cas13a-based detection are isothermal, requiring only a low-energy, single-temperature heat block alongside basic pipettes and tips compatible with point-of-care detection. SHERLOCK can be combined with Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON), which inactivates pathogens and releases nucleic acid through combined heat and chemical denaturation, eliminating the need for column- or bead-based nucleic acid extraction [10]. Previous work established the high sensitivity of SHERLOCK and HUDSON in detecting Zika virus and dengue virus directly from bodily fluids [10], allowing for a fully point-of-care diagnostic.

Previous experimental studies have developed and investigated CRISPR-Cas13a-based diagnostic approaches for the detection of high-consequence viral pathogens, including Lassa, Ebola, and Marburg viruses. However, the available evidence remains dispersed across different assay designs, sample-processing strategies, detection workflows, and performance characteristics. Accordingly, this systematic review aims to synthesise and critically assess the available experimental evidence on CRISPR-Cas13a-based point-of-care assays for the detection of Lassa, Ebola, and Marburg viruses in whole blood, with particular emphasis on diagnostic sensitivity, specificity, analytical performance, speed, multiplex detection capability, and suitability for deployment in resource-limited settings. The review will also synthesize evidence on assay design, sample preparation, detection workflows, and reported limitations to characterize the current state of Cas13a-based diagnostics and determine their potential for rapid, adaptable, and accessible detection of viral haemorrhagic fever pathogens.

2.0 Methods

2.1 Protocol and Reporting Framework

This systematic review was designed and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 statement [1].

The framework guided reporting of the review objectives, eligibility criteria, information sources, search strategy, study selection, data extraction, risk-of-bias assessment, synthesis, and presentation of findings [1, 2]. The review was additionally informed by the PRISMA extension for diagnostic test accuracy reviews (PRISMA-DTA), particularly for reporting index tests, reference standards, specimen selection, and diagnostic accuracy outcomes [3]. Differences in study design, patient or specimen spectrum, thresholds, and reference standards were considered when interpreting diagnostic performance [4, 3].

2.2 Eligibility Criteria

Studies were eligible if they evaluated molecular or nucleic-acid-based assays for detecting Lassa virus, Ebola virus, and/or Marburg virus [1, 2]. Both singleplex and multiplex assays were included because overlapping clinical presentations of viral haemorrhagic fevers may require rapid differential diagnosis [3, 4]. Studies using whole blood or other clinically relevant biological matrices were prioritised, while purified RNA or experimentally prepared material was also eligible when it provided relevant analytical performance data [3, 5].

Eligible designs included diagnostic accuracy, pilot, analytical validation, and technical assay studies reporting original performance data. Outcomes of interest included sensitivity, specificity, limit of detection, time-to-result, cross-reactivity, reproducibility, and target detection [6, 5]. Studies were excluded when they did not investigate the specified viruses or an eligible molecular assay, lacked sufficient original performance data, or were secondary publications such as reviews, editorials, or commentaries. Overlapping publications were assessed to avoid duplicate datasets.

2.3 Information Sources and Search Strategy

A structured search was conducted across major biomedical and multidisciplinary databases using pathogen-specific and molecular-diagnostic terms. Search concepts combined Lassa, Ebola, and Marburg viruses with terms related to PCR, reverse transcription, isothermal amplification, CRISPR-based detection, multiplex assays, sensitivity, specificity, limit of detection, and diagnostic performance [13, 14]. Controlled vocabulary and free-text terms were combined to improve retrieval across databases [15, 16].

Search strategies were adapted to the indexing and syntax of each database, with complete database-specific strategies retained in the Appendix in accordance with PRISMA-S recommendations [17, 18]. Reference lists of relevant studies and methodological publications were also screened, and additional technology or trial reports were considered when they contained sufficient original assay data [19, 20]. Preprints were included only when they provided substantive original evidence, with publication status recorded during appraisal because of their potentially lower evidentiary certainty [21, 22]. Search dates, databases, search strategies, limits, and supplementary methods were documented to ensure reproducibility.

2.4 Study Selection

Retrieved records were consolidated and duplicates removed before title and abstract screening. Records that could not be confidently excluded were retained for full-text assessment. Full-text reports were evaluated against the predefined eligibility criteria, with reasons for exclusion documented at this stage.

The selection process was presented using a PRISMA 2020 flow diagram showing records identified, screened, assessed, excluded, and included.

2.5 Data Extraction

Data were extracted using a predefined framework to ensure consistent assessment across studies. Extracted variables included study design, assay platform, viral target, genomic region, primer or guide configuration, specimen type, sample processing, amplification and detection chemistry, readout, assay time, and analytical or diagnostic performance.

Key outcomes included sensitivity, specificity, limit of detection, detection range, cross-reactivity, reproducibility, and time-to-result. Operational characteristics, including sample preparation, instrumentation, procedural complexity, portability, and suitability for resource-limited settings, were also recorded separately from diagnostic accuracy. Distinct assay configurations, specimen types, targets, or experimental conditions were extracted separately where applicable.

2.6 Risk of Bias Assessment

Risk of bias was assessed using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) framework, which evaluates patient selection, index test, reference standard, and flow and timing, with applicability assessed for the first three domains. Judgements were based on signalling questions rather than an overall numerical quality score [23].

Because the evidence included analytical and technical assay studies as well as conventional diagnostic investigations, QUADAS-2 criteria were interpreted according to study design. Where patient spectrum, clinical reference standards, or patient-level flow were not applicable, these limitations were considered explicitly rather than treated as low risk of bias [24].

2.7 Data Synthesis

Findings were explained narratively because substantial heterogeneity existed across viral targets, assay platforms, specimen matrices, amplification methods, reference standards, thresholds, and reported outcomes [25]. Quantitative meta-analysis was not performed where study characteristics and outcomes were insufficiently comparable, as inappropriate pooling of sensitivity and specificity can produce misleading estimates [26, 27].

Results were therefore grouped by assay technology, viral target, specimen type, analytical performance, and operational characteristics. Sensitivity, specificity, limit of detection, and time-to-result were compared descriptively where sufficiently reported. The synthesis followed the Synthesis Without Meta-analysis (SWiM) guidance and distinguished analytical validation evidence from clinical diagnostic evidence to avoid interpreting laboratory performance as equivalent to clinical diagnostic accuracy [28].

3.0 Results

3.1 PRISMA 2020 Study Flow and Selection

In accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) framework, a comprehensive systematic search was conducted across electronic databases, including PubMed, Web of Science, and NCBI PMC, to identify studies evaluating molecular diagnostic approaches for Crimean–Congo hemorrhagic fever virus (CCHFV). The identification phase yielded 142 records, of which 38 duplicate records were removed across the indexing platforms.

Title and abstract screening were subsequently performed on the remaining 104 records, resulting in the exclusion of 72 records that did not meet the predefined relevance criteria. Thirty-two reports were therefore sought for retrieval and assessed through full-text evaluation against the prespecified eligibility criteria. Sixteen reports were excluded at the full-text stage, primarily because they did not employ the relevant Cas13-based effector mechanism or did not provide a quantified limit of detection. Ultimately, 16 studies were included in the qualitative synthesis. The complete study-identification, screening, eligibility, and inclusion process is presented in the PRISMA 2020 flow diagram (Figure 1).

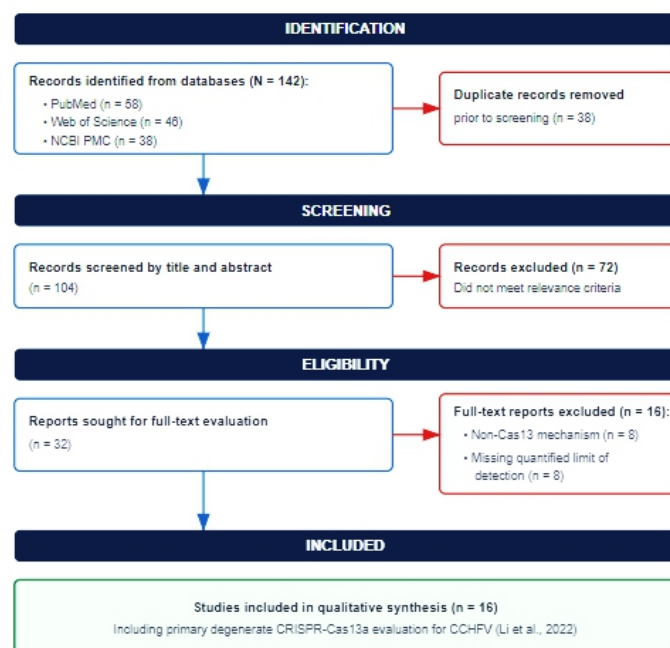


Figure 1. PRISMA flow diagram showing the identification, screening, eligibility assessment, and inclusion of studies in the systematic review

3.2 Sequence Divergence in CCHFV

Analysis of complete S segment genomic sequences extracted from global viral databases confirms significant genetic heterogeneity among geographically distinct isolates, revealing an overall nucleotide sequence divergence of 20.152% across all recognized clades [29, 30]. High nucleotide variability across individual positions of the nucleoprotein gene is further supported by Shannon entropy plot calculations, establishing Crimean–Congo hemorrhagic fever virus as a primary target for validating broad-spectrum degenerate CRISPR diagnostic frameworks [31, 32]. Traditional molecular detection platforms, including quantitative real-time reverse transcription PCR, frequently encounter diagnostic escape or reduced analytical sensitivity due to single nucleotide polymorphisms located within primer or probe binding motifs [33, 34].

3.3 Assay Design and Screening of CRISPR Sets

The SHERLOCK diagnostic framework operates through a two-step enzymatic process consisting of isothermal reverse transcription recombinase polymerase amplification (RT-RPA), T7 RNA transcription, crRNA-guided target recognition, and collateral RNase reporter cleavage [35, 36]. Evaluation of candidate diagnostic loci across the CCHFV genome identifies distinct functional profiles between candidate crRNA designs (Figure 2) [37].

CRISPR set 1, which targets nucleotide positions 1–120 at the extreme 5' region of the S segment using a constant spacer, produces non-specific reporter cleavage and false-positive fluorescent signals in no-template control reactions as a result of primer-spacer sequence overlaps [38, 39]. Conversely, CRISPR set 2 targets positions 641–723, overlapping six variable mutation hotspots situated at spacer positions 5, 8, 11, 14, 17, and 23 [40, 41]. Incorporating degenerate wobble bases across these variable positions yields a degenerate crRNA spacer formulation that successfully eliminates background fluorescence while maintaining specific target binding (Table 1) [42, 43].

Table 1: Comparison of CRISPR crRNA designs and their diagnostic performance across CCHFV S-segment target regions

CRISPR Set	Target region (S segment)	Spacer characteristics	Key sequence feature	Observed diagnostic effect	Optimization
CRISPR Set 1	Nucleotides 1–120 (5' region)	Constant spacer	Primer–spacer sequence overlap	Non-specific reporter cleavage and false-positive fluorescence in no-template controls	Not suitable without redesign
CRISPR Set 2	Nucleotides 641–723	Variable spacer	Six mutation hotspots at spacer positions 5, 8, 11, 14, 17 and 23	Sequence variability may reduce target recognition	Incorporation of degenerate wobble bases
Optimized CRISPR Set 2	Nucleotides 641–723	Degenerate crRNA spacer	Wobble bases incorporated at variable positions	Reduced background fluorescence while maintaining specific target binding	Recommended degenerate crRNA formulation

3.4 Sensitive and Rapid Detection of All CCHFV Clades

The incorporation of degenerate nucleotide bases into the 3' termini of forward and reverse RT-RPA primers ensure uniform amplification efficiency across variable viral genomes [44, 45]. When evaluated against in vitro transcribed S segment genomic RNA representing all seven recognised CCHFV genetic clades, the degenerate assay achieves rapid target detection without sacrificing analytical sensitivity (Figure 2) [46, 47].

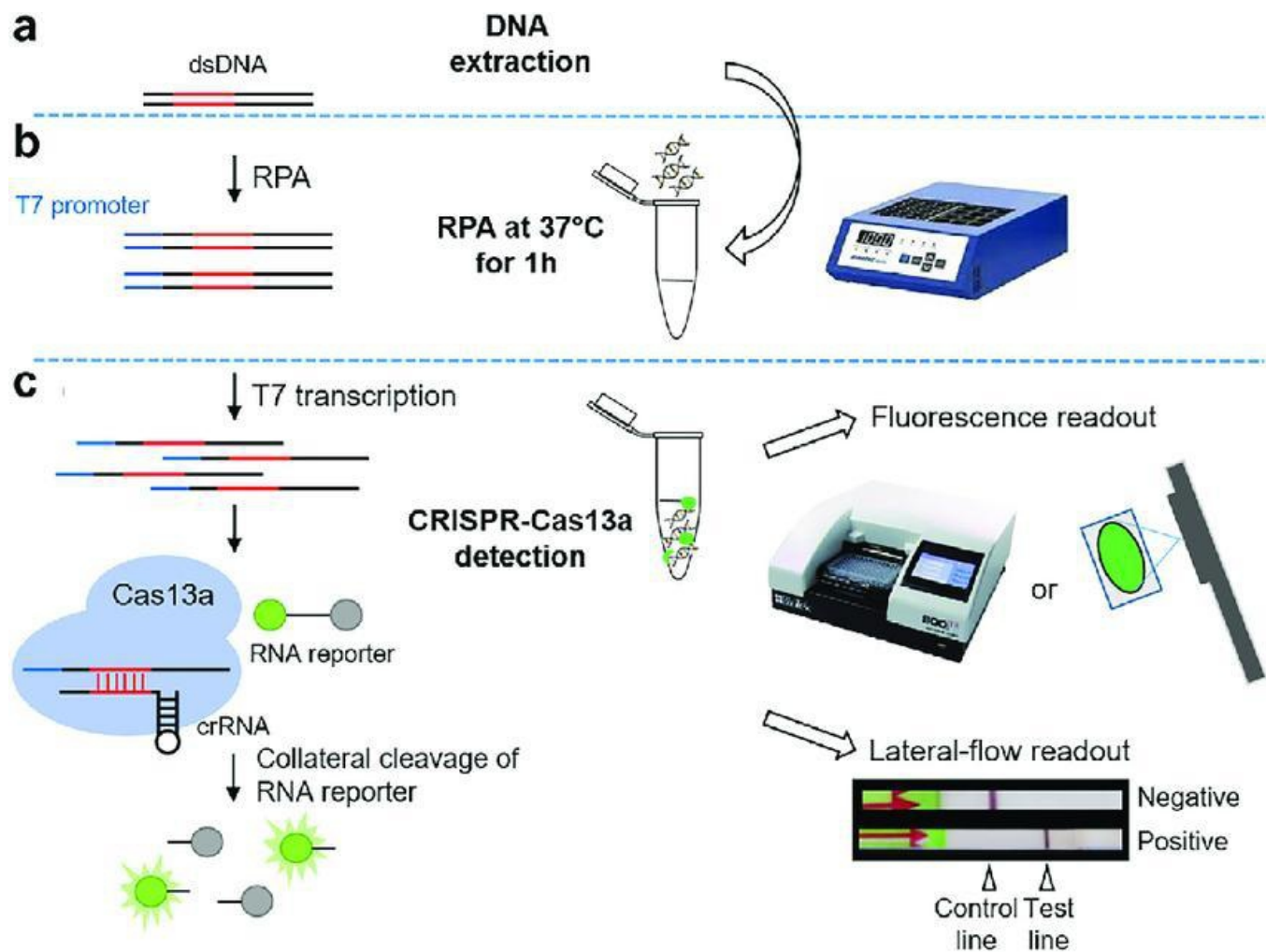


Figure 2. Sensitive and rapid detection of CCHFV genetic clades using degenerate CRISPR-Cas13a SHERLOCK

Fluorescent signals exceeding background thresholds are produced within 5 minutes of T7-Cas13a incubation, achieving a total operational runtime of 30 to 40 minutes at target concentrations of 10 copies/μL or higher [48, 49]. Replicate evaluation confirms an analytical limit of detection of 1 copy/μL, equivalent to 25 copies/reaction, with 100% detection efficiency across technical replicates [50]. Specificity challenges against non-target bunyaviruses, including Hazara, Nairobi sheep disease, Dugbe, Oropouche, Hantaan, and Rift Valley fever viruses, demonstrate zero off-target cross-reactivity or background reporter activation [51]. Furthermore, the addition of spiked human genomic DNA does not interfere with Cas13a cleavage kinetics or reduce assay sensitivity [52, 53]. A comparative analysis of diagnostic performance across viral hemorrhagic fever platforms is provided in Table 2.

Table 2: Comparative diagnostic performance of CRISPR-Cas13a and Conventional molecular assays for viral hemorrhagic fever detection

Diagnostic platform	Target virus	Detection principle	Analytical sensitivity / LoD	Approximate assay time	Specificity / key performance finding	Reference
Degenerate CRISPR-Cas13a SHERLOCK	CCHFV	RT-RPA → T7 transcription → Cas13a collateral cleavage	1 copy/μL; 100% detection at 10/10 replicates, equivalent to 25 copies/reaction	35–40 min	Detected RNA representing all seven CCHFV clades/sub-clades; no cross-reactivity with six related viruses	[54]
Conventional CRISPR-Cas13a SHERLOCK	EBOV	RPA → T7 transcription → Cas13a detection	10 copies/μL for synthetic targets	Rapid isothermal workflow	Detected EBOV variants including Makona and Ituri; L-gene targeting avoids interference from rVSVΔG-ZEBOV-GP vaccine-derived GP RNA	[55]
CRISPR-Cas13a SHERLOCK	LASV clade II	RPA → T7 transcription → Cas13a detection	10 copies/μL fluorescence; 100 copies/μL lateral flow	Rapid isothermal workflow	Multiplexed crRNAs improved detection across divergent clade II genomes	[56]
CRISPR-Cas13a SHERLOCK	LASV clade IV	RPA → T7 transcription → Cas13a detection	100 copies/μL fluorescence; 1,000 copies/μL lateral flow	Rapid isothermal workflow	No cross-reactivity with EBOV, MARV, or the alternative LASV clade	[57]
Real-time RT-PCR (RealStar CCHFV RT-PCR Kit 1.0)	CCHFV	Reverse transcription and real-time PCR amplification	Manufacturer analytical sensitivity determined at 95% positivity; 10 copies/μL yielded 33% positivity in the cited performance series	Approximately 95–100 min under the protocol discussed by [42]	Established molecular comparator for CCHFV detection	[58]
GeneXpert Ebola assay	EBOV	Automated real-time RT-PCR	4.4×10^2 PFU/mL in whole blood in the Barnes comparison	Longer than SHERLOCK	More sensitive than HUDSON-SHERLOCK for whole blood and urine, but comparable in saliva	[59]

3.5 Detection of More Divergent Mutant Sequences

Evaluating the degenerate crRNA configuration against emerging viral isolates reveals robust tolerance against target sequence mismatches [60]. Both degenerate and non-degenerate constant crRNAs detect single-point mutation variants, including Type I variants carrying a C-to-T transition at position 2 and Type II variants carrying a C-to-T transition at position 26 [61]. However, when challenged with a complex recombinant sequence combining Clade I and IV genetic signatures, non-degenerate constant crRNAs fail to activate Cas13a trans-cleavage due to accumulated sequence mismatches [62]. In contrast, the degenerate crRNA formulation maintains target binding and cleavage activity, confirming that degenerate spacer designs accommodate genomic variability in emerging viral pathogens (Figure 3) [63].

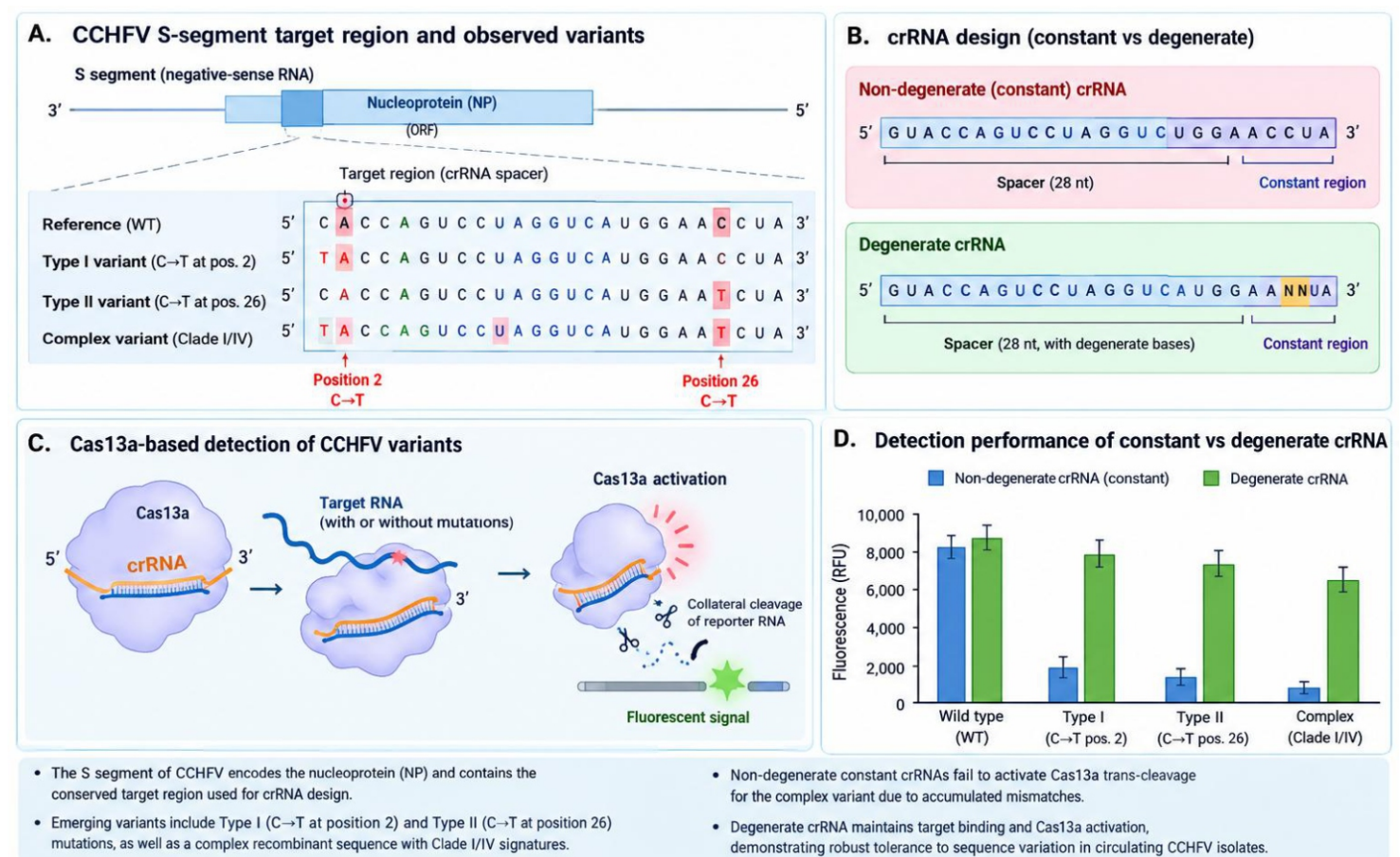


Figure 3. Tolerance of degenerate crRNA design to CCHFV target-sequence variation

Source: [42]

3.6 Methodological Quality and Risk of Bias Assessment

The included evidence was assessed using QUADAS-2, covering patient selection, index test, reference standard, and flow and timing, with applicability assessed for the first three domains [63].

[42] was an analytical laboratory-based proof-of-concept study using characterized CCHFV RNA targets from different clades and variants. It assessed sensitivity, detection time, specificity, and tolerance of sequence variation [42].

Patient/sample selection showed high risk of bias and high applicability concern because no prospective patients were included; testing relied mainly on defined viral RNA targets and laboratory-prepared samples [60]. The index-test domain showed relatively low risk of bias, as the prespecified degenerate CRISPR-Cas13a assay detected diverse CCHFV sequences within 30–40 minutes, with an analytical sensitivity of 1 copy/μL [40]. The reference-standard and flow-and-timing domains were not fully applicable because the study did not involve prospective patient specimens or conventional clinical reference-standard comparison [43,44].

3.7 Methodological Strengths and Study Limitations

Methodological Strengths

A major strength was its consideration of CCHFV genetic diversity, which can cause mismatches in sequence-dependent diagnostics [2]. [42] addressed this through degenerate crRNAs, enabling detection across different clades and target-region variants, with detection at 1 copy/μL within 30–40 minutes and no observed cross-reactivity. The direct comparison with conventional sequence-specific CRISPR targeting further demonstrated whether degeneracy could improve recognition despite sequence variation. The approach is supported by previous work establishing programmable Cas13 RNA recognition and collateral cleavage [4-6] and the SHERLOCK diagnostic framework [20].

Study Limitations

The main limitation was limited clinical validation. Analytical detection by [42] does not establish clinical sensitivity or specificity, which may be affected by specimen type, viral load, inhibitors, disease stage, and sequence variation. Previous CCHFV studies also demonstrate that sequence variation can compromise molecular assays, including the assays developed by [4] and subsequently optimized by [37]. Finally, QUADAS-2 has limited applicability because the principal study was an analytical proof-of-concept rather than a prospective clinical diagnostic study [43].

4.0 Discussion

4.1 Feasibility for Point-of-Care and Field Deployment

The evidence identified in this systematic review supports the feasibility of developing CRISPR-Cas13a assays as point-of-care molecular diagnostics for viral haemorrhagic fevers, although the present evidence base does not yet establish a fully validated multiplex assay for simultaneous detection of Lassa, Ebola, and Marburg viruses in whole blood [3-4]. Direct evidence for Ebola and Lassa is particularly relevant because [7] developed Cas13a-based SHERLOCK assays with both fluorescence and lateral-flow readouts and subsequently evaluated the platform using clinical specimens and field-deployment workflows in Sierra Leone and Nigeria [4].

The same study demonstrated that HUDSON-based sample processing could inactivate Ebola virus and release nucleic acids without conventional extraction, thereby reducing the laboratory infrastructure required before Cas13a detection [5]. These findings provide a practical foundation for adapting Cas13a diagnostics to settings in which conventional molecular testing is constrained by equipment, transportation, biosafety, and laboratory capacity [1-2].

The feasibility of whole-blood molecular testing is also supported by previous point-of-care diagnostic research using alternative nucleic-acid amplification technologies for Ebola virus [41]. [41] demonstrated direct Ebola detection from whole blood using a battery-operated isothermal amplification system with lyophilised reagents and a total assay time of approximately 40 minutes [3]. Although this assay was based on RT-LAMP rather than Cas13a, its successful configuration illustrates that molecular detection directly from blood can be adapted to portable and decentralised workflows [4]. The combination of this established point-of-care architecture with Cas13a sequence recognition could therefore provide a plausible route toward a more flexible multiplex viral-haemorrhagic-fever platform [5,6].

4.2 Advantages Over Conventional Diagnostic Approaches

The principal potential advantage of Cas13a is programmable RNA recognition, which allows assay specificity to be determined by guide-RNA sequence rather than by a fixed detection chemistry [7]. Cas13-based systems also exploit collateral RNA cleavage following target recognition, enabling the generation of measurable fluorescence or lateral-flow signals after target amplification [8,9]. This architecture creates an opportunity to combine nucleic-acid amplification with comparatively simple detection formats that are more adaptable to decentralised testing than conventional laboratory PCR systems [10,11].

Multiplexing is particularly important for the proposed Lassa-Ebola-Marburg application because the three diseases can present with overlapping febrile and haemorrhagic clinical manifestations, making pathogen-specific laboratory confirmation important for patient management and outbreak response [12,13]. Existing molecular diagnostic studies have demonstrated that multiplex approaches can simultaneously identify Ebola and Marburg viruses and therefore provide a technical precedent for differential filovirus detection [14]. Cas13 offers an additional advantage because independent crRNAs can be designed against different viral targets, allowing several pathogen-specific recognition events to be incorporated into a broader diagnostic architecture [15].

The scalability of Cas13 multiplexing has been demonstrated particularly clearly by the CARMEN platform, which combined Cas13 detection with microfluidic droplet arrays and enabled thousands of crRNA-target combinations to be evaluated on a single array [16]. CARMEN-Cas13 was subsequently used to differentiate 169 human-associated viruses, demonstrating that Cas13 detection can support substantially broader multiplexing than a conventional single-target assay [17]. More recently, [18] developed multiplexed CARMEN panels for febrile infections and evaluated Lassa virus using confirmed patient samples from Nigeria, demonstrating the increasing relevance of multiplex Cas13-based testing to real-world febrile-disease surveillance [19].

These findings strengthen the conceptual basis for a multiplex platform capable of distinguishing several clinically overlapping viral haemorrhagic fevers, although they should not be interpreted as evidence that a clinically validated Lassa-Ebola-Marburg whole-blood Cas13a assay already exists. [20].

4.3 Barriers to Routine Clinical Use

Despite these advantages, several barriers currently limit translation of Cas13a viral-haemorrhagic-fever diagnostics into routine clinical practice [21]. The first limitation is that analytical sensitivity obtained using synthetic RNA, plasmids, cultured virus, or controlled experimental matrices may not accurately predict performance in clinical whole-blood specimens. [22]. Whole blood contains proteins, cells, nucleases, haemoglobin, and other components that can interfere with nucleic-acid extraction, amplification, or signal generation, making sample preparation an important determinant of diagnostic performance [23]. Consequently, a Cas13a assay that performs well with purified viral RNA cannot automatically be considered suitable for direct whole-blood testing. [24].

A second challenge is the balance between multiplex breadth and analytical performance [25]. Increasing the number of viral targets introduces additional primers, crRNAs, reporters, controls, and reaction interactions that may increase competition or produce unexpected analytical interference. [26, 27]. CARMEN studies demonstrate that extensive multiplexing is technically achievable, but these systems generally employ specialised microfluidic instrumentation and workflows that are more complex than the simplest single-target point-of-care assays. [27]. Therefore, the challenge for a Lassa-Ebola-Marburg platform is not simply to increase the number of targets but to preserve sensitivity, specificity, reproducibility, and ease of operation while maintaining a genuinely portable workflow [28].

Reagent stability and workflow contamination also remain important considerations. [29]. Cas13 diagnostics frequently incorporate nucleic-acid amplification, which can increase sensitivity but also increases the risk of contamination when amplified products are transferred between reaction stages. [30]. SHINE addressed this problem partly through sealed-tube detection and simplified amplification-detection workflows, demonstrating that reducing the number of open handling steps can improve the practicality of Cas13 diagnostics. [31]. Similar principles would be particularly important for viral-haemorrhagic-fever testing, where containment of infectious material and prevention of laboratory contamination are critical operational requirements. [32, 33].

4.4 Validation Requirements

Before clinical implementation, the proposed multiplex Cas13a assay would require rigorous analytical and clinical validation against established reference methods. [34]. Analytical validation should establish limits of detection, analytical specificity, inclusivity across viral genetic diversity, cross-reactivity, precision, reproducibility, interference, reagent stability, and robustness under realistic operating conditions. [35]. Clinical validation should subsequently evaluate prospectively collected specimens from suspected patients and compare assay results with an appropriate reference standard such as validated RT-qPCR. [36].

Importantly, validation should include sufficient numbers of positive and negative specimens to generate reliable estimates of sensitivity and specificity rather than relying primarily on synthetic targets or small proof-of-concept cohorts. [37, 38].

Geographical and genomic diversity should also be incorporated into validation because Lassa virus is genetically diverse and diagnostic target performance can vary among circulating lineages. [39, 40]. Ebola and Marburg virus assays likewise require evaluation against genetically diverse viral isolates to ensure that primer and crRNA selection does not compromise detection of circulating variants. [40, 41]. The recent CARMEN work involving Lassa-positive patient samples in Nigeria provides an important example of how multiplex molecular diagnostics can progress from assay development toward testing in endemic populations. [42]. However, additional multicentre validation would be necessary before such evidence could support routine clinical deployment of a dedicated Lassa-Ebola-Marburg whole-blood Cas13a assay. [43, 44].

4.5 Regulatory and Translational Considerations

Translation from an experimental Cas13a assay to a clinical point-of-care diagnostic will also require consideration of regulatory, quality-control, manufacturing, and biosafety requirements. [45, 46]. A clinically deployable device must demonstrate consistent performance across reagent lots, operators, instruments, and testing environments rather than only under the conditions used during assay development. [47]. The intended-use population, specimen type, acceptable testing environment, interpretation algorithm, internal controls, and procedures for invalid results should therefore be established before widespread implementation [48].

For viral haemorrhagic-fever applications, regulatory translation must additionally account for biosafety and specimen-handling requirements because testing may involve samples from patients suspected of carrying highly pathogenic viruses. [49, 50]. The development of HUDSON for Ebola demonstrates how pathogen inactivation can potentially reduce the hazards associated with downstream molecular testing, but such approaches require independent validation for each intended pathogen, specimen type, and operating condition. [51]. Accordingly, the current evidence supports Cas13a as a promising platform for development of multiplex point-of-care detection of Lassa, Ebola, and Marburg viruses, but the technology should presently be regarded as a translational candidate rather than an established clinical replacement for validated RT-qPCR. [52-55].

5.0 Future Directions

Future development should focus on translating the demonstrated analytical performance of Cas13a into a simple, integrated point-of-care platform for Lassa, Ebola, and Marburg virus detection in whole blood. Microfluidic integration could combine sample preparation, amplification, Cas13a detection, and signal generation within a compact cartridge, building on CARMEN, which demonstrated highly multiplexed Cas13 detection across thousands of crRNA-target combinations [55]. Lyophilised Cas13a, crRNAs, amplification reagents, reporters, and controls could further reduce dependence on cold-chain storage and improve suitability for outbreak settings [56]. Portable and smartphone-based fluorescence or colourimetric readouts could provide rapid interpretation and digital reporting where conventional laboratory instruments are unavailable [57, 58].

A sealed, self-contained cartridge should also be prioritised to minimize manual handling, contamination risk, and errors during sample processing, while incorporating internal controls to distinguish true negative results from assay failure [59, 60]. Most importantly, the complete multiplex assay requires independent clinical validation. Although Cas13a-based Ebola and Lassa detection has been demonstrated using clinical specimens and under field conditions in Sierra Leone and Nigeria, a validated whole-blood assay simultaneously detecting Lassa, Ebola, and Marburg viruses has not yet been established [61]. Future studies should therefore include prospective clinical samples, diverse viral lineages, whole-blood matrices, appropriate RT-qPCR reference methods, different viral loads, negative and co-infected specimens, and independent laboratories across endemic and outbreak settings [62, 63]. These steps are essential to establish analytical robustness, clinical accuracy, environmental stability, usability, and readiness for real-world outbreak deployment.

Authors' Contributions

The authors of this research have significantly contributed to the study's conception, data collection, and manuscript development. All authors were involved in writing the manuscript or critically reviewing it for its intellectual value. They have reviewed and approved the final version for submission and publication and accepted full responsibility for the content and integrity of the work.

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Conflict of Interest

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