CLINICAL IMPLICATIONS OF BASIC RESEARCH

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A CRISPR Way to Diagnose Infectious Diseases

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During the past 20 years, tremendous advances in methods involving polymerase-chain-reaction (PCR) assays and DNA sequencing have transformed clinical virology and microbiology laboratories. These new methods allow accurate and rapid diagnosis of a wide array of infectious diseases and facilitate the monitoring of responses to the treatment of infections, such as those caused by human immunodeficiency virus and cytomegalovirus. However, there remains an important gap in our diagnostic armamentarium: rapid, reliable, easy-to-use, inexpensive diagnostic tests that can be conducted at the point of care. For years there have been calls for these types of tests in areas with limited resources, but such tests may have importance across a wide range of settings, providing results that can affect clinical decisions in real time.

To this end, Gootenberg et al.[1] have reprogrammed an endonuclease that associates with CRISPR (clustered regularly interspaced short palindromic repeat) sequences in the DNA of prokaryotes (these sequences are part of prokaryotes' adaptive immune system)[2-4] to achieve single-molecule analytical sensitivity for rapid nucleic acid detection. The researchers exploited a behavior of the Cas13a enzyme called "promiscuous RNAse activity": once the enzyme cleaves an RNA target (such as a virus-specific sequence to which it is specifically guided by a complementary RNA), it can bind to and degrade other RNA fragments, such as those linked to fluorescent tags that serve as reporters (Fig. 1). Combining the described Cas13a-based detection with isothermal amplification of both DNA and RNA targets in a single tube allows for rapid and real-time detection of targets, even at low concentrations, and permits the differentiation of specific variants in either RNA or DNA.

To determine whether this method is likely to provide the sensitivity and specificity required for the diagnosis of infectious diseases, Gootenberg et al. constructed lentiviruses containing fragments of the genomes of the Zika virus (ZIKV) and the dengue virus (DENV). The system detected ZIKV sequences at concentrations as low as 2 attomolar (aM) and could distinguish ZIKV from DENV, an important characteristic for clinical applications (Fig. 2). The authors then explored the potential use of this method in the field by lyophilizing the reagents and then rehydrating them in a spot on paper.[5] The paper-based system worked but resulted in a lower overall fluorescent signal for ZIKV and a higher background signal for DENV, such that the detection limit was reduced by about 1 $\log_{10}$ (20 aM). The performance of the detection method was also assessed with the use of serum and urine samples from four patients infected with ZIKV; the test detected ZIKV in all four clinical samples, with RNA concentrations ranging from 8.25×10^5 copies per milliliter to 1.25×10^3 copies per milliliter, as verified by means of quantitative real-time PCR. No data regarding assay specificity were presented for DENV.

The authors also showed how the method, designated SHERLOCK (specific high-sensitivity enzymatic reporter unlocking), identified bacterial pathogens by targeting the 16S rRNA gene V3 region. Starting from an isolated colony, SHERLOCK could detect *Escherichia coli* and *Pseudomonas aeruginosa* and distinguish these species from *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*. In addition, SHERLOCK could readily differentiate clinical isolates of *K. pneumoniae* that possess either carbapenemase or New Delhi metallo-beta-lactamase-1 resistance genes. Finally, the specificity of SHERLOCK was

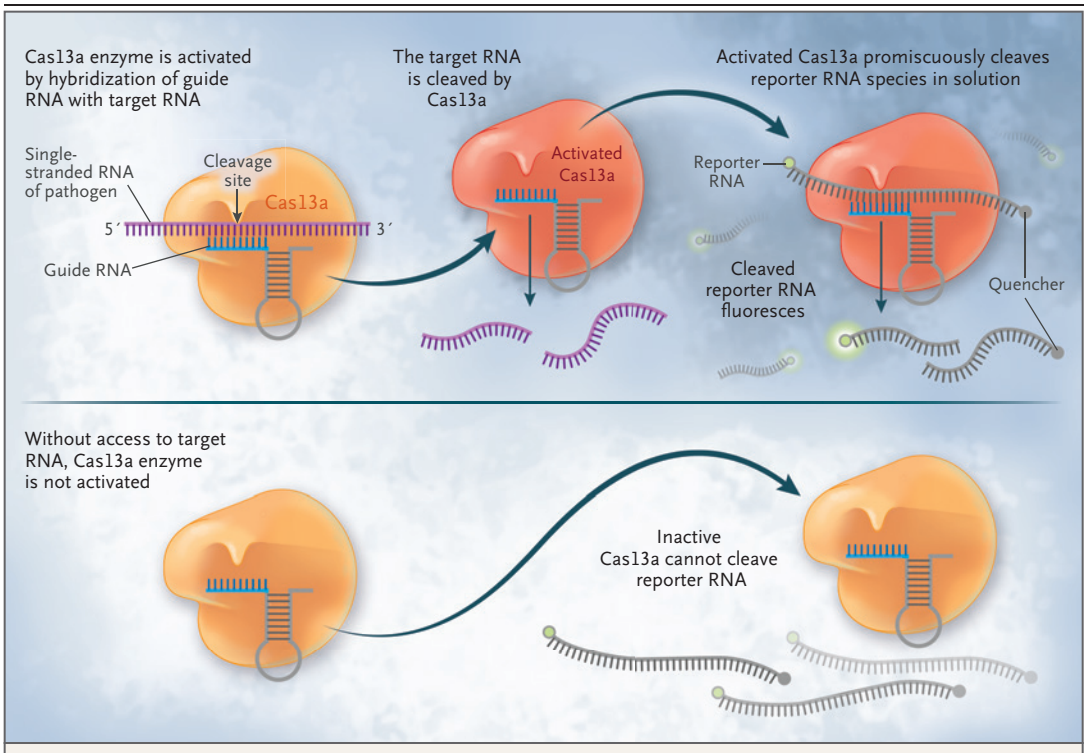

Figure 1. The Mechanism of SHERLOCK.

The SHERLOCK reaction combines a preamplification of DNA with recombinase polymerase amplification (RPA) or a preamplification of RNA with reverse-transcription RPA with subsequent Cas13a-directed collateral detection. During preamplification, T7 RNA polymerase promoters are added to allow the transcription of amplified DNA to RNA. This RNA can then be detected through incubation with Cas13a, complementary CRISPR RNAs, and fluorescent RNA sensors. On binding a target RNA sequence, the Cas13a enzyme becomes activated and promiscuously cleaves other RNA species in solution, a phenomenon known as the collateral effect. RNA sensors with a fluorescent reporter molecule on the 5′ end and a quencher molecule on the 3′ end are cleaved by activated Cas13a, generating a fluorescent signal. In the absence of Cas13a activation, cleavage of the reporter RNA and generation of fluorescence do not occur. The combination of amplification steps and this detection system allows for attomolar detection with single-base specificity within 1 to 2 hours.

enhanced by introducing synthetic changes to the guide RNA that led to the generation of one or more mismatched bases when hybridized with target RNA. This modified assay allowed for the discrimination of targets that differed in only a single base pair and successfully distinguished such targets as African and American strains of ZIKV, different serotypes of DENV, five health-related gene alleles from human saliva, and various cancer-related mutations in suspensions of cell-free DNA. The latter represents one of the most powerful applications of SHERLOCK. The method also has several important characteristics that make it feasible for use in point-of-care testing, including the speed with which a paper-based test can be designed and synthesized (only a few days), the estimated cost of the reagents and materials (less than $1 per test), the stability of the lyophilized reagents, and the speed with which the assay can be performed (within 1 to 2 hours).

Although the results described by Gootenberg et al. are encouraging, a more rigorous assessment of the performance characteristics of the SHERLOCK system is needed for each of the described applications. Moreover, the process of bringing any new technology to market and introducing it into clinical practice is challenging. The test would need to have performance characteristics that are similar to those of the current laboratory methods used to analyze clinical samples from patients and yet be designed with

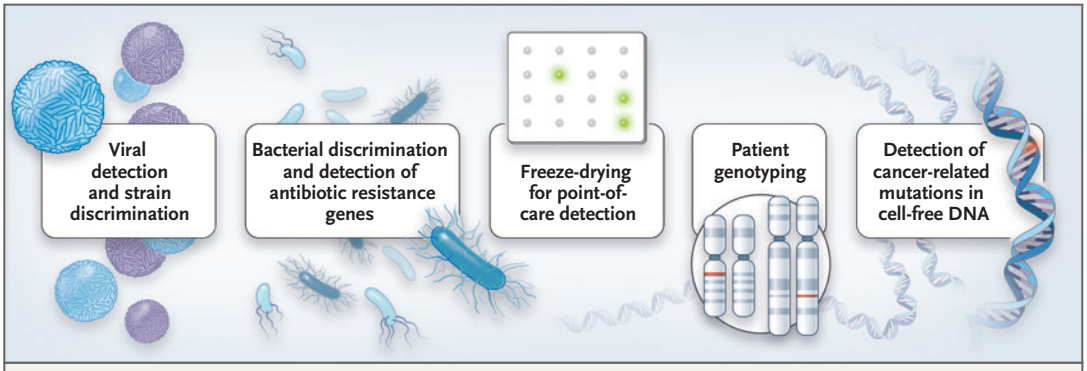

Figure 2. Versatility in Application.

Gootenberg et al.[1] showcased the application of SHERLOCK in several scenarios. These include detecting a single molecule of Zika virus and distinguishing closely related strains; discriminating different species of bacteria and detecting antibiotic-resistance genes; freeze-drying for point-of-care detection; performing rapid genotyping of patient samples; and detecting cancer-related mutations in cell-free DNA.

the simplicity needed for use at the point of care by clinicians or nonlaboratory personnel. The regulatory bar for the clearance of point-of-care tests by the Clinical Laboratory Improvement Amendments is quite high, and the process of obtaining a waiver can be long, complex, and expensive. A less intuitive challenge is the acceptance and uptake of a new test in clinical practice. In the past, some traditional diagnostic methods, such as viral culture and rapid antigen-detection tests, were so limited in their overall performance characteristics that new molecular technologies were quickly incorporated into clinical use. Now, however, tests must be shown to improve clinical care or to be cost-effective; obtaining the funding for and performing the relevant studies would be challenging. Although there is a clear need for rapid and inexpensive point-of-care tests, and although the excitement about the possibility that the SHERLOCK technology may, like PCR, represent a breakthrough in terms of the speed and the accuracy of detection and differentiation of many microbial pathogens, we eagerly await more detailed studies to determine whether this innovative method can fill the diagnostic "gap."

Disclosure forms provided by the authors are available at NEJM.org.

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