UJEMI PEARLS

A CRISPR-Cas13 system based diagnostic tool for arboviruses

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SUMMARY The unpredictability of the emergence and re-emergence of arboviruses underscores the need for enhancing global preparedness in surveillance capacity for tracking and predicting the growth of arboviral epidemics and case-specific arboviral disease management. In particular, the development of a more advanced clinical diagnostic tool is important for low-resource countries with a higher chance of occurrence of new arboviral epidemics. Despite rapid improvements, the current diagnostic approaches still have limitations and are incapable of meeting the increasing demand for an ideal diagnostics for routine clinical diagnosis of arboviruses. Furthermore, the global incidence of common arboviruses like mosquito-borne dengue viruses (DENV) and Zika viruses (ZIKV) continues to increase dramatically over the years due to globalization, natural and demographic changes. The co-circulation of these arboviruses and different serotypes of the same arbovirus presenting similar clinical symptoms in the tropical regions of the world have further challenged the limits of current diagnostics. In addition to being a programmable gene-editing platform, the recent CRISPR-Cas system has demonstrated itself to be a promising diagnostic tool through its capability to directly detect viruses in clinical samples in an inexpensive, rapid, sensitive and specific manner. The SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) platform exploits the CRISPR-Cas13 endonucleases system in prokaryotes. It has the unique ability to specifically cleave the target followed by subsequent non-specific cleavage of all transcripts in proximity of the target, known as collateral effects, which enables the system to detect the viral genome target upon amplification of the non-specific cleavage of reporter molecules in proximity of the target. This article will explore the currently approved molecular and serological diagnostic tools for arboviruses (specifically ZIKV and DENV) and compare them with the SHERLOCK platform by investigating their advantages and disadvantages in a clinical setting. This article will further explore the current potential and limitations of the SHERLOCK platform to be developed as a universal point-of-care test for arboviral infection, which may drastically change the future of diagnostics for infectious and non-infectious diseases.

INTRODUCTION

Arthropod-borne viruses (arboviruses) have taken the world by surprise in becoming the major public health concern globally. In particular, Zika virus (ZIKV) emerged decades after its first discovery in the 2013-2017 epidemics in the Pacific islands and Latin America, and dengue virus (DENV) has become the most rapidly spreading global endemic in the tropics recently [1-4]. In addition, the emergence and re-emergence of arboviruses has been unpredictable with respect to the disease transmission and outcomes [1]. Arboviruses are difficult to accurately and rapidly diagnose due to being asymptomatic in 20% to 40% of cases, and if symptomatic, they manifest similar non-specific symptoms in the acute stage among themselves and with other common tropical parasitic and bacterial infections such as malaria, including headache, myalgia, and joint pain. Moreover, patients often are simultaneously co-infected with other infectious diseases [5]. To further complicate the clinical diagnosis, arboviruses often share a similar mode of transmission in urban and suburban regions and each of them often consists of multiple serotypes due to high mutation rate of the viral genome [1, 5-8].

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As a result, arboviruses have been extensively studied. In particular, the arboviruses borne by *Aedes* spp. mosquitoes such as ZIKV and DENV belong to the genus *Flavivirus*; members of this genus impose the greatest health impacts [5]. They are studied as a model for predicting and preparing for potential new arboviral outbreaks, and for understanding biological pathogenesis in order to develop therapeutic options and epidemiological surveillance [6]. ZIKV is genetically divided into the African and Asian strains, and has been associated with neurological complications [4, 5, 7, 8]. DENV includes four serotypes, designated DENV-1, DENV-2, DENV-3 and DENV-4; they can cause life-threatening dengue hemorrhagic fever or dengue shock syndrome as a result of hetero-serotypic secondary DENV infection due to antibody-dependent enhancement (ADE) [5, 6, 9].

Increasing global human traffic, urbanization, and global warming have continuously caused the geographical expansion of arbovirus vectors and viral epidemics worldwide as well as the emergence of co-circulation of different arboviruses. Multiple serotypes of the same arboviruses transmitted by the same arthropod have challenged the public health and economic burden of endemic countries, underscoring the need for advanced and universal serotype-specific diagnostic tools for arboviruses in all healthcare settings since patients with early detection and proper medical care have a fatality rate less than 1% [8, 10].

A newly developed system, based on Clustered Regularly Interspaced Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) proteins, has been demonstrated to be a promising diagnostic candidate [11-13]. CRISPR-Cas, a component of the natural adaptive immune system found in bacteria and archaea to fight against invasive genetic elements [11, 12]. The system is based on short sequences captured from exogenous DNA fragments of previous infections. These sequences are transcribed into CRISPR RNA (crRNA), which, in conjunction with Cas enzymes, guide the degradation of similar nucleotide sequences via complementary base pairing [11]. The exploitation of this mechanism with arboviral genetic material has been proposed for the development of a point-of-care molecular diagnostic tool for arboviruses due to its capacity for rapid target detection directly in clinical samples in an inexpensive, sensitive and specific manner [11-13].

RESEARCH QUESTIONS

Arbovirus is now a major global health concern; as a result, global awareness and preparedness for potential arboviral disease outbreaks have increased drastically in the last decade. Despite the rapid development of medical diagnostic tools including the currently approved nucleic acid- and protein-based technology such as reverse transcription polymerase chain reaction (RT-PCR) and serological tests, respectively, limitations exist. The CRISPR-Cas system based SHERLOCK has recently been identified as a potential diagnostic technology. However, further improvements of the system are required and proposed to increase its clinical applicability. In order to determine whether SHERLOCK can outperform the currently available diagnostic tools and to determine its potential for being a point-of-care test for arboviruses, this review focuses on the following research questions:

1. What are the limitations of RT-PCR and serological tests for arboviruses?
2. What is the clinical applicability of SHERLOCK?
3. What are the potential limitations of SHERLOCK and how can they be addressed to improve its applicability as a universal medical diagnostic of arboviral diseases?

PROJECT NARRATIVE

What are the limitations of currently approved RT-PCR and serological tests for arboviruses? Currently, diagnostic approaches for common arboviruses such as ZIKV and DENV are based on nucleic acid- and protein-based technology, such as RT-PCR and serological assays, respectively [5]. Real-time RT-PCR is rapid, specific and sensitive for direct detection and amplification of targets in clinical samples based on primers targeting the highly conserved regions of viral genomes. In the case of ZIKV, primers are designed to target the flanking 5′ and 3′ non-coding regions of genome, as well as the nonstructural proteins (NS) [14]. Viruses in serum are detectable up to 5 days after the onset of disease

symptoms. Recently, studies have found that viruses are detectable up to 14 days after onset of symptoms in urine samples [5]. Despite the low viral burden in blood, the assay is sensitive as the limit of detection of different PCR assays ranges from 10^1 to 10^3 viral copies/ml, whereas viremia in blood ranges from 10^2 to 10^6 viral copies/ml in the acute stage of infection [14, 15]. In addition, the assay is highly specific such that it is able to distinguish between the African and Asian strains of ZIKV in samples from multiple geographical locations, and minimize false-positive results because of cross-reaction with other co-circulating arboviruses such as DENV [15]. The average percentages of positive and negative agreement in serum across all FDA-approved PCR assays are 97% and 95%, respectively [14]. In particular, the Trioplex real-time RT-PCR assay is multiplexed for ZIKV, DENV, and chikungunya viruses; multiple samples can be simultaneously tested per round of reaction with primers targeting different common tropical infectious agents, achieving high efficiency [14]. However, these assays are expensive as laboratory equipment and trained personnel are required. It is not an ideal solution for resource-poor endemic regions, as sometimes samples need to be shipped internationally for diagnosis. In addition, both DENV and ZIKV have a short window of time for detection, as viremia exists only in the acute phase of the infection, which is often left unnoticed due to its flu-like symptoms [5].

Beyond the acute phase, serological assays on serum samples are used [5]. The serological assays are based on detection of antibodies against flavivirus antigens about one week following the infection, primarily against E and NS1 protein [14]. Although the IgM antibody titers decrease over time, they remain detectable in over 80% of patients for about six months [14]. In addition, patients remain seropositive for IgG against the infected flavivirus for decades after infection [5]. Hence, most assays are unable to differentiate between primary and secondary flavivirus infection. Moreover, due to the high structural homology of viral antigens among different serotypes of the same virus and across different co-circulating flaviviruses, false positives are frequent from high cross-antigenicity [5, 14]. In general, serological assays demonstrate a high positive agreement, ranging from 83.3% to 100% but as low as 30.9% for negative agreement since it is more prone to interference [14]. As a result, subsequent confirmatory tests are required. Nevertheless, the serological assays remain the most commonly used diagnostic tools, especially in low-resource countries and during outbreaks, due to their cheap price, rapid result production and easy administration.

What is the clinical applicability of SHERLOCK? The CRISPR-Cas system is diverse and SHERLOCK uses the Cas13 protein of the Type VI system of Cas family [16]. The Cas13 protein is an RNA-guided RNA endonuclease that cleaves single-stranded RNA (ssRNA) targets with the aid of single-stranded crRNA via complementary base-pairing [10]. It contains two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains that function as ribonuclease (RNase) [16, 17]. These domains are only activated through a conformational change upon binding of the target-crRNA complex, and cleave the target [16]. However, the HEPN domains will remain enzymatically active after the target cleavage, and non-specifically cleave any nearby RNA transcripts, known as collateral effect [10, 16].

In combination with HUDSON (heating unextracted diagnostic samples to obliterate nucleases) which extracts nucleic acids after both heat and chemical inactivation of RNases in body fluids and cell lysis, and RPA (recombinase polymerase amplification) which amplifies the released genetic material, SHERLOCK is able to directly detect the viral genome of interest from clinical samples of patients' bodily fluid without extensive sample preparation and does not require expensive lab equipment and trained personnel [18, 19]. The recently updated version allows the diagnostic results to be visually displayed on a paper strip via lateral-flow based technology, which further enhances its portability [19]. It is estimated that each reaction costs about 60 cents, and produces results within two hours of sample input. This is ideal as a point-of-care diagnostic tool [19].

Moreover, SHERLOCK is desirable for arboviral disease diagnosis due to its high sensitivity and specificity. By exploiting the unique cleavage property of Cas13, SHERLOCK is extremely sensitive as the limit of detection is as low as 2 attomolar,

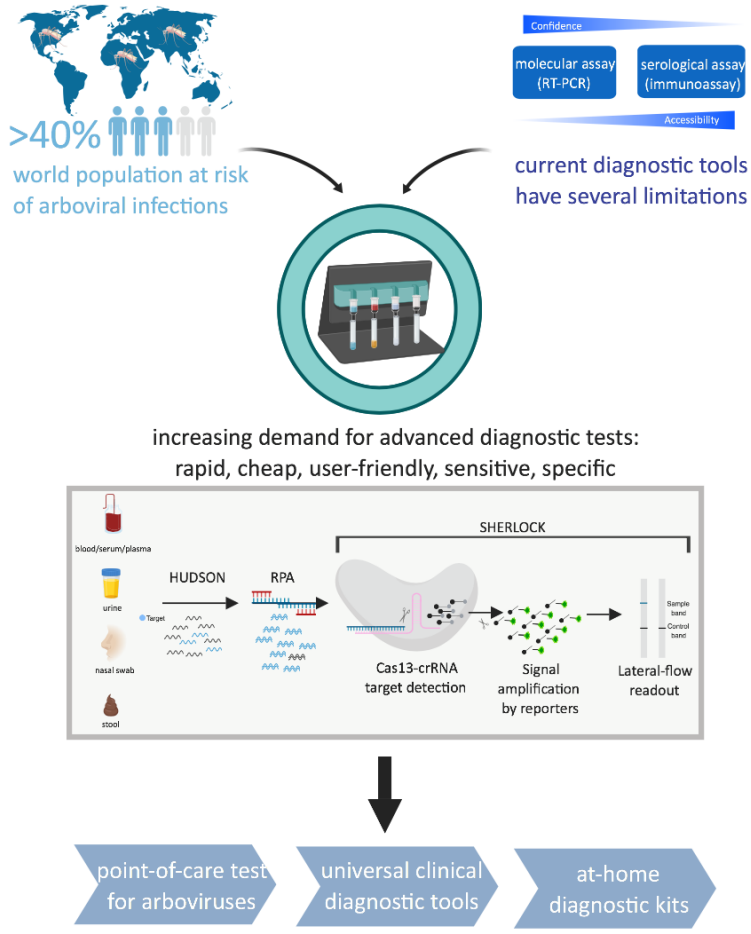

FIG. 1 Schematic for a novel CRISPR-Cas13-based technology that has great potential for future development into an ideal universal point-of-care diagnostic tool for arboviruses and other pathogens. This figure underscores the increasing demand for a more advanced diagnostic test as a result of the worldwide emergence of arboviruses and limitations of current diagnostic tools in early detection and disease monitoring. SHERLOCK, a CRISPR-Cas13-based technology is able to produce diagnosis outputs in a rapid, cheap, user-friendly, sensitive and specific manner. It also has a great potential for further development into point-of-care tests for arboviruses, universal clinical diagnostic tools for infectious agents, as well as at-home diagnostic kits available for the general public.

exceeding significantly above the current diagnostics [18]. The signals are amplified upon detection of the target by adding reporter molecules such as fluorescent reporters into the HUDSON-treated clinical samples, or biotins in lateral-based paper readouts, exploiting the collateral effect [18, 19]. In terms of specificity, the platform can reliably distinguish between DENV, ZIKV, yellow fever viruses, and other commonly co-circulating flaviviruses [18]. Specifically, the platform is able to differentiate among the four DENV serotypes, and ZIKV African strain from the American strain (evolved from the Asian strain) [18-20]. The determination of DENV serotypes is critical for patients that are re-infected with DENV, since patients are at a greater risk of severe dengue complications when infected with a different serotype as a result of ADE [9, 21, 22]. Previous infection triggers a strong and life-long antibody response against the infecting DENV serotype. The memory antibodies can cross-react with DENV of a different serotype of the secondary infection to form a virus-immune complex; the complex, instead of neutralizing the virions, aids viral entry and replication, aggravating the infections [9]. Detecting the specific ZIKV strain in patients is especially vital for pregnant women, as only the Asian strains have been associated with fetal birth-defect [23]. As a result, with early diagnosis, healthcare workers are able to predict the disease outcome in patients and manage their treatment appropriately.

Collectively, these features of SHERLOCK demonstrate its potential as a diagnostic tool for arboviruses as it is sensitive and specific as RT-PCR, and inexpensive and user-friendly as serological assays.

What are the potential limitations of SHERLOCK and how can they be addressed to improve its applicability as a universal medical diagnostic of arboviral diseases? However, several limitations need to be addressed with SHERLOCK in order to fully realize its potential as a medical diagnostic tool for arboviral diseases. The major limitation of SHERLOCK is that it is not amenable to multiplexing. Currently, the updated system is capable to simultaneously detect up to three different RNA viruses. However, this does not

meet the criteria of an ideal arbovirus diagnostic tool since they are known to be genetically diverse. For example, the *Flavivirus* RNA genome has a high mutation rate; multiple serotypes and strains of the same virus exist, like ZIKV and DENV [5-8]. In addition, increasing globalization has led to different arboviruses and various strains of the same arbovirus co-circulating in the same region. Therefore, multiplexing capability is crucial for arboviral diagnosis. Perhaps the platform could incorporate more Cas13 enzyme variants in the reaction cocktail, as demonstrated in SHERLOCK v2 [19]. SHERLOCK v2 is able to detect three different ssRNA targets by incorporating three different Cas13 with distinct cleavage preferences in the reaction mixture, selected from currently biochemically identified Cas13 enzymes [19]. Since the field of CRISPR-Cas system is advancing rapidly and more Cas proteins are being discovered, there is a great potential to find more Cas13 proteins with distinctive cleavage sites with minimized cross-reactivity for wide arrays of arbovirus. Each enzyme is selected targeting a different serotype of a specific arbovirus such as in the case of DENV [16]. Therefore, a cocktail with multiple enzymes can be used to differentiate arboviruses and other co-circulating pathogens in the same region and making the platform multiplex. However, increasing the number of enzymes in the reaction cocktail inevitably increases the price per reaction slightly, as each protein costs about $0.003 per reaction [19].

Flaviviruses, as well as other single-stranded RNA viruses, have a high mutation rate primarily due to the lack of exonuclease proofreading activity in their RNA-dependent RNA polymerase [24, 25]. This high mutation rate is beneficial for the viruses; it is selected evolutionarily since it promotes higher genetic variability, increasing the chance of beneficial mutations for adaptation to changing environments. However, it also renders the diagnosis more difficult [25]. For example, molecular diagnostic tools rely on complementary base-pairing. Although crRNA targets the non-structural proteins of the viral genome that are less prone to mutation, the risk of viral escape from detection still exists as a result of lacking complementarity between the crRNA and targeted region [26]. The limitation can be theoretically resolved by using the platform in conjunction with a WHO-level next-generation sequencing technology database which is established from compilation of acquired samples worldwide, with the goal to monitor arboviral mutations and epidemiology. Subsequently, further computational meta-analysis can be performed to understand the genomic variability and genotype the mutations to update the diagnostic and monitor changes in locally circulating strains in order to readily produce a guide crRNA for SHERLOCK during epidemics or routine diagnosis. However, the proposed solution is ideal but costly. Hence, simultaneous active research in investigating the host-pathogen interactions is required. An ideal target for SHERLOCK will be a specific, conserved host nucleic acid biomarker, which is resistant to viral mutations. With the discovery of such biomarkers and production of their complementary crRNA, the SHERLOCK platform can resolve its susceptibility to viral mutations.

CONCLUSIONS

Currently, over 40% of the world's population is at risk of arboviral infections. The emerging high-profile arboviruses worldwide like DENV and ZIKV exhibit similar acute-stage disease symptoms, and region and mode of transmission [27]. The global burdens of arboviruses have underscored the importance of developing detection tools as early diagnosis can greatly improve the disease outcome in patients and minimize the disease transmission [8]. With the advancement of new technologies, promising progress has been made. The recently developed SHERLOCK platform is a novel diagnostic and screening tool that has great potential for early detection and monitoring of arboviral diseases. The platform is based on the CRISPR-Cas13 system, which is able to produce diagnostic information in a rapid, cheap, convenient, highly sensitive and specific manner. This is ideal for a field diagnosis tool, especially in resource-poor countries where it is most needed. However, the technology still requires further improvements to address its lack of multiplexing ability and susceptibility to viral mutations; I proposed solutions including using multiple enzymes per cocktail of reaction, as well as using the platform with a well-established next-generation sequencing database while actively searching for specific, conserved host biomarkers to overcome both limitations respectively. As a result,

SHERLOCK can be further engineered into a universal diagnostic tool, applicable for standard diagnosis of a wide array of infectious agents.

In addition, a more exciting future extension of SHERLOCK is to expand its application not only as a clinical diagnostic tool, but also potentially as a point-of-care tests available at local pharmacies or even as a personal at-home test kits purchasable by the general public. Such technology will reshape the field of medical diagnosis since it is highly in demand as arboviral diseases are often left unnoticed or misdiagnosed due to its flu-like symptoms in the acute phase, preventing patients from receiving early diagnosis and proper medical care on time. Therefore with the at-home test kit, people can conveniently and rapidly screen themselves when at risk or in suspicious of the diseases without travelling to the doctor's office or hospitals. However, future research and development of the platform is required to make the extension plausible. One important barrier is sample treatment. Although nucleic acids can be directly detected and amplified from the clinical samples with the current platform, samples still need to be treated with heat and chemical enzymes [19].

The field of CRISPR-Cas system is rapidly advancing and the development of SHERLOCK exemplifies the potential of the field. SHERLOCK enables a new approach in the field of clinical diagnosis and research for infectious and non-infectious diseases. In the future, resource-poor countries can easily access high-standard point-of-care diagnosis. With further engineering of the technology, at-home diagnostic kit will be accessible to the public across the world.

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