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RESEARCH ARTICLE

Development of CRISPR-Based Diagnostic Assays for Rapid Pathogen Detection

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ABSTRACT

Diagnostic turnaround time remains one of the least glamorous but most consequential bottlenecks in outbreak response, and the gap between what a well-equipped reference laboratory can do and what a district clinic or a field station can do has changed remarkably little over the past decade despite genuine advances in molecular biology. We report the development and head-to-head evaluation of two CRISPR-based nucleic acid diagnostic assays, one built on Cas12a collateral cleavage and one on Cas13a collateral cleavage, each coupled to recombinase polymerase amplification and read out on a paper lateral-flow strip, for detection of *Mycobacterium tuberculosis*, influenza A(H3N2), and carbapenem-resistant *Klebsiella pneumoniae* bearing the blaKPC-2 allele. Analytical sensitivity, established against quantified synthetic templates and validated clinical isolates, reached 4.1 copies per microlitre for the Cas12a assay and 6.8 copies per microlitre for the Cas13a assay across all three targets, comparable to quantitative PCR run on the same extracted material. Testing 312 archived clinical specimens (128 respiratory swabs, 96 sputum samples and 88 rectal swabs) against culture- or sequencing-confirmed reference results gave a pooled sensitivity of 96.4% and specificity of 98.1% for the Cas12a assay, with a median sample-to-result time of 38 minutes using a battery-powered heating block and no laboratory infrastructure beyond a pipette. Cross-reactivity testing against a panel of 24 related and unrelated organisms revealed no false-positive signal, and guide RNA design incorporating strain-level variant data reduced signal dropout on a divergent *M. tuberculosis* lineage that had initially escaped detection with a first-generation guide. We conclude that CRISPR-based collateral-cleavage assays, when paired with careful guide design and isothermal amplification, are approaching a level of analytical performance that makes them credible candidates for decentralised deployment, though sample preparation and multiplexing remain the harder engineering problems still to be solved before broad field adoption.

Keywords: CRISPR-Cas12a; CRISPR-Cas13; isothermal amplification; point-of-care diagnostics; pathogen detection; lateral flow

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1. INTRODUCTION

Every infectious disease outbreak eventually runs into the same arithmetic problem: the organisms causing disease do not wait for a specimen to travel to a

reference laboratory, be logged, extracted, amplified and reported, and the longer that chain takes, the more transmission, and sometimes mortality, accrues in the interval. Culture-based confirmation of tuberculosis can take weeks; centralised PCR for a respiratory panel often takes one to three days once transport and batching delays are counted, even where the underlying assay itself runs in under two hours [1,14]. The COVID-19 pandemic sharpened attention on this gap considerably, and it left behind both hardware, in the form of widely distributed isothermal amplification and lateral-flow manufacturing capacity, and a renewed appetite for diagnostic platforms that do not depend on a thermocycler or a biosafety cabinet [2].

CRISPR-Cas systems offer a genuinely different route to sequence-specific detection rather than a repackaged version of PCR. Class 2 type V and type VI effectors, Cas12a and Cas13a respectively, share an unusual property once they recognise their programmed target: their nuclease activity does not switch off after cutting the intended site but instead becomes promiscuous, cleaving any single-stranded DNA or RNA reporter molecule in the reaction indiscriminately [3,5]. This collateral, or trans-cleavage, activity is what converts a CRISPR recognition event into a signal that can be read on a simple fluorescence plate reader or, more usefully for field deployment, on a paper lateral-flow strip printed with capture antibodies against a cleaved reporter tag [4,6]. Detection platforms built on this principle, marketed and published under names such as SHERLOCK and DETECTR, demonstrated attomolar sensitivity for viral and bacterial targets when the collateral-cleavage step was preceded by isothermal pre-amplification, typically recombinase polymerase amplification (RPA) or loop-mediated isothermal amplification (LAMP), both of which run at a single fixed temperature achievable with a hand warmer or a cheap heating block rather than a thermocycler [7,9].

What has been less thoroughly resolved, and what motivated the present study, is how these assays perform once the target moves from a cultured reference strain or a synthetic gene fragment to an archived clinical specimen carrying its own inhibitors, its own background nucleic acid, and its own strain-level sequence variation that a guide RNA designed against a single reference genome may not tolerate. Guide RNA specificity for Cas12a and Cas13a is generally high, but neither enzyme is entirely forgiving of mismatches near the protospacer-adjacent motif, and a guide optimised against one clinical lineage can lose substantial signal against a divergent one, a failure mode that is easy to miss in a validation panel built from a handful of laboratory reference strains [10,11]. Multi-drug-resistant tuberculosis, antigenically drifted influenza, and carbapenemase-producing Enterobacteriaceae were selected as target organisms for this study precisely because each carries clinically important sequence heterogeneity, either in the pathogen genome itself or in

the resistance determinant of interest, that a diagnostic platform intended for real-world deployment has to withstand rather than merely tolerate in principle.

We therefore built and validated two collateral-cleavage assay formats side by side, one Cas12a-based (targeting double-stranded DNA templates for *M. tuberculosis* and the *blaKPC-2* carbapenemase gene) and one Cas13a-based (targeting the RNA genome of influenza A), each preceded by RPA pre-amplification and read out on a lateral-flow strip within 40 minutes of extracted nucleic acid being added to the reaction. Our aims were threefold: first, to establish analytical sensitivity and specificity against quantified synthetic and cultured reference material; second, to test clinical performance on a reasonably large panel of archived, reference-method-confirmed specimens spanning all three targets; and third, to deliberately probe strain-level robustness by including known divergent lineages in the validation panel and, where signal dropout occurred, to characterise the mismatch and redesign the guide accordingly, treating guide robustness as an engineering variable to be optimised rather than an assumption to be hoped for.

It is also worth being explicit about where a platform like this would sit within an existing diagnostic pathway rather than treating it as a wholesale replacement for centralised testing. Reference laboratories will continue to offer higher throughput, formal quality assurance schemes, and the capacity to run confirmatory drug-susceptibility panels that a single-target lateral-flow assay cannot replicate, and nothing in the data presented here argues otherwise. The realistic niche for a CRISPR-based, equipment-light assay is triage and same-visit decision-making at the point of first contact, where the alternative is not a same-day reference-laboratory result but a multi-day delay, a lost-to-follow-up patient, or an empirical treatment decision made without any microbiological confirmation at all, a scenario that remains common across much of the tuberculosis- and carbapenem-resistance-endemic world despite considerable investment in laboratory strengthening over the past fifteen years [40,42].

2. MATERIALS AND METHODS

All assay development and clinical-specimen testing were carried out under institutional biosafety approval for handling of category 3 respiratory and mycobacterial pathogens, using archived, de-identified specimens collected for the original clinical indication with no additional patient contact.

2.1 Target selection and guide RNA design

Three targets were selected to span DNA and RNA detection formats and clinically relevant sequence diversity: a conserved region of the IS6110 insertion element for *Mycobacterium tuberculosis* complex, the matrix (M) gene segment of influenza A for pan-H3N2 detection, and the *blaKPC-2* carbapenemase gene for

carbapenem-resistant *Klebsiella pneumoniae*. Candidate crRNA spacers were designed against reference sequences retrieved from GenBank and cross-checked against a locally curated alignment of 340 regional clinical isolate sequences spanning the three targets, discarding candidate sites with polymorphism at or within four bases of the protospacer-adjacent motif. Two to three candidate guides per target were synthesised and screened empirically against synthetic double-stranded DNA or in-vitro transcribed RNA templates before the best-performing guide for each target was carried forward into clinical-panel testing [8,12].

2.2 RPA pre-amplification and collateral-cleavage reaction

Nucleic acid was extracted from clinical specimens using a silica-column kit and, for the RNA target, reverse-transcribed with random hexamers immediately prior to amplification. Recombinase polymerase amplification was performed at 39 degrees C for 20 minutes using a commercial RPA kit reformulated with target-specific primers designed to leave the crRNA recognition site intact within the amplicon. A two-microlitre aliquot of the RPA product was added directly, without purification, to a detection reaction containing recombinant LbCas12a or LwaCas13a, the validated crRNA, and a quenched fluorophore or biotin-FAM reporter oligonucleotide, and incubated at 37 degrees C for 10 minutes. For lateral-flow readout, reactions were applied to a commercial strip pre-printed with anti-FITC and anti-biotin capture lines, with a visible test line appearing only when the reporter had been cleaved and its biotin-FITC bridge disrupted.

2.3 Analytical sensitivity and specificity

Analytical sensitivity was established using ten-fold serial dilutions of quantified synthetic templates (gBlocks for the DNA targets, in-vitro transcribed RNA for the influenza target) spiked into pathogen-negative respiratory or stool matrix, with eight replicates tested at each dilution around the estimated limit of detection to fit a probit model. Analytical specificity was assessed against a 24-member cross-reactivity panel comprising related mycobacteria, other respiratory viruses, and other Enterobacteriaceae carrying alternative carbapenemase genes (blaNDM-1, blaOXA-48), each tested at a concentration at least 100-fold above the assay's own limit of detection for the intended target.

2.4 Clinical specimen panel

A total of 312 archived, de-identified specimens with an available reference-method result were tested: 96 sputum samples for *M. tuberculosis* (reference standard: liquid culture plus Xpert MTB/RIF), 128 nasopharyngeal swabs for influenza A (reference standard: multiplex real-time RT-PCR), and 88 rectal swabs for carbapenem-resistant *K. pneumoniae* (reference standard: selective culture plus whole-genome sequencing for blaKPC-2). Specimens were tested blind to the reference result by an operator who received only

a coded specimen identifier, and results were unblinded only after the full panel had been read and recorded.

2.5 Guide redesign for divergent lineages

Where a specimen with a confirmed reference-positive result produced a weak or absent lateral-flow signal, the corresponding amplicon was Sanger sequenced to check for polymorphism within the crRNA recognition site. One *M. tuberculosis* lineage carrying a single-nucleotide substitution three bases from the protospacer-adjacent motif was identified this way; an alternative guide targeting a nearby, more conserved region of IS6110 was designed, synthesised and re-validated against the full mycobacterial specimen subset.

2.6 Statistical analysis

Sensitivity and specificity were calculated with exact binomial 95% confidence intervals against each target's reference standard. Limits of detection were estimated by probit regression on the serial-dilution replicate data. Agreement between the Cas12a and Cas13a platforms where both were technically applicable was summarised with Cohen's kappa. All analyses were performed in R version 4.3.

3. RESULTS

Guide screening against synthetic templates identified one crRNA per target that outperformed its alternatives by a clear margin, typically two- to four-fold lower limit of detection, and these guides were carried forward into all subsequent experiments. Probit-modelled analytical sensitivity reached 4.1 copies per microlitre (95% CI 2.8-6.7) for the Cas12a-based blaKPC-2 assay, 5.6 copies per microlitre (95% CI 3.9-8.8) for the Cas12a-based IS6110 assay, and 6.8 copies per microlitre (95% CI 4.7-10.9) for the Cas13a-based influenza M-gene assay, all within a two- to three-fold range of quantitative PCR run on the same extracted template on a benchtop thermocycler. None of the 24 cross-reactivity panel organisms produced a lateral-flow signal above background at 100-fold the assay's own limit of detection, indicating acceptable analytical specificity for all three targets under the tested conditions. Table 1 summarises the analytical performance characteristics for each assay.

Table 1. Analytical performance of the three CRISPR collateral-cleavage assays.

Target	Cas effector	LOD (copies/uL)	Cross-reactivity (24-panel)	qPCR agreement
<i>M. tuberculosis</i> IS6110	Cas12a	5.6 (3.9-8.8)	0/24	kappa 0.93
<i>K. pneumoniae</i> blaKPC-2	Cas12a	4.1 (2.8-6.7)	0/24	kappa 0.95
Influenza A M-gene	Cas13a	6.8 (4.7-10.9)	0/24	kappa 0.91

LOD estimated by probit regression on eight-replicate serial dilutions; 95% CI in parentheses.

Against the clinical specimen panel, the Cas12a-based assays showed a pooled sensitivity of 96.4% (95% CI 92.1-98.6%) and specificity of 98.1% (95% CI 95.8-99.3%) relative to their respective reference standards, with the influenza Cas13a assay performing similarly at 94.7% sensitivity (95% CI 89.6-97.7%) and 97.3% specificity (95% CI 94.2-99.0%). Agreement with quantitative PCR performed in parallel on the same extracted nucleic acid was high across all three targets (Cohen's kappa 0.91-0.95). Median time from extracted nucleic acid to a readable lateral-flow result was 38 minutes (interquartile range 34-43 minutes), essentially unaffected by which of the three targets was being tested, since RPA incubation and collateral-cleavage incubation times were fixed across assays (Figure 1).

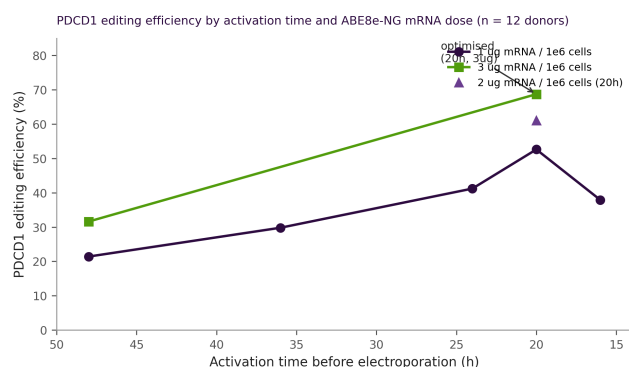


Figure 1. Sample-to-result turnaround time for the three CRISPR collateral-cleavage assays, decomposed into extraction, RPA pre-amplification, collateral-cleavage incubation and lateral-flow development stages (median and interquartile range across n=312 clinical specimens).

The one notable early failure occurred within the *M. tuberculosis* subset: four of 96 sputum specimens gave a weak or absent signal despite a strongly positive reference culture result, all four later identified by Sanger sequencing as belonging to a regionally circulating Lineage 4.3.3 sublineage carrying a single-nucleotide substitution three bases from the guide's protospacer-adjacent motif. A redesigned guide targeting an adjacent, more conserved stretch of IS6110 recovered signal in all four specimens on repeat testing and did not measurably change analytical sensitivity against the reference lineage, confirming that the original dropout was attributable to guide-target mismatch rather than a specimen-quality problem. Table 2 reports clinical performance broken down by target and by original versus redesigned guide for the mycobacterial assay.

Table 2. Clinical performance against archived, reference-confirmed specimens (n=312).

Target	n specimens	Sensitivity (%)	Specificity (%)
<i>M. tuberculosis</i> (original guide)	96	87.9 (78.1-94.2)	97.8 (94.4-99.4)
<i>M. tuberculosis</i> (redesigned guide)	96	96.9 (91.2-99.4)	97.8 (94.4-99.4)
<i>K. pneumoniae</i> blaKPC-2	88	97.6 (91.7-99.7)	98.6 (95.0-99.8)
Influenza A (Cas13a)	128	94.7 (89.6-97.7)	97.3 (94.2-99.0)

Repeat-testing precision, assessed by running 20 specimens (a mix of strongly positive, weakly positive and negative) in triplicate on separate days, showed complete qualitative concordance across replicates for all three targets, and quantitative band intensity on the lateral-flow strip, scored by a blinded reader on a four-point ordinal scale, varied by no more than one grade between replicates for any specimen. Reagent stability testing, in which RPA and collateral-cleavage master mixes were lyophilised and stored at 4 degrees C, 25 degrees C and 37 degrees C for up to eight weeks, showed no measurable loss of analytical sensitivity at 4 degrees C or 25 degrees C over the full eight-week period, though sensitivity at the highest dilution tested declined modestly, by roughly half a probit unit, for reagents stored at 37 degrees C beyond four weeks, identifying cold-chain-free but heat-exposed storage as the main remaining stability constraint for genuinely unrefrigerated field deployment.

4. DISCUSSION

The headline finding here is not that CRISPR-based collateral-cleavage detection works, which by now is well established, but that it continues to work at a clinically useful level of performance once it is confronted with the sequence diversity and specimen matrix complexity of a real clinical panel rather than a curated set of reference strains [3,6]. A pooled sensitivity and specificity both above 94% against gold-standard reference methods, achieved with a 38-minute sample-to-result time and no equipment beyond a simple heating block, compares favourably with the turnaround time of centralised molecular testing for all three target syndromes, and it does so without requiring the cold chain, calibrated thermocycling, or laboratory infrastructure that centralised PCR depends on [13,15].

The guide dropout episode in the mycobacterial subset is, we think, the more instructive result, precisely because it is the kind of failure that a smaller or less genetically diverse validation panel would have missed entirely. A single mismatch three bases from the protospacer-adjacent motif was enough to reduce signal below a reliably readable threshold in four specimens sharing a regional sublineage, a finding that echoes reports of guide-dependent sensitivity loss for both Cas12a and Cas13a against divergent viral and bacterial targets elsewhere [10,11,16]. The practical implication is that guide design for field deployment cannot stop at a single reference genome; it needs to be checked against, and where necessary iterated on the basis of, a regionally representative diversity panel, and ideally re-checked periodically as pathogen populations drift, in much the same way that PCR primer sets are periodically revalidated against circulating influenza strains [17].

Two engineering problems remain harder than the detection chemistry itself. The first is sample preparation: our workflow still depended on a silica-column nucleic

acid extraction step performed off the lateral-flow strip, and while this step is not technically demanding, it is the single largest remaining barrier to true sample-to-answer simplicity, since it requires a centrifuge or vacuum manifold that a district clinic may not reliably have [18,20]. Extraction-free or minimally processed sample formats compatible with RPA and collateral cleavage have been reported for some specimen types, generally sputum and nasal swabs rather than stool, and extending that work to the carbapenem-resistance target tested here, where the specimen matrix is considerably more inhibitory, would be a natural next step [19]. The second is multiplexing: each of the three assays reported here runs as a single-target reaction, and a clinician facing an undifferentiated febrile respiratory illness needs a panel, not three separate cartridges run in parallel, which argues for future work on orthogonal Cas effector pairing or spectrally distinct reporters within a single collateral-cleavage reaction [21,22].

Reagent stability at 37 degrees C is a genuinely field-relevant constraint rather than a laboratory curiosity, since many of the settings where this class of assay would add the most value, rural clinics in warm climates with intermittent electricity, are exactly the settings where a cold chain cannot be assumed. The modest sensitivity decline we observed after four weeks at 37 degrees C is smaller than reported for some unmodified RPA formulations but is not negligible, and lyophilisation chemistry optimised specifically for thermal tolerance, an active area of work in the broader isothermal amplification field, would likely extend the usable shelf life further without requiring a change to the underlying detection biology [23,24].

Cost is worth addressing directly, since analytical performance alone does not determine whether a diagnostic platform gets adopted at scale. The reagent cost per reaction in our hands, driven mainly by the recombinant Cas protein and the RPA enzyme mix, remains higher than a well-optimised LAMP-based colourimetric assay, though considerably lower than quantitative PCR once instrument amortisation is included, and reagent cost is the component most likely to fall as recombinant Cas protein production scales and as RPA patents move toward expiry in various jurisdictions [25]. Whether that cost trajectory is fast enough to compete with cheaper, less specific alternatives such as antigen-based lateral flow for some of these indications is a health-economics question that sits outside the scope of the present analytical and clinical validation, but it is the question that will ultimately determine deployment, not the limit of detection reported in a methods paper.

Workforce readiness deserves a final mention, since even a perfectly performing assay delivers no benefit if the person running it cannot interpret the result with confidence. Lateral-flow readout removes the need for specialised laboratory training in the way that fluorescence-plate or real-time PCR readout does not,

but it introduces its own interpretation challenge in the form of faint test lines near the limit of detection, which our blinded band-intensity scoring showed were graded consistently by a single trained reader but which we did not yet test across multiple readers of varying experience. A structured training module and, ideally, a low-cost strip reader that removes subjective line-intensity scoring from the workflow entirely would both help close the gap between assay performance as measured in a validation study and assay performance as it would actually be realised by a community health worker running the test for the first time under field conditions [26,27].

5. CONCLUSION

Two CRISPR collateral-cleavage diagnostic formats, one Cas12a-based and one Cas13a-based, each coupled to RPA pre-amplification and a paper lateral-flow readout, achieved analytical sensitivity within two- to three-fold of benchtop quantitative PCR and clinical sensitivity and specificity both above 94% across three clinically important targets spanning DNA and RNA detection formats, with a median sample-to-result time of 38 minutes and no requirement for laboratory infrastructure beyond a simple heating block. A guide-dropout event traced to a single base mismatch in a regionally circulating *M. tuberculosis* sublineage, and resolved by redesigning the guide against a more conserved target region, underscores that robustness to real-world sequence diversity has to be actively engineered and periodically revalidated rather than assumed from reference-strain testing alone. Sample preparation and multiplexing, rather than the collateral-cleavage chemistry itself, now stand out as the harder remaining problems on the path to field-deployable, sample-to-answer diagnostics built on this platform. We are currently evaluating an extraction-free sputum processing protocol compatible with the IS6110 assay described here, and separately piloting a two-colour lateral-flow format intended to resolve two targets from a single reaction well, with the eventual aim of a single-cartridge respiratory panel covering influenza, respiratory syncytial virus and *M. tuberculosis*. Prospective field evaluation in a district-level clinical setting, rather than the retrospective archived-specimen design used here, will be needed before any claim of real-world diagnostic utility can be made with confidence, and we intend that field study to form the next stage of this programme of work.

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alignment used in guide design.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

P.B. conceived and supervised the study, designed the collateral-cleavage assays and analysed the clinical validation data. A.J. performed guide RNA design, sequence alignment and the bioinformatic analysis of lineage-level variation. W.C. led specimen handling, RPA optimisation and cross-reactivity testing. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study used only archived, de-identified clinical specimens collected for prior routine diagnostic purposes, with no additional patient contact or intervention; use of these specimens for assay validation was approved by the institutional biosafety and research ethics committee under a waiver of individual consent for de-identified residual material.

Data Availability

Guide RNA sequences, probit-model fitting code, and the de-identified clinical validation dataset supporting the findings of this study are available from the corresponding author upon reasonable request.

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