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EDITORIAL

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Developing efficient diagnostic tools is essential for the timely detection and management of infectious diseases like Monkeypox (Mpox). The CRISPR/Cas (clustered regularly interspaced short palindromic repeats/Cas protein) system has gained significant attention for its precision and versatility. Derived from a natural bacterial defense mechanism, CRISPR/Cas acts as a molecular scissors [1]. CRISPR/Cas uses CRISPR RNA (crRNA) to guide proteins like Cas 3 [2], Cas 9 [3], Cas12 [4], and Cas13 [5] to specific DNA or RNA sequences. For point-of-care (POC) testing, Cas12 and Cas13 are widely utilized RNA-guided nucleases that leverage crRNA to precisely recognize and cleave target nucleic acid sequences, enabling highly specific and sensitive diagnostics. Cas12, targeting double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA), requires a protospacer adjacent motif (PAM) sequence (e.g., TTTV for Cas12a) for precise binding. Upon target recognition of target DNA, Cas12 undergoes activation and exhibits collateral cleavage, indiscriminately degrading nearby ssDNA reporters, generating a detectable fluorescence or colorimetric signal. In contrast, Cas13 exclusively targets single-stranded RNA (ssRNA) and does not require a PAM but instead recognizes a protospacer flanking sequence (PFS). Once activated, Cas13 undergoes nonspecific collateral cleavage of surrounding ssRNA, enabling real-time RNA virus detection through fluorescence or lateral flow assays [6]. Here, we discuss opportunities and challenges in implementing CRISPR-based POC testing for Mpox detection.

1. Development of multiplexed assays by simultaneous detection of multiple pathogens

The development of multiplexed assays is crucial for simultaneously detecting multiple infectious targets because they enable rapid turnaround time and require minimal sample volume, treatment, and inform treatment and isolation plans when co-infections of multiple pathogens are detected in the epidemic region. Instead of running separate tests for different poxviruses, a single multiplex assay can identify multiple strains. For example, in 2022, Mpox clade I and II spread throughout Cameroon [7]. Similarly, multiplex assays can be applied to detect other pathogens, such as SARS-CoV influenza and respiratory syncytial virus (RSV) co-infections, HIV, HBV, and HCV co-infections [8], Zika, Chikungunya, and Dengue co-infections, etc. Multiplexing of targets using nucleic acid testing (NAT)-coupled assays and enzyme-linked immunosorbent assays (ELISAs) are commonly used for multiplex detection. However, ELISAs show less specificity and a limited magnitude of dynamic range [6]. NAT-based multiplexing is becoming popular because it shows high specificity and sensitivity. PCR, LAMP, and RPA are among the most widely used techniques for multiplexing. PCR and RPA require $2n$ ($n = 1,2, \dots, n$) primers and n TaqMan probes for PCR, while RPA uses n proprietary probes (exo, nfo, and fgo). Similarly, LAMP requires $4n$–$6n$ primers and n probes. Designing these primers and probes is complex and involves rigorous checks for cross-reactivity. Although LAMP and RPA are isothermal reactions, PCR requires a thermocycler for temperature control, adding complexity. These factors make POC testing challenging for all these methods. In addition, CRISPR-based multiplexing, such as Cas12 and Cas13, allows for multiplex detection using their collateral cleavage activity, eliminating the need for multiple primers [9]. It requires only n crRNA based on their PAM site and n probes. Cas proteins such as PsmCas13b, LwaCas13a, CcaCas13b, and AsCas12a with differential trans-cleavage substrate preferences for distinct dinucleotide repeats (AU, UC, AC, and GA) located on target are used in CRISPR multiplexing [10]. For Mpox virus detection, Cas12 can be used directly with the target DNA [11], but for Cas13, the target DNA first needs to be

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transcribed into ssRNA using T7 transcription [12]. The current limitation of CRISPR-based multiplexing is that it would require pre-amplification because of its low sensitivity for low viral load, limited number of Cas protein with unique substrate preferences, and cross-reactivity for homologous target sites. Multiplexed Cas13 detection can be combined with multiplexed pre-amplification methods, such as RPA, enabling the simultaneous detection of multiple targets in a single reaction [6]. Probe-free readouts from electronic and electrochemical sensors also have potential. Electronic detection achieves single molecular sensing but requires length-encoded targets for multiplexing to have distinguished specificity [11].

2. Rapid customization for emerging variants by adaptive diagnostic platforms

To address the challenges of implementing CRISPR-based POC testing for Mpox, rapid customization is essential for maintaining diagnostic accuracy, sensitivity and specificity as the virus evolves. CRISPR platforms enable quick adaptation by modifying crRNA sequences to target new mutations, with simplified assays overcoming the limitations of traditional methods like PCR that require specialized equipment and expertise. These systems, enhanced by the sequence-independent collateral cleavage activity of Cas enzymes, can be rapidly updated to detect emerging viral strains. Key challenges include streamlining regulatory approvals through modular CRISPR designs, where only crRNA sequences are updated, which can accelerate deployment while meeting WHO ASSURED criteria: affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable to end-users [13]. The modular design of these tests means that only the crRNA sequence needs to be updated to target new variants, which could accelerate regulatory approval and facilitate the rapid deployment of new diagnostic tools. Researchers are leveraging conserved regions of the MPXV genome for broad detection and variable regions for strain-specific identification [6]. CRISPR–Cas12a-based nucleic acid detection methods [11], such as DETECTR [4], RAA-Cas12a-MPXV [14], and those integrated with high-sensitivity signal transduction technology offer promising approaches for Mpox detection [15,16]. CRISPR-based methods like HOLMES [17], Cas12aVDet [18], and DIgital CRISPR approach (WS-RADICA) [19] also hold potential for future Mpox detection applications. This technology is particularly promising for underserved communities, enabling the simultaneous detection of multiple viral strains in a single test. This approach enables efficient screening, timely detection, and swift public health responses, ensuring effective outbreak management in resource-limited settings. These advancements position CRISPR-based POC tests as transformative tools, enabling timely and accurate Mpox detection to manage outbreaks effectively.

3. Enhancing reagent stability and shelf-life

To facilitate the deployment of CRISPR–Cas technologies in resource-limited settings without reliable cold chain infrastructure, enhancing reagent stability and shelf-life is critical. Lyophilization (freeze-drying) is a key method, where water is removed from reagents to allow room-temperature storage without significant activity loss [20]. Interestingly, untreated lyophilized reagents showed diminished signal compared to pre-lyophilization. To mitigate this and improve stability for Mpox detection, Wang *et al.* added 0.1% BSA as a cryoprotectant [12]. This streamlined assay preparation, which includes viral lysis and lyophilization, improved user convenience, reduced processing time, and enhanced suitability for field applications. Stabilizing excipients like sucrose and mannitol protect proteins and nucleic acids during the process, while destabilizing agents such as polyethylene glycol and potassium chloride are removed to enhance stability. Despite these advances, challenges persist, including limited stability of some lyophilized master mixes at room temperature due to protein aggregation without nonionic surfactants [20]. Paper-based devices offer an alternative for reagent stability, embedding reagents onto substrates for easy transport and incorporation into POC testing tools [13]. Kairo-CONAN, a CRISPR–Cas3-based portable Mpox diagnostic platform, leverages this approach along with gold nanoparticles, utilizing a disposable hand warmer for heat and freeze-dried reagents for ambient stability, enabling detection via lateral flow assay [6,21]. Storage conditions also remain crucial, while lyophilization can allow for room temperature storage, as refrigeration or freezing may still be required for some reagents and biological samples like bodily fluids to maintain integrity [6]. Finally, the purity of excipients is essential, as contaminants like peroxides in surfactants can oxidize proteins. Continued research into optimized formulations and innovative preservation methods ensures CRISPR technologies reach broader applications with enhanced stability and functionality. While stabilizing additives and removing destabilizing agents before lyophilization are essential steps, ongoing efforts are needed to overcome remaining limitations, such as limited shelf life and reduced sensitivity.

4. Integration with digital health technologies

Integrating CRISPR-based diagnostics with digital health technologies leverages mobile applications and cloud platforms to transmit test results in real-time to public health databases, enabling rapid disease hotspot identification and intervention monitoring. Wang *et al.* developed a vest pocket device (SCOPE CPod) to monitor CRISPR-based Mpox detection, which is a smartphone-based platform using a Bluetooth device [12]. Similarly, newly developed platforms like MASTR Pouch [22] and CRISPR Cube [23] extend portable digital testing capabilities. In addition, various artificial intelligence (AI)-based diagnostic methods classify Mpox alongside other pox diseases. Hybrid AI models, including ensemble learning and metaheuristic optimization, improve the classification of Mpox and other pox diseases. CNN-based models with transfer learning effectively distinguish Mpox from other skin diseases [24]. AI models, such as InceptionV3, have achieved high accuracy over 95% in classifying Mpox, Chickenpox, and Measles [25]. Comparatively, Mpox shares symptoms with Smallpox and Cowpox but is generally less severe. ML methods include few-shot learning, ensemble learning, hybrid approaches, transfer learning, CNNs, RNNs, reinforcement learning, unsupervised learning, and supervised learning. However, most AI technologies rely on image analysis , which may not capture molecular or genetic variations of Mpox.. To advance detection, scientists need to develop AI systems integrated with direct sensing platforms that capture real-time signals. Portable instruments and wearable devices offer on-site testing and real-time monitoring [14]; however, challenges such as affordability, infrastructure limitations, and device accessibility hinder widespread adoption in resource-limited settings. Addressing these gaps will further optimize public health responses, strengthen global health security, and enhance disease surveillance.

5. Concluding remarks

Despite the promise of CRISPR-based detection for Mpox, key challenges remain, particularly in addressing pre-amplification requirements for low viral loads, potential cross-reactivity in multiplex assays, and maintaining reagent stability at ambient temperatures. Future efforts should optimize multiplex assays, integrate sample preparation and assay preparation steps, enhance reagent formulations for room-temperature storage, and refine integrated digital health solutions to accelerate equitable, rapid, and accurate outbreak response.

Author contributions

Md. Ahasan Ahamed drafted the original manuscript, and Weihua Guan planned the concept/design of the article and revised it critically for important intellectual content.

Disclosure statement

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