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CRISPR diagnostics: Underappreciated uses in perinatology

Adam Pan*, and Katherine L. Kraschel

Solomon Center for Health Law & Policy, Yale Law School, United States

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ABSTRACT

CRISPR-based therapeutics have the potential to revolutionize the treatment of hereditary diseases, but current efforts to translate research to the bedside face significant technical, regulatory, and ethical hurdles. In this article, we discuss an underappreciated application of CRISPR: diagnostic testing, and argue that: (1) CRISPR diagnostics are poised to disrupt diagnostic practices including perinatal screening and (2) since CRISPR diagnostics pose minimal technical, regulatory and ethical hurdles (unlike CRISPR therapeutic uses) they are likely to be clinically relevant before CRISPR-based therapies, and thus warrant medical community's attention.

Introduction

In the past seven years, a previously niche field of research has taken the world by storm. The technology, known as clustered regularly interspaced short palindrome repeats, or CRISPR, relies on an ancient bacterial immune response that was first characterized in 1993.[1] While there were only 86 CRISPR-related research papers in 2011, there were well over 2000 in 2016.[2] The prodigious increase in publications may be attributed to discoveries in 2012 and 2013 by two research groups that along with an enzyme known as Cas9, CRISPR could be used to create a revolutionary gene-editing platform.[3] Since then, the scientific community has been alight with the myriad potential uses for CRISPR. When confronted with such exciting innovation, our instinct often drives us to focus on the most ambitious and world-changing applications, and the community has naturally gravitated towards gene therapy and other interventional techniques. However, given the paradigm-shifting nature of CRISPR therapeutics, sufficiently understanding their clinical and *in vivo* consequences to safely enable their application may remain an unfulfilled aspiration for decades to come. Moreover, clinical or *in vivo* applications of CRISPR are beset by a complex, rich, and intellectually interesting combination of legal and ethical issues, making much of the discourse to date one of hypothesized risks and conundrums.[4]

In addition to its thought-provoking therapeutic uses, CRISPR is a promising diagnostic tool. As a diagnostic, the CRISPR system is not used with a goal to alter gene expression in surviving, CRISPR-altered cells, but instead harnesses the power of CRISPR to efficiently and specifically detect nucleic acids and produce measurable downstream effects in cellular samples that are otherwise usually discarded. Unlike therapeutic applications, diagnostic applications of CRISPR are typically noninvasive and do not carry the risk of directly harming the patient. Perhaps because of the very fact that they do not implicate as many ethical quagmires, CRISPR's utility as a diagnostic is arguably under-discussed in the literature and popular press. This is problematic given that CRISPR diagnostic implications are more likely to become a reality much sooner than many clinical uses more broadly

*Corresponding author.
E-mail address: Adam.Pan@yale.edu (A. Pan).

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Table 1 – Comparison of the two major clinical applications of CRISPR and their respective potential benefits, risks, and regulatory barriers.

	CRISPR *ex vivo* & *in vivo* therapeutics	CRISPR diagnostic screening
Potential clinical benefits	Treatment for: • Certain genetic diseases in specific tissues • Non-genetic diseases through techniques such as CAR-T therapy • Treatment for broad class of genetic diseases in many tissues	Screening that is: • Specific • Sensitive • Inexpensive • Noninvasive • Field deployable • Field deployable
Clinical risks	• Off-target editing may lead to unintended and irreversible mutations and possible cytotoxicity. • Immune response/cytokine reaction	• Direct risks to patient unlikely • Possible sensitivity and specificity issues (false positives/negatives) may lead to adverse medical decisions
Regulatory barriers	FDA Biologics license application and clinical trials	Premarket Approval (PMA) in high-risk instances or FDA Premarket Notification 510(k) for most *in vitro* diagnostics or

discussed, and are of particular interest to perinatology. In this article, we discuss the current landscape of CRISPR applications in perinatology and argue that more attention should be paid to the impact of CRISPR diagnostic technology in the field. (Table 1)

What is CRISPR?

CRISPR refers to an adaptive immune response in archaea and bacteria that is used to target and degrade viral DNA.[iv] This is achieved through an endonuclease (e.g. Cas9), a protein capable of cleaving DNA in a highly specific way.[iv] By reengineering this immune response to target specific segments of genetic material, researchers could make highly precise genetic modifications to virtually any type of cell, including human cells.[iv] This is the basis of CRISPR therapeutic and diagnostic platforms. A key component of CRISPR platforms is designing the so-called guide RNA, or gRNA, to very precisely target specific nucleic acids, such as those genetic sequences that are only present in particular diseases or conditions. It is important to note that much of the potential associated with CRISPR technologies is not necessarily due to the enablement of new modes of genetic engineering, but that it far supersedes prior modes of genetic engineering in efficiency, speed, and cost and thus allows for more nuanced and precise applications of existing gene editing strategies.

Diagnostic use of CRISPR

The promise of CRISPR diagnostics

Diagnostic uses of CRISPR technologies promise huge gains in access and clinical effectiveness. The recent Zika outbreak illustrates the disruptive power of CRISPR technologies in perinatal diagnostics. Between 2015 and 2016, several countries, including the United States, experienced a widespread outbreak of Zika fever.[5] Researchers quickly discovered that while the symptoms of Zika fever were mild in adult patients, the virus could be transmitted from an infected pregnant woman to her fetus, resulting in severe birth abnormalities including microcephaly.[6] Diagnosis of Zika virus infection was difficult, as the clinical signs for an infection overlapped significantly with other arboviruses, such as dengue fever or West Nile virus.[7] Accurate diagnosis required either blood or urine tests using complex and expensive and/or time-intensive platforms (such as dengue virus-specific IgM ELISA, plaque-reduction neutralization tests, or RT-PCR.)[8] When the American Red Cross attempted to test roughly 4 million blood samples for the virus, the resulting laboratory tests came to a cost of nearly $42 million, or about $10 per test.[9]

This year, *Science* published three articles demonstrating new and improved diagnostic uses for CRISPR. The first paper, published by researchers at the Broad Institute, a collaborative research center of Harvard University and the Massachusetts Institute of Technology, described an update to their SHERLOCK diagnostic platform first described last year.[10] Dubbed SHERLOCKv2 (Specified High-Sensitivity Enzymatic Reporter UnLOCKing), the CRISPR-based diagnostic platform could identify multiple viruses in a single sample—such as Zika and dengue—while exceeding the sensitivity of SHERLOCKv1 by a factor of one hundred.[11] The SHERLOCK platform is based on Cas13 (a molecular relative of the Cas9 enzyme used in many CRISPR therapeutic platforms that cuts RNA rather than DNA). With the help of gRNA, Cas13 can target and cut specific RNA sequences.x Once Cas13 has cut the target RNA, it does what researchers have termed "collateral cleavage" and cuts other RNA sequences. This collateral cleavage effect is used to degrade labeled RNA also added to the sample that produces a signal once it is cleaved, indicating the presence of the sequence targeted by the gRNA cleaved by Cas13. When the platform is combined with nucleic acid amplification, it can detect single-base mismatches at attomolar (10^{-18} moles/litre) sensitivity. Since Zika and Dengue are close genetic relatives, the ability of the SHERLOCKv2 platform to distinguish between the two viruses highlights the power of CRISPR platforms' specificity.

SHERLOCKv2 isn't just limited to Zika and Dengue—the researchers state that the technique is easily adaptable to a variety of diseases.[xi] The test can be integrated directly onto a paper strip in a way similar to home pregnancy tests. [xi] The

test is just as simple to use and, according to the researchers, could be manufactured for a few dollars each.[xi] A companion piece published on the same day demonstrated a complimentary protocol, HUDSON, which when combined with SHERLOCK enabled instrument-free detection of Zika and Dengue in less than two hours.[12] The paper also described how the method allows for rapid design and testing of mutations of the viruses. [xi]

In the same issue of *Science*, researchers at University of California Berkeley described an alternative CRISPR-based diagnostic tool.[13] The platform, called DETECTR, is based on many of the same enzymes used by SHERLOCK, but uses fluorescent markers to enable detection.[xiii] The publication described a system designed to detect HPV16 and HPV18 (which it can do at a 100% and 92% accuracy, respectively), but like SHERLOCK, the test could easily be repurposed to detect Zika as well. [xiii] In addition, the DETECTR team claims that each test costs less than one dollar and takes around one hour to complete—significantly cheaper than the tests currently used by the American Red Cross.[14]

These articles showcase the utility of CRISPR as a powerful diagnostic tool that is both accurate and inexpensive. While the researchers characterized SHERLOCK and DETECTR as general-purpose tools, these techniques are particularly well-suited to applications in perinatology. The applicability to Zika and Dengue screening is clear,[15] but the technology also serves as a natural evolution to current prenatal genetic screening techniques such as amniocentesis, chorionic villus sampling, and cell-free fetal DNA testing, continuing the trend of increasingly non-invasive testing. Use of a CRISPR-based detection platform could enable perinatologists to take smaller samples resulting in less invasive procedures,[16] reduce turnaround time,[17] or even enable diagnosis and reporting within a single outpatient visit.

While it is understandable that therapeutic and other *in vivo* uses of the CRISPR-Cas9 platform have captivated a significant amount of public and bioethical discourse, and it is important for the bioethical and legal landscape to proactively get out in front of the issues rather than react to their clinical use, this focus has overshadowed the more immediate and critical use of CRISPR technology in diagnostics, which is of particular relevance in perinatology.

Potential safety concerns for CRISPR diagnostics

The precision of any CRISPR-based system is critical in order to avoid targeting inadvertent nucleic acid sequences, more commonly known as "off-target" genetic edits.[iv] The prevalence and reduction and elimination of off-target effects of CRISPR remains a highly active area of research.[18]

It is likely that the clinical consequences of off-target effects of CRISPR diagnostics will be minimal. For example the SHERLOCK system showed discrimination on the basis of single nucleotide mismatches.[x] Since CRISPR diagnostics rely on an amplified downstream effect of cutting a particular nucleic acid sequence, if a relatively small number of the targeted sequences are cut "off-target" but the vast majority of targeted sequences are cut as intended, these inadvertent changes will likely only affect the sensitivity of the test. Unlike in the case of therapeutic and other *in vivo* applications of CRISPR (CRISPR-Cas9 in particular), off-target cleavage in CRISPR diagnostic platforms do not run the risk of inflicting a patient with a lifelong uncertainty or ailment since the technology never interacts with any of their surviving cells. However, this does not mitigate all risk. While unlikely given CRISPR's precision, in the event of false negatives or false positives, patients could fail to seek necessary treatment or pursue unnecessary, potentially harmful medical interventions. However, these risks are the same as those associated with any conventional diagnostic test, and unlike therapeutic applications of CRISPR, do not implicate novel considerations that must be based on a better understanding of CRISPR's effect on the human body.

Regulatory pathway for CRISPR diagnostics

To be used clinically, diagnostics are regulated and approved as a medical device by the U.S. Food and Drug Administration (FDA). Most CRISPR diagnostics will likely go through the regulatory approval pathway for *in vitro* diagnostics (IVD). Like other medical devices (but unlike drugs or biologics) the FDA classifies IVDs based on risk, and their regulatory approval pathway is generally determined by their risk classification.[19] All IVDs must be registered, are subject to listing requirements, and most adhere to adverse-event reporting requirements.[20] On the other end of the spectrum, IVDs classified as high-risk are subject to FDA premarket approval (PMA) which involves review for safety and effectiveness and most often includes clinical trials.[xix] Moderate-risk IVDs are either approved via the PMA pathway or cleared by what's known as the 510(k) pathway by showing "substantial equivalence" to an already-approved IVD. [xix] Notably, the 510(k) pathway generally does not require clinical trials and thus sidesteps the time-intensive Investigational Device Exemption required to test the IVD.

At this point in time, it is not entirely clear which pathway will apply for CRISPR diagnostics in order for them to make it to market. It will, like other devices, depend upon their intended use – that is, what disease or condition the CRISPR diagnostic seeks to diagnose/identify. Those used to identify higher-risk diseases are more likely to be classified as high-risk devices and be required to go through the full PMA process. Diagnostics for higher-risk diseases can result in greater harm in the event of an erroneous diagnosis, for false-negatives that could result in the patient not seeking life-saving treatment, or a false-positive causing significant emotional distress or seeking unnecessary, expensive and/or risky treatments. Even after other CRISPR diagnostics are approved, the expedient 510(k) clearance pathway may not be available for CRISPR diagnostics used to diagnose higher-risk conditions. However, lower-risk diagnostics might be able to access the 510(k) process.

Zika diagnostics and genetic testing kits provide some insight into the level of review that may be expected for CRISPR diagnostics. During the recent Zika outbreak, the FDA noted that the "Zika virus may have serious implications for certain populations."[21] It notified at least five companies that their Zika diagnostic tools were "high risk" tests, but did not specify if the test was subject to PMA or 510(k).[22] Zika PCR-based diagnostics were given Emergency Use Authorization

in 2016, which allows the FDA to short circuit the standard approval process for device approval during an outbreak, reducing clearance to weeks or even days.[23] Once the Department of Health and Human Services declares an end to an outbreak, the diagnostic must be submitted through either the PMA or 510(k) process.

When the FDA approved 23andMe (a direct-to-consumer genetic information test) and exempted the test from full PMA review it was careful to delineate between devices approved for the purposes of gathering information (such as 23andMe) versus those used to provide diagnosis.[24] "Excluded from today's marketing authorization and any future, related exemption are genetic health risks tests that function as diagnostic tests. Diagnostic tests are often used as the sole basis for major treatment decisions, such as a genetic test for BRCA, for which a positive result may lead to prophylactic (preventative) surgical removal of breasts or ovaries." [xxiv]

Pieced together, these two examples suggest that diagnostic CRISPR tests – used by clinicians for diagnosis may require full PMA review (particularly those that diagnose serious conditions) while others used to diagnose less-serious conditions may be cleared through 510(k).[25] However, whether through PMA or 510(k) review, the regulatory review process for CRISPR diagnostics is fairly straight-forward, and since risks associated with CRISPR diagnostics are the same as with conventional diagnostics, their path from bench to clinic will likely have fewer detours and hazards than their therapeutic counterparts. As such, CRISPR diagnostics are expected to be clinically relevant in the near future.

Therapeutic uses Of CRISPR

The potential for therapeutic CRISPR applications

In order to illustrate the relative virtues of CRISPR diagnostics, we briefly discuss the promises and risks associated with CRISPR's potential therapeutic uses. Unlike CRISPR diagnostics that rely on *ex vivo* patient samples that will ultimately be discarded, most CRISPR-related therapeutics in clinical trials focus on gene editing of extracted somatic cells (non-reproductive cells) that are then transplanted back into the patient (*ex vivo* trials). A smaller number focus on editing cells left intact within the patient's body (*in vivo* trials). The CRISPR molecular machinery is similar to that used in the context of diagnostics, but the much-discussed Cas9 platform is used, which facilitates editing or removing DNA without "collateral cleavage," thus facilitating survival of the edited cells and their progeny.[iii]

One type of *ex vivo* therapy employs CRISPR to produce altered chimeric antigen receptor T-cells (CAR T-cells), a promising technology that seeks to use the human body's immune cells to treat cancer.[26] In CAR T-cell therapy, human immune cells known as T-cells are extracted from the body and edited to target the patient's cancer cells. The modified T-cells are then reintroduced into the patient's body, where they attack the patient's cancer. More recent versions of CAR T-cell therapy can program the modified T-cells with an "off switch," enabling deactivation of the introduced cells at the end of treatment.[27] As will be discussed further below, While the FDA has approved two CAR T-cell therapies over the past year, new CRISPR-based CAR T-cell therapies have yet to pass regulatory muster.[28]

Somatic *in vivo* gene editing is applicable to an even larger spectrum of diseases. Unlike with *ex vivo* techniques, there is no concern that the target cell must survive outside of the patient's body, or that modified cells must survive and thrive after being reintroduced into the patient.[29] Furthermore, *in vivo* gene editing enables therapeutics that target multiple types of tissue. However, *in vivo* techniques also face unique challenges, such as the difficulty of designing agents for *in vivo* delivery, and the potential immune response due to the delivery vector, which are often of viral.[30] Proof of concept studies in mice and rats have demonstrated successful *in vivo* correction of mutations causing Duchene muscular dystrophy,[31] retinitis pigmentosa,[32] and hypercholesteremia,[33] and researchers have even shown that CRISPR can be used to combat infectious disease.[34] Examples of human studies include a CRISPR-based therapeutic for Leber Congenital Amaurosis, the most common cause of congenital childhood blindness, which is currently under development by Editas Medicine,[35] as well as MilliporeSigma's recent two-year study to use CRISPR to repair the gut bacteria of malnourished children.[36]

Potential safety concerns for CRISPR therapeutic interventions

Despite their potential therapeutic promise significant debate continues over the safety of therapeutic uses of CRISPR.[37] As previously discussed, therapeutic uses of CRISPR can expose patients to off-target effects, for example, when CRISPR-altered cells are transplanted back into a patient during a CAR-T cell therapy.[38] Off-target effects are analogous to genetic mutations: they can result in point mutations, deletions, insertions, inversions, and translocations. Thus, like genetic mutations, the result of an off-target edit run the gamut from benign silent mutation, to deleterious mutation that results in significant cytotoxicity.[39] The effects of introducing harmful mutations may be grave, and while "[o]ne off-target cut does not equal cancer," researchers remain wary of any unintended consequences precisely because the results of off-target mutations are difficult to predict.[40] Furthermore, the repeated DNA damage caused by CRISPR-Cas9 has recently been hypothesized to activate a p53-mediated response that could increase the risk of cancer.[41] Despite its reputation for precision, there is conflicting evidence regarding the likelihood of off-target mutations caused by CRISPR gene editing,[42] and researchers have been working hard to develop refinements to the CRISPR/Cas9 platform to reduce the incidence of off-target effects.[43] In sum, there remains significant uncertainty over the nature and magnitude of safety risks associated with CRISPR therapeutics.

Regulatory hurdles for CRISPR therapeutics

As is the case with diagnostics, FDA regulates the approval and sale of CRISPR-related drugs and therapeutics. Specifically, CRISPR research is subject to oversight by the Center for Biologics Evaluation and Research (CBER).[44] Approval of a CRISPR-based therapeutic requires the successful submission

of a Biologics License Application.[45] As with any other biologic, most CRISPR-based therapeutics will undergo three phases of clinical trials to receive approval from the FDA.[46] The three-phase process includes screening for safety in a small group of healthy individuals, efficacy (usually as compared to a placebo) in a larger group of research participants with the disease, and finally more robust, multi-dose studies of safety and efficacy in a group of several hundred to a few thousand participants with the disease. [xlvi] The FDA has yet to approve a CRISPR gene therapy and recently put one of the first CRISPR-related Phase I/II clinical trials on hold.[47] This biologic approval process is much more expensive, labor intensive, and time-consuming than the device approval (particularly the 510(k) process). In addition to FDA regulatory requirements, gene-therapy protocols such as CRISPR therapeutics, have up until now been subject to review by the Recombinant DNA Advisory Committee (RAC), a committee established under the National Institutes of Health for the purpose of advising the director of the NIH on research related to recombinant DNA. Finally, all gene-editing research funded by the National Institutes of Health (NIH) are overseen by Institutional Biosafety Committees, which are typically organized similarly to Institutional Review Boards, and are directly accountable to the NIH Office of Biotechnology Activities.

The FDA and NIH have recently acknowledged the substantial difficulties faced by researchers and corporations in getting regulatory approval for gene-editing therapies when they issued proposed guidelines for streamlining the review of gene therapy trials.[48] Although the title of the proposal suggests an increase in RAC oversight, the new guidance would effectively eliminate RAC review, and further eliminate NIH reporting requirements relating to gene-therapy protocols. In an accompanying perspective letter, the FDA Commissioner and NIH Director argued that these elements of NIH review are already subsumed by the FDA's regulatory framework, and that RAC review has already been effectively phased out—the RAC only reviewed three of 275 gene therapy protocols since a 2016 NIH guidance that sets the current standard for RAC review. They further acknowledged that gene therapy still faces some significant scientific and safety challenges, including delivery and off-target effects, but concluded that the FDA's regulations "are now well suited to gene therapy." While this proposal may result in significantly less paperwork for researchers, it ultimately signals that the FDA intends to continue to follow its existing framework for establishing safety and efficacy for CRISPR therapeutics.

Conclusion

The rapid development of and controversy over therapeutic uses of CRISPR have overshadowed another avenue of CRISPR research that may have a greater clinical impact in the near future: diagnostics. While CRISPR-based gene therapies may revolutionize perinatal care and transform the treatment of long-feared congenital conditions to routine interventions over the course of the next few decades, there are still significant scientific, regulatory, and ethical roadblocks that engender skepticism in the technology. It will be years, or even decades, before the first CRISPR-based therapeutics reach the bedside.

Conversely, the trajectory of CRISPR's unsung heroes – its diagnostic platforms – face a much clearer path to regulatory approval and their use is fast-approaching. Moreover, given the platforms' relative affordability and flexibility, they have the potential to be inexpensive and responsive to new and changing diseases like Zika. Finally, diagnostic applications may allow clinicians to appreciate the power of CRISPR-based platforms without implicating the current issues that plague therapeutic applications and provide an immediate and positive use of the technology—something that may ultimately aid the adoption and uptake of CRISPR-based therapeutics in the future.

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