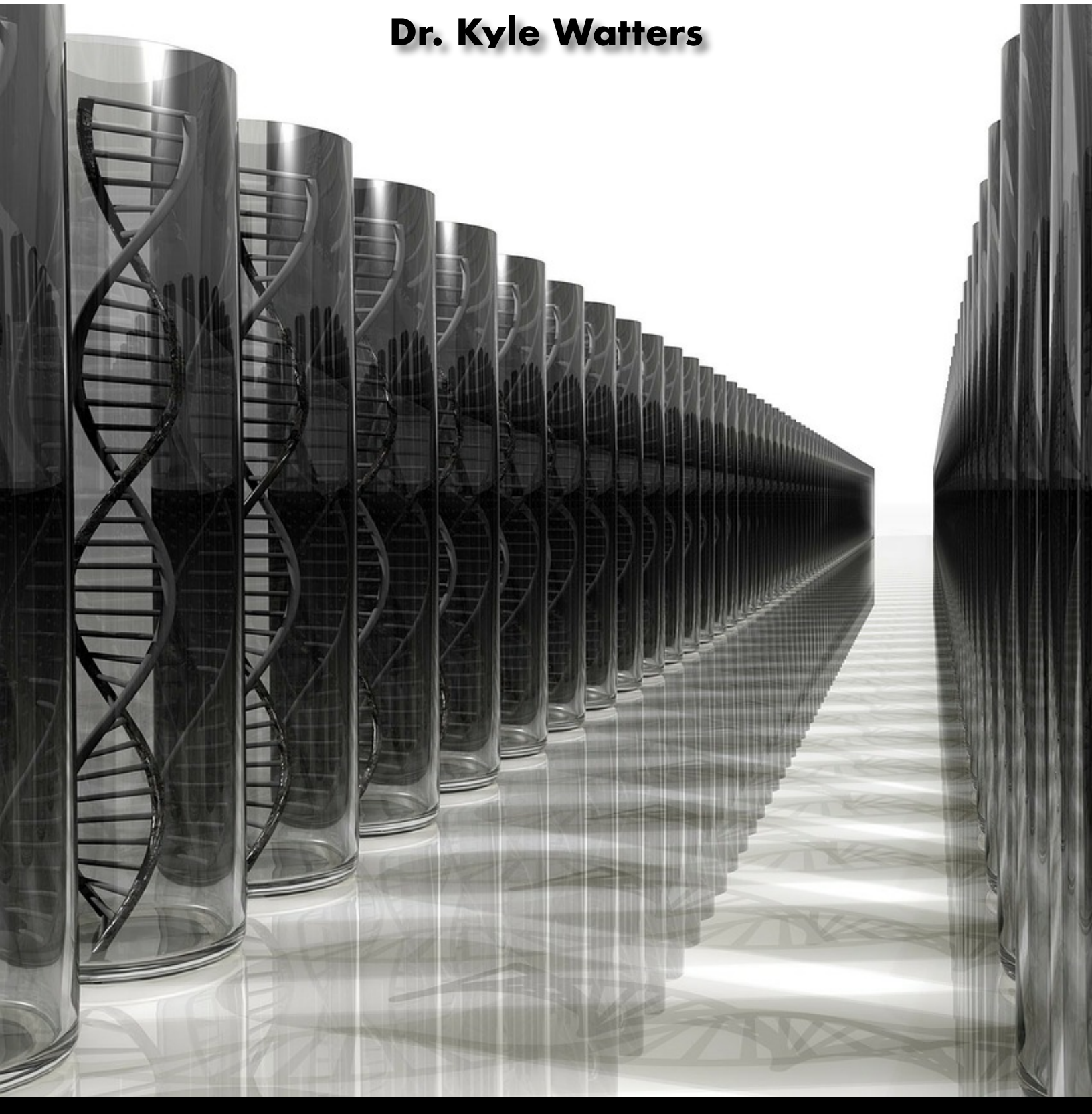
THE CRISPR REVOLUTION: POTENTIAL IMPACTS ON GLOBAL HEALTH SECURITY

Dr. Kyle Watters



STUDY OVERVIEW

Researchers from George Mason University and Stanford University initiated a two-year multidisciplinary study, E*diting Biosecurity*, to explore critical biosecurity issues related to CRISPR and related genome editing technologies. The overarching goal of the study was to present policy options and recommendations to key stakeholders, and identify broader trends in the life sciences that may alter the security landscape. In the design of these options and recommendations, the research team focused on how to manage the often-competing demands of promoting innovation and preventing misuse, and how to adapt current, or create new, governance mechanisms to achieve these objectives.

The four study leads and seven research assistants for *Editing Biosecurity* were assisted by a core research group of fourteen subject-matter experts with backgrounds in security, the life sciences, policy, industry, and ethics. The centerpiece of the study was three invitation-only workshops that brought together the study leads and the core research group for structured discussions of the benefits, risks, and governance options for genome editing. To support these workshops and the final report, the study leads prepared two working papers on risk assessment and governance, respectively, and commissioned five issue briefs on key topics. The authors assume full responsibility for the report and any errors or omissions.

Issue Briefs and Working Papers

Perello E. *CRISPR Genome Editing: A Technical Policy Primer*. Editing Biosecurity Issue Brief No. 1. Arlington, VA: George Mason University; December 2018.

Carter SR. *Genome Editing, the Bioeconomy, and Biosecurity*. Editing Biosecurity Issue Brief No 2. Arlington, VA: George Mason University; December 2018.

Watters K. *Genome Editing and Global Health Security*. Editing Biosecurity Issue Brief No 3. Arlington, VA: George Mason University; December 2018.

Esvelt K. *Gene Drive Technology: The Thing to Fear is Fear Itself*. Editing Biosecurity Issue Brief No 4. Arlington, VA: George Mason University; December 2018.

Vogel KM, Ouagrham-Gormley SB. Anticipating emerging biotechnology threats: A case study of CRISPR. *Politics and the Life Sciences*. 2018 Oct 23:1-7.

Koblentz GD, Kirkpatrick J, Palmer MJ, Denton SW, Tiu B, and Gloss K. *Biotechnology Risk Assessment: State of the Field*. Editing Biosecurity Working Paper No 1. Arlington, VA: George Mason University; December 2017.

Kirkpatrick J, Koblentz GD, Palmer M, Denton SW, and Tiu B, *Biotechnology Governance: Landscape and Options*. Editing Biosecurity Working Paper No. 2. Arlington, VA: George Mason University; March 2018.

All of the working papers, issue briefs, and a list of the project's participants are available at the project's website: https://editingbiosecurity.org/.

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KYLE WATTERS BIOGRAPHY

Dr. Kyle Watters is a postdoctoral researcher in the lab of Dr. Jennifer Doudna at University of California Berkeley, where he is studying the interplay between CRISPR prokaryotic defense systems and countermeasures used by invading mobile genetic elements. The DARPA SafeGenes program currently funds his work with the goal of improving the biosafety and biosecurity concerns involved with the use of CRISPR associated gene editors. Prior to joining the Doudna Lab, Kyle received his Ph.D. in Chemical Engineering from Cornell University for his work on RNA structure-function relationships and RNA synthetic biology. He received his B.S. in Chemical Engineering from Rensselaer Polytechnic Institute.

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Abstract

Biotechnology and medicine are constantly battling threats to global health security. Many modern techniques exist for detecting and predicting where outbreaks might occur and between small molecule drugs, like antibiotics, biologics, and vaccines, much progress has been made in treating diseases globally. However, outbreaks still frequently occur, and many pathogens exist for which there are no treatment. In this review, we paint a picture of the challenges faced in maintaining global health security and how a new wave of gene editing technologies can overcome them. We discuss a number of technologies that are actively developing CRISPR as a treatment or diagnostic tool as well as how CRISPR tools can affect drug development indirectly and speculate on what the near future for these research directions holds. Last, some of the risks associated with gene editing technologies and the efforts scientists are undertaking to mitigate them are covered. Overall, CRISPR and gene editing technologies are likely to have a positive influence on our global health security outlook, both as a form of treatment and detection tool as well as a force for increasing the rate of biological and drug discovery.

Keywords: CRISPR, gene editing, global health security, diagnostics, health, biotechnology

Introduction

The global community suffers from a range of both known health burdens, as well as emerging and unanticipated outbreaks that create an additional stress on health systems. With the high degree of worldwide connectivity across political and geographical boundaries, these burdens and outbreaks are able to rapidly grow from local to global concerns.

Malaria, TB, and HIV are examples of the most lethal, continuing global health disease burdens. Seasonal flu, a periodic burden, is anticipated on an annual basis, and poses a significant threat due its combined ability to quickly spread and mutate. Additionally, a great many other communicable diseases are capable of doing immense global harm if they are able to escape their regional confines. Despite our awareness of these major health burdens, unanticipated outbreaks still occur. For example, while we have not yet experienced (or identified) a major engineered biothreat used against humans in modern times, we may anticipate the debilitating impact that one could have, especially with multiple state actors having maintained bioweapon development programs in recent history (Gilsdor and Zilinskas, 2005). We are lacking in complete solutions for these and many other diseases, yet the fact that they are identified, expected, and at least partially understood creates opportunities in research and development (R&D) to develop diagnostics, prophylactics, and treatments.

Potential Sources of Biological Outbreaks

In general, the following types of biological outbreak threats need to be addressed when considering global health security:

1. Release of a well-known agent with a high capacity for dispersion and/or harm

Presenting one of the most recognizable threats, this type of outbreak is the subject of a great deal of monitoring and discussion by the World Health Organization (WHO). Each year, the WHO publishes their list of blueprint priority diseases that, in 2018, is topped by diseases including Crimean-Congo hemorrhagic fever (CCHF), Ebola/Marburg virus, and Lassa fever (Edmond and Eickhoff, 2008). These high priority diseases are determined on a number of factors, but most importantly on their infectivity, severity, transmissibility, and available diagnostics/countermeasures. For example, CCHF exhibits a high mortality rate, is transmissible by both tick bite and contaminated bodily fluids, and has no effective treatments or vaccines (Whitehouse, 2004).

The lack of countermeasures for WHO blueprint priority diseases is a major factor in their high priority designation and motivates the development of new treatments. The rapid development of facile gene editing tools (TALENs, ZFNs, CRISPR/Cas) is helping to speed up development, but are currently best-suited for targeting DNA, which limits efforts to directly target the causative agents, as the WHO priority list is dominated almost exclusively by RNA viruses.

A large outbreak with one of these types of agents would most likely occur through via unintentional spreading by a contaminated vector in close proximity to a large number of people (e.g. an improperly decontaminated Ebola researcher traveling by commercial aircraft) after coming in contact with an environmental source. While good safety practices and strict rules for those coming in contact with these agents can greatly help combat unintentional spreading, these types of outbreaks still occur. For example, the recent outbreak of Ebola in West Africa is believed to have originated from contact between an infected bat and a young boy, who spread the disease in the human population (WHO Ebola Response Team, 2016).

2. Known agent with new properties

This type of threat creates a new variant of an existing agent that has not been observed by the medical community. These variants can be brought about by both natural evolution/ mutation or intentional manipulation. Considering first natural evolution, these variants can appear as a response to environmental pressure or from the natural propensity of certain pathogens to evolve quickly. The rapid mutation rate of the influenza virus exemplifies this type of biothreat from a known agent, and has caused multiple outbreaks in recent history including the 2009 swine flu pandemic (Smith et al. 2009, SteelFisher et al. 2010) and the outbreak of a highly pathogenic avian flu in 2004 (Chen et al., 2005). The recent Zika outbreak in the Americas is another illustrative example, where an infection that was generally considered mild

obtained mutations that allowed it to spread rapidly and led to cases of Guillain–Barré syndrome and birth defects (WHO, 2016). Another looming problem for the global health community is the rise of antimicrobial resistance due to the increased use of antibiotics (Spížek et al., 2010).

Separate from natural evolution, the intentional manipulation of a pathogen's genetic material could pose a potential source of biological outbreak. Intentional manipulations could include weaponizing mutations to create a new or modified bioweapon that give an agent enhanced virulence by increasing antibiotic/antiviral resistance, widening the host range, overcoming host defenses, etc. For example, two controversial studies in 2012 demonstrated that avian influenza could be engineered to transmit between ferrets – the standard model for human infection/transmission in influenza research (Herfst et al. 2012, Imai et al. 2012).

In terms of understanding the introduction of factors enhancing virulence, CRISPR/Cas genetic tools are likely best employed to understand the evolving host-pathogen relationship from the host perspective via gene screening, as CRISPR/Cas tools have not been used for engineering viruses, as many viral engineering tools already exist.

3. Appearance of an unknown agent

Last, the biggest challenge in identifying the cause of an outbreak comes from an unknown agent. These new agents could be the result of either a completely undiscovered pathogen, or an organism misclassified as non-pathogenic. In the 2018 WHO list of blueprint priority diseases, this type of threat was simply titled 'Disease X' (Edmond and Eickhoff, 2008), as a broadly encompassing term for any disease that could pose an epidemic threat but is currently unknown to science or medicine.

Examples in recent history include, among others, the appearance of Legionnaires' disease in 1976 (Winn, 1988) the SARS outbreak in China in 2003 (Zhao et al., 2003), and the identification of Middle East Respiratory Syndrome coronavirus (MERS-CoV) in 2012 (Zaki et al., 2012).

Challenges in Identifying, Diagnosing, and Treating Outbreaks

With the variety of agents that pose potential global health risks, mitigating these risks is a major challenge. While the logistical challenges associated with implementing new technological solutions across various geographic and political borders are considerable, the development of new technologies arguably lies at the heart of maintaining global health security. The ability to identify, diagnose, and treat infections is critical to minimizing the harm done by known global health burdens and unexpected outbreaks. Below, some of the major challenges in technological development are discussed.

Identifying and Diagnosing New Threats

In reference to the three potential sources of biological outbreaks above, each presents a different level of difficulty in identifying the threat present. The release of a well-known agent is easier to identify since a wealth of information is likely available about the agent's composition, its mechanisms of replication, and the symptoms of infection. Developing tools to identify new threats from mutated/altered agents faces increased challenges relative to well-known or wild-type agents, given that mutations could interfere with the ability to detect the pathogen if they occur in regions critical for developed assays. Thus, development of robust assays that can account for mutations, acquisitions, genetic drift, etc. are essential for early detection of a potential outbreak.

The greatest challenge comes in identifying the causative agents behind outbreaks when the agent is not already considered a biothreat. If the agent is a completely unknown species/strain, a wide panel of assays are required to narrow down the type of threat and additional sequencing/biochemistry/microbiology is potentially necessary to fully evaluate the threat present. The challenge is somewhat different if the threat is from a manipulated or mutated non-pathogen, as the symptoms of infection may be generally mild (as was the case with Zika (WHO, 2016)) or resemble those of another known agent, especially if an unknown agent acquired pathogenic DNA/RNA from a known agent. Further, potential contamination by co-occurring organisms could lead to incorrect conclusions about the threat. A classic example is the misidentification of *Haemophilus influenzae* as the causative agent of 'the flu' before the discovery of the influenza virus (15). Technologies that can rapidly identify many potential threats are critical for a timely response to a potential outbreak.

Developing New Treatments

Generally, there are two major routes to treating infection that are generally employed. First, antibiotics and antivirals are small molecules that bind a specific site in a biological molecule to disrupt the pathogen's life cycle. Alternatively, vaccines or monoclonal antibodies are used to treat prophylactically (although some diseases have a period when vaccines are still effective after infection) by priming the human immune system to recognize a specific agent. Ideally, either course of treatment is designed to cover as many strains of an agent as possible, given that one of the biggest challenges in developing effective treatments is the tendency for pathogens to mutate and recombine.

Influenza is a good example of a pathogen that is difficult to treat with these approaches (Fig. 1). It is an RNA virus made up of eight unique genomic segments that can reshuffle during co-infection to drive diversity on top of the high mutation rate afforded by its RNA-dependent RNA polymerase (Samji, 2009). Over the course of roughly 80 years of research into influenza two major drugs were found to be effective: amantadine and oseltamivir/zanamivir (better known as Tamiflu/Relenza, respectively) (Barik, 2012). However, overuse of amantadine has now led to near complete resistance of influenza to the drug, leaving only one class of drugs available to treat infections (Hussain et al., 2017). Vaccines for influenza have been available since it was

discovered, with improved strain monitoring and technology to improve the effectiveness of the vaccine in more recent years. However, the vaccine's effectiveness is still fairly low due to the mutation rate of the virus, either from wild strains developing escape mutants, or mutations occurring during growth in eggs (Zost et al., 2017). Despite large worldwide investments into influenza research and the tools available to modern biology, mutation and recombination events present a major challenge in developing new treatments.

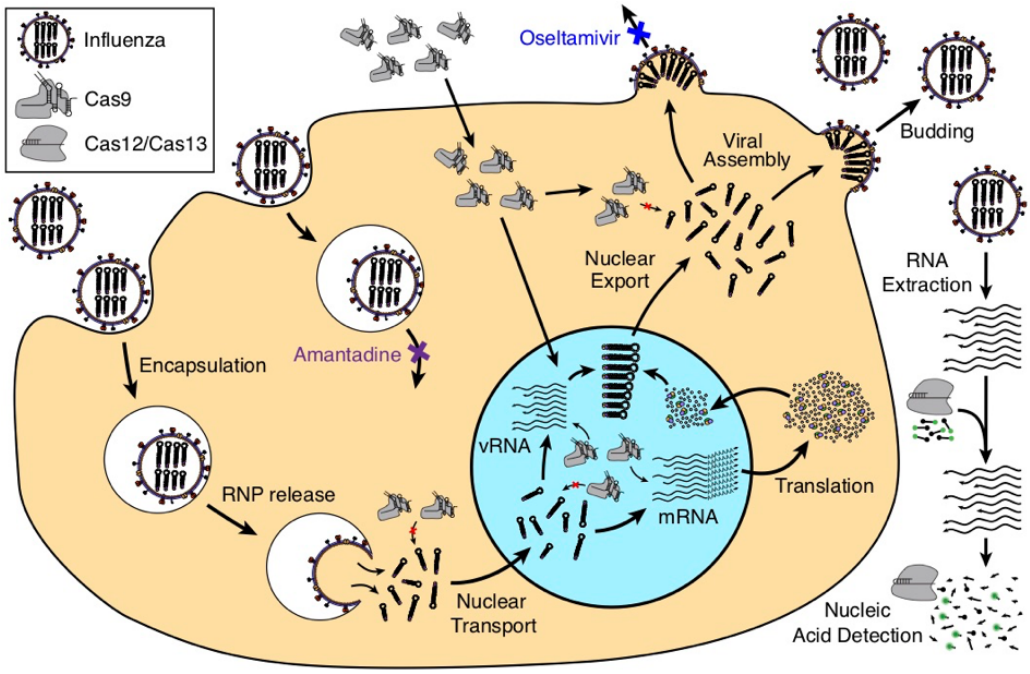

Figure 1. The influenza viral replication pathway involves binding to the cell surface, encapsulation, release of the ribonucleic protein (RNP) complexes, transport of the RNPs to the nucleus, generation of new RNPs, packaging, and release. Currently, two drugs have been developed to inhibit influenza replication: amantadine (purple) that blocks RNP release and oseltamivir (blue) that blocks budding of new viruses. CRISPR and gene editing have the possibility to be used to directly target viral RNAs to inhibit replication, although packaging in RNPs is likely to block access to the RNA for CRISPR targeting. Last, CRISPR can also be used in a diagnostic setting to detect viral RNAs, down to specific strains using techniques like SHERLOCK (Myhryold et al., 2018) and DETECTR (Chen et al., 2017b).

More generally, drug and vaccine development contain many other challenges that need to be addressed by future technologies. Much faster and cheaper R&D strategies for drug and vaccine development are needed to improve our preparedness against potential biothreats.

Last, a general challenge faced by biomedical community is the need to use diagnostic tools and treatments in the field. In the developed world access to state-of-the-art laboratory space is fairly easy, but many of the emerging biothreats are coming from developing countries. Thus, developing new technologies that are cheap, readily portable, and require minimal lab equipment are key for addressing new biothreats in a timely manner.

Identifying, Diagnosing, and Treating Infectious Diseases with Gene Editing Technology

The recent development of gene editing technologies including TALENs (Sanjana et al., 2012), ZFNs (Urnov at al., 2010), and CRISPR-Cas (Sander and Joung, 2014), has fundamentally changed the direction of biomedical research by providing new tools that can accurately edit an organism's genome, which may belong to a human, pathogen, or animal model. Many variations of these technologies exist as well, using modified versions of each system's characteristic editing protein. Below, a number of applications of gene editing technology will be discussed with a focus on CRISPR for its widespread adoption and ease of use, although many of these applications could theoretically be extended to TALENs and ZFNs as well.

In recent years, CRISPR effectors including Cas9, Cas12, and Cas13 have been used to develop a plethora of tools useful for basic science as well as biomedical research. Each of these three Cas proteins is somewhat different, although each is a single large protein that uses a guide RNA to direct it to a specific nucleic acid target sequence: Cas9 cleaves double stranded DNA to leave blunt ends (Gasiunas et al. 2012, Jiang and Doudna 2017, Jiang et al. 2015) (Fig. 2A), Cas12 recognizes double stranded DNA to activate a nuclease that can leave a staggered cut or nonspecifically degrade single-stranded DNA (Chen et al. 2017b, Zetsche et al. 2015, Swarts et al. 2017) (Fig. 2B), and Cas13 is a non-specific RNase that is activated when it matches its guide RNA to a target (East-Seletsky et al. 2016, Abudayyeh et al. 2016, Gootenberg et al. 2017, Smargon et al. 2017, Yan at al. 2018) (Fig. 2C). Cas9 in particular has seen major use in the rapid development of new animal disease models, drastically cutting the time and cost of development. Between Cas9, Cas12, and Cas13, many engineered forms exist that may contain one or more of the following modifications: complete or partial abrogation of nuclease activity (dCas) (Larson et al., 2013), fluorescent tagging (Chen et al., 2013), ability to be activated by chemical or light stimuli (Oakes et al. 2015, Polstein and Gersbach 2015), and the ability to recruit chromatin remodeling factors (Amabile et al., 2016). These modifications have formed the basis of a number of new tools whose applications are discussed further below.

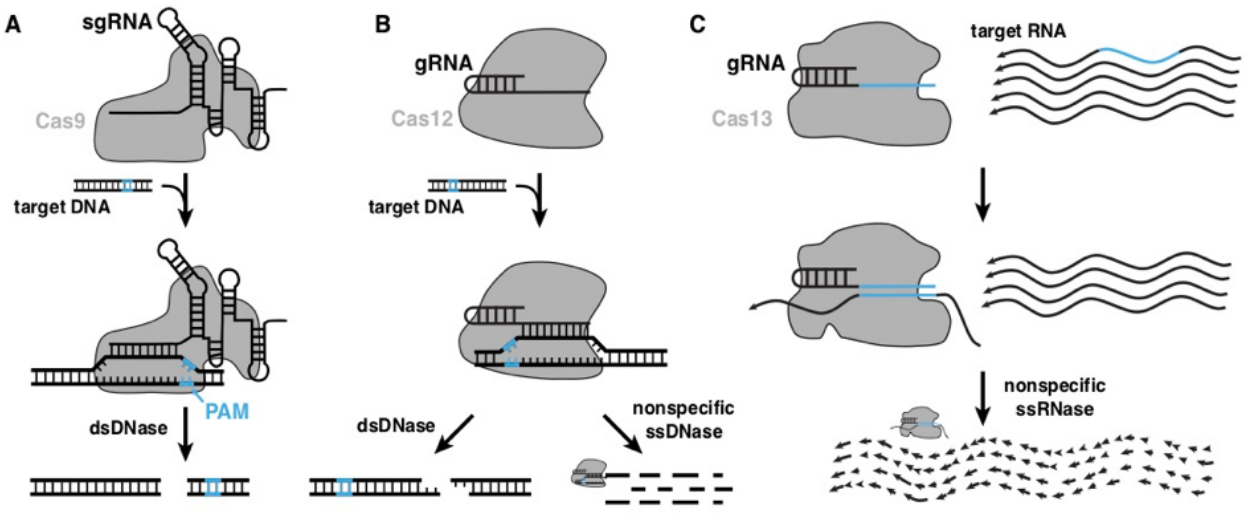

Figure 2. Differences between Cas9, Cas12, and Cas13. A) The Cas9 protein binds to two RNAs that can be fused into a single guide RNA (sgRNA). The Cas9 RNP complex recognizes a DNA sequence matching the spacer sequence next to a matching protospacer adjacent motif (PAM) sequence, unwinds the DNA, and performs a blunt cut near the PAM sequence. B) Cas12 binds a small guide RNA (gRNA) that recognizes a double stranded DNA target, unwinds the DNA, and cuts both strands with a staggered tube distal from the PAM sequence. Binding to a DNA target, either single- or double-stranded, can also activate non-specific DNAse activity that cleaves ssDNA nonspecifically. C) Cas13 forms a complex with its gRNA to find and bind a matching RNA sequence, which activates a nonspecific nuclease that nonspecifically cleavage all surrounding single-stranded RNA.

Identifying and Diagnosing New Threats

One of the most beneficial uses of CRISPR for pathogen research has been the use of CRISPR interference to identify host factors critical to the agent's replication cycle. With CRISPR interference, a catalytically dead Cas9 (dCas9) fused to a chromatin remodeling protein is guided to the beginning of a gene to silence expression (Amabile et al., 2016). By using a library of gRNAs covering the host organism's genome, individual genes or gene clusters can be knocked down in parallel to identify which genes' silencing leads to a survival phenotype (Parnas at al., 2015). Thus, in one straightforward experiment a list of genes involved in pathogen biogenesis can be obtained. Similarly, CRISPR interference knockdowns can be used to work out the function of an agent's genes without needing to make recombinant virus or create transgenic cell lines or animals both of which can be time-consuming.

It is worth noting here that many of these knockdown assays were previously possible using RNA interference (RNAi) pathways (Mohr and Perrimon, 2012), possibly more effectively than CRISPR even, but CRISPR affords the additional flexibility of being able to make inheritable genetic changes by switching to the wild-type Cas9 protein for gene editing or with epigenetic markers (Amabile et al., 2016). These genetic changes can be made with a high degree of precision allowing for targeted gene knockouts to study the effect of host gene functions in the pathogen life cycle.

A particularly interesting application of CRISPR technology is to diagnose the presence of a specific agent from a patient sample. There are two similar strategies that are currently employed, SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) (Gootenberg et al. 2017), which uses Cas13, and DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) (Chen et al., 2018), which uses Cas12. Briefly, input RNA or DNA from a sample containing a potential agent is amplified and converted to RNA with recombinase polymerase amplification that is then exposed to Cas13 with a guide RNA to probe for the presence of a specific sequence in the agent. If that sequence is present, Cas13's non-specific RNase activity activates, cleaving a reporter RNA that releases a fluorescent signal. The authors additionally show that SHERLOCK can be reconstituted on paper for cheaper deployment into the field to detect Zika and Dengue virus in human samples (Gootenberg et al., 2017). The workflow for DETECTR is essentially the same, except that Cas12 is used instead of Cas13 to recognize and cleave DNA sequences instead of RNA sequences (Chen et al., 2018). Using

DETECTR, the authors demonstrated the ability to distinguish between two closely related strains of human papillomavirus (HPV).

Developing New Treatments

While CRISPR has proven valuable for the development of new tools for identification and diagnosis, much of the excitement surrounding CRISPR technologies comes from its use as a gene editing tool, providing the potential for developing new treatments. In DNA editing applications, base changes are created by the way that eukaryotic cells repair their DNA after a cleavage event. Two possible modes of DNA repair occur: 1) non-homologous end joining (NHEJ) in which the cell simply tries to 'paste' the broken DNA strands back together, typically resulting in insertions or deletions, and 2) homology directed repair (HDR) in which the cell uses a homologous sequence to determine what bases are missing/damaged in the break (Zaboikin at al., 2017) (Fig. 3). The two repair mechanisms result in fundamentally different outcomes, with NHEJ frequently resulting in non- or missense mutations, while HDR can either fully repair the break or accept a donor DNA to insert new sequences. Thus, only supplying a Cas protein and guide RNA will usually result in NHEJ repair and lead to the silencing of a gene. To create intentional insertions, mutations, or deletions a donor DNA must be supplied to use the HDR pathway and avoid the random result of NHEJ repair, which competes with HDR. Both of these types of editing are employed to make genetic changes depending on the desired outcome (Ibid.).

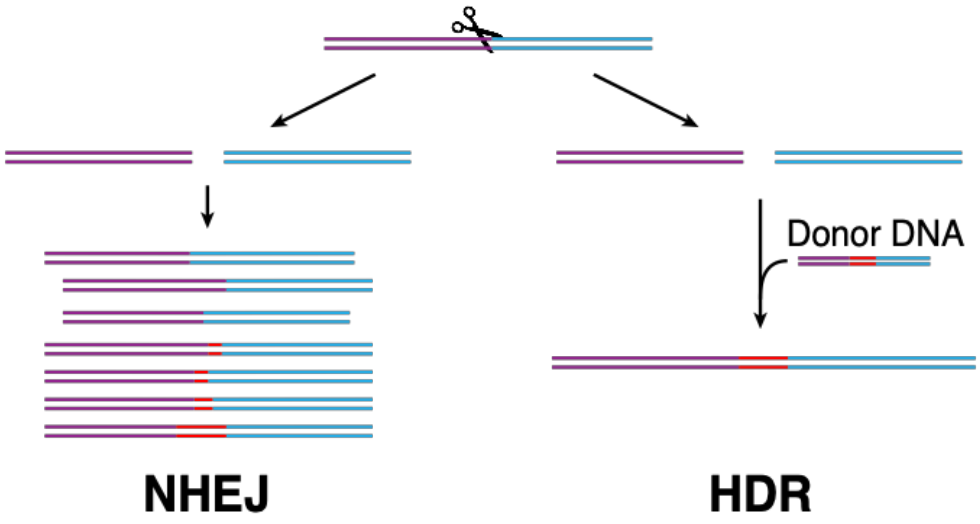

Figure 3. Differences between non-homologous end joining (NHEJ) and homology directed repair (HDR). After a double-stranded break, there are two major mechanisms of cell-mediated repair: NHEJ and HDR. If a donor DNA is present with homolog to each side of the break, the HDR pathway will lead to templated repair. Thus, HDR can lead to highly specific editing for biotechnology applications. In competition with HDR is NHEJ, which sticks the broken DNA ends together, typically involving the generation of insertions or deletions (indels). These indels are usually disruptive to a gene if in a coding region.

Alternatively, CRISPR effectors have been coupled with deaminases to allow individual bases to be edited without the need for a double strand break (Komor at al. 2016, Hess et al. 2017) (Fig. 4). These base editors prevent the need to rely on NHEJ and can introduce specific point mutations into a DNA sequence without the need to supply an extraneous template for HDR. Currently, a number of base editors exist using Cas9 (Komor et al. 2017, Gaudelli et al. 2017) or Cas12 (Li et al., 2018) that are able to convert AT base pairs to GC or vice versa. Base editing has also been demonstrated with Cas13 (Cox et al., 2017), although there is disagreement in the field as to its effectiveness (Vogel et al., 2018).

A major challenge in gene editing is the possibility of permanent unwanted edits. To get around this issue, researchers have developed other CRISPR tools that do not edit the genome permanently, but instead recruit chromatin remodeling proteins (Gilbert at al. 2013, Mandegar at al. 2016) or methyltransferases (Amabile et al., 2016) to alter expression levels of the CRISPR targeted gene. Depending on the method used, the alterations to gene expression levels can be long-lasting, resulting in phenotypic changes without the need for genotypic changes.

Another strategy to silence genes without a genotypic alteration is to target the mRNA product instead of the gene itself. As described above, Cas13 can be used to target individual mRNA transcripts for cleavage (Yan et al. 2018, Abudayyeh et al. 2017, Konermann et al. 2018) or base editing (Cox et al., 2017). Additionally, some work with Cas9 has demonstrated that certain homologs can target RNA (Strutt at al. 2018, Dugar et al. 2018), including the hepatitis C virus in eukaryotic cells (Price et al., 2015).

One of the biggest changes in biomedical research brought about by gene editing, and especially CRISPR, is the ability to create new cell and animal models quickly and efficiently. Unlike ZFN and TALEN technologies, CRISPR does not require redesign of the effector nuclease, only the guide RNA, which is much simpler. Synthesizing guides is relatively inexpensive and many can be tested in a short period of time. Further, the high activity of Cas nucleases results in a higher probability of making a desired mutation/change, shortening screening times which becomes more significant as the maturation time of the model organism increases (Smalley 2016, Ma and Liu 2015). The faster turnaround time of new model organisms benefits biological research as a whole, allowing for more appropriate testing environments and less time spent building research materials and more time collecting new data. Similarly, the development of monoclonal antibodies (mAbs) and vaccines for prophylactic treatments can benefit from CRISPR/Cas9 through increased rates of cell line generation as well as the ability to perform genomic screens to identify what epitopes are targeted by mAbs (Zotoya et al., 2016).

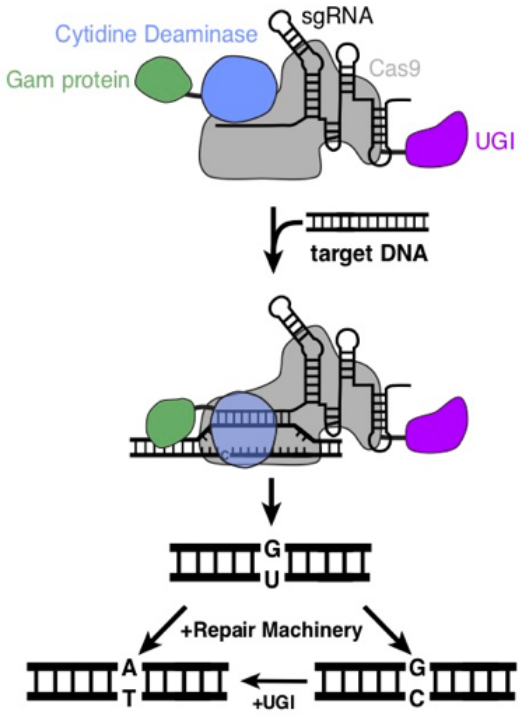

Figure 4. Basics of a C→T Cas9 base editor. A catalytically dead Cas9 is fused to a phage Gam protein (green), cytidine deaminase (blue), and uracil glycosylase inhibitor (UGI; purple) to create one version of base editor (46). After binding to a DNA target, the deaminase accesses the unwound, single-stranded DNA and converts C→T for cytidine deaminase or A→I for adenosine deaminase. The edited base is then either corrected back by the repair machinery or the pairing nucleotide is changed to correct the base matching. The Gam protein and UGI are included to help bias the repair toward the desired edited product and minimize off-target effects.

While CRISPR has not been used as a treatment in a living human at the time this article was written, multiple clinical trials are expected to commence in the near future (Le Page, 2017). One challenge to overcome however, is the need to deliver the Cas protein and gRNA to large number of cells, but only a specific subset, while avoiding triggering the immune system. A number of studies are exploring different delivery options (Song, 2017), but CRISPR/Cas tools still face a number of specific challenges. For example, recent studies have demonstrated that a large fraction of patients tested had antibodies for SpyCas9 and SauCas9, two commonly used Cas9 editors, as well as anti-specific T-cell against SauCas9 (Charlesworth et al., 2018). However, as the authors point out, Cas editors not exposed in the serum (such as those packaged in a delivery vehicle) are safe from antibodies. The bigger challenge comes from the antigen-specific T-cells, which can clear cells that contain the Cas gene editor if the degraded editor is presented on the cell surface (Ibid.). Other recent work has demonstrated that improper preparation of the gRNAs for Cas9 or Cas12 can also result in an immune response (Kim at al., 2018). These problems associated with delivery transcend CRISPR and gene editing itself, however, and are part of a larger challenge of biologic drugs and treatments.

Future Potential for Gene Editing Solutions in Global Health Security

The last five years since the publication describing the basic mechanisms of Cas9 DNA cleavage (Jinek et al., 2012), new techniques and tools are constantly being published across all biological fields highlighting how pervasive CRISPR has become.

Identifying and Diagnosing New Threats

A major factor in containing outbreaks is the speed at which a potential outbreak can be identified and the causative agent be detected in the affected population. Recent work describing paper-based agent detection (Pardee et al., 2016) is particularly exciting, and recent use of Cas12 and Cas13 to detect both RNA or DNA from potential agents is poised to have a major impact on outbreak response and general agent monitoring in the field (Myhryold et al. 2018, Gootenberg et al. 2017, Chen at al. 2018). Future improvements to increase the detection limit (Gootenberg et al., 2018), increase signal:noise strength, and shorten assay run time may make these types of paper assays a standard field method for agent detection.

Another major area where gene editing technologies will continue to have a major impact is the discovery and understanding of agent replication mechanisms. CRISPR screens to identify key genes in these pathways are likely to be used in an increasing amount to quickly identify what factors of infection are important to consider when developing treatments (Fellmann et al., 2016).

Developing New Treatments

The potential for gene editing, especially CRISPR technologies, to impact the development and use of new treatments is tremendous. Gene editing technologies have a major advantage over traditional drugs in that they can target the genetic basis of a disease, and do not require the development time and cost to understand and block interactions between biological molecules with complex three-dimensional topography. While it is very challenging to predict small molecule interactions, designing gRNAs is very simple. DNA accessibility does play a role in CRISPR/Cas targeting, but individual genes can be targeted with a gRNA library that is much smaller than a typical commercial small molecule library. Additionally, improving the efficacy and delivery of a traditional drug (biological or small molecule) requires each drug molecule be engineered separately, however, improvements in gene editing technology transcend individual gene targets and can be broadly applied to many treatments by simply switching the gRNA target sequence.

The first human recipient of ZFNs to treat Hunter Syndrome has begun a new period of gene editing in humans, with multiple trials using CRISPR/Cas scheduled for the near future (Le Page, 2017). Additional work on curing a variety of genetic diseases with CRISPR, such as muscular dystrophy (Lee et al., 2017), is underway (Yang et al., 2016). This progress on treating

genetic diseases then feeds back to impact global health security efforts. For example, retroviruses like HIV can be removed from the population by excising the viral DNA from the genome, which has already been tested in mice (Yin at al., 2017). There is more work to be done, however, as concerns have been raised about the possibility of escape mutations driving further evolution of the virus (Song, 2017). Alternatively, viral infections could be combated by editing the human genome to resist viral infections altogether. Again, considering HIV, gene editing could be used to truncate the CCR5 receptor to mimic the CCR5-Δ32 mutation that provides innate immunity to the virus (Pancino et al., 2010).

Directly targeting viruses is also a potential route to treating disease outbreaks (Soppe and Lebbink 2017, White et al., 2015). CRISPR-based antivirals are particularly exciting due to the ease of generating large numbers of guide RNAs relative to the difficulty of developing small molecule drugs that pathogens will likely eventually evolve resistance to. The adaptation of a prokaryotic antiviral system (i.e. CRISPR) for use in humans for antiviral purposes is still in its early stages, but future successes could provide a completely new method for rapidly combating emerging viral diseases. We are also likely to see a large expansion in the use of RNA targeting and editing. Many of CDC's select agents are RNA viruses that do not convert to DNA, requiring direct RNA targeting to be cleaved. Additionally, concerns over accidental edits to patient genomes and the degree of permanency implied make direct RNA targeting or editing very attractive.

Other Uses for Gene Editing Pertaining to Global Health Security

Gene-editing technologies also have a number of relevant applications to global health security outside of the human body. While not covered in this review, gene drives have the potential to control vectors and minimize the possibility of certain outbreaks, either by eliminating the vector entirely or by editing the vector's genome to remove its ability to carry the agent (Hammong et al., 2016).

Environmental surveillance systems could also be developed to monitor the presence of agents. Biosensing circuitry has been a major focus of the synthetic biology community (Slomovic et al., 2015), which could be incorporated into crop plants, etc. using CRISPR technologies to provide a real-time detection system for regional agents of concern.

Challenges and Risk Assessment for Gene Editing Applications

As with many major scientific advances, there are a number of challenges and risks associated with developing/using CRISPR gene-editing technologies, discussed below.

Scientific Challenges

Progress in gene-editing research faces of a number of the standard challenges expected in science, but two in particular are the most notable. First, measuring the degree of off-targeting

that occurs (level of unwanted editing) is fairly challenging. A number of techniques have been developed so far, such as GUIDE-seq (Tsai at al., 2015), CIRCLE-seq (Tsai et al., 2017), VIVO (Akcakaya at al., 2018), and Digenome-seq (Kim et al., 2015), but the need to separate naturally occurring insertions/deletions/mutations from those caused by gene-editing off-targets remains a major challenge of the field.

Improving editors to minimize off-target effects is another challenge that is currently being addressed. Efforts into improving the accuracy of *S. pyogenes* Cas9 have been fruitful, resulting in a hyper-accurate Cas9 variant (Chen et al., 2017a), but took years of development including other variants that saw lower on-target activity (Kleinstiver et al., 2016). While future engineering of accurate Cas effectors can build on the lessons learned so far, each new nuclease must be refactored limiting the speed of development.

An additional challenge in using gene editing technology for treatments of biothreat agents is the high propensity of those agents to mutate. Mutation of bases key to CRISPR targeting, such as those in the seed region of target sequence or the protospacer adjacent motif (Makarova at al., 2017) could potentially lead to inactive treatments. Care must be taken to choose guide RNAs targeting the most conserved regions of a gene to avoid mutation issues, while working within the restrictions placed by the PAM sequence required by each effector. Similarly, the appearance of single nucleotide polymorphisms within the human population presents a challenge when designing treatments, as certain guide sequences many only result in proper targeting in a subset of the global population.

Last, the problem of delivery is probably the biggest scientific hurdle that must be cleared to make CRISPR gene-editing a commonplace treatment. Small molecule drugs, when properly designed, have the advantage of high stability and ease of transport into cells. Biologics like Cas:gRNA complexes are susceptible to proteases and nucleases in the body, which can quickly degrade foreign biological molecules. A number of solutions have been proposed (Song, 2017), but are further complicated by the large size of most Cas effectors relevant for gene-editing (Shmakov at al., 2017), which are more difficult to package into traditional vectors, like the adeno associated virus (Bak and Porteus, 2017). The problem of Cas protein complex packaging and delivery is likely to dominate the scientific challenges faced as the research community moves forward in developing CRISPR gene-editing tools. Thus, efforts toward treating infectious disease with CRISPR/Cas can benefit from the lessons learned during the ongoing development of treatments for genetic diseases.

Risks to Human Health and Biosecurity Associated with Gene-Editing Tools

Within the scientific community, one of the biggest risks associated with using gene-editing tools is the possibility of off-target mutations/insertions/deletions. While Cas effectors tend to be highly accurate, off-target modifications do occur and could potentially lead to a lethal or cancerous outcome. Much work has been done to try minimize this possibility by improving the accuracy of the editors or using tools that target RNA, instead of DNA, to avoid accidentally making an unwanted and permanent change to the genome.

A risk that comes with directly targeting agents is the possibility of stimulating its intrinsic mutation rate (Wang at al., 2016). As previously stated, many of the CDC select agents are RNA viruses, which as a group tend to rapidly mutate, making vaccine and traditional drug development difficult. Targeting specific strains, or incomplete clearing virus from the host, could result in an artificial selection for viruses with mutations or sequences that avoid CRISPR targeting. The same would be true for other pathogens as well. Overuse of CRISPR may in fact spur mutation further, possibly limiting the effectiveness of other traditional treatments, including vaccines.

Another risk is the potential for misuse of the widely available CRISPR tools. Harm could be done intentionally by nefarious actors or unintentionally by unwitting do-it-yourself biologists. For example, an unintentional modification of bacteria from poorly designed experiments or improper biocontainment could create a new pathogen that could be released into the environment. With DIY biologists, it is unlikely that those interested in carrying out editing *in vivo* are unlikely to have performed the expected progression of using increasingly higher organisms to minimize risk.

A last threat to biosecurity is if gene-editing tools are themselves used as a weapon. This novel type of bioweapon could target specific subpopulations in a geographic/local manner or by targeting single nucleotide polymorphisms present in select groups. Whether used offensively or defensively, this type of bioweapon could allow other bioweapons to be used in a focused manner to avoid the collateral damage that is typically associated with biological weapons; a potential reason we have not seen many biological weapons used in the modern era. Ultimately, however, CRISPR gene editors could be used bluntly as a bioweapon if a delivery mechanism could be devised.

Societal Challenges

The perception of gene editing by the public will play an important role in how CRISPR technology will used by the biomedical community. In the United States editing genes in babies, to prevent genetic disease for example, remains unpopular (Blendon at al. 2016, Scheufele at al. 2017, Funk et al. 2016), although editing to prevent infection by various agents was not explicitly asked about. Similarly, in considering the American public, the National Academies of Science and Medicine concluded that the public was uneasy about germline editing and caution should be taken, although provided a path forward with strict requirements (NASEM et al., 2017). They also conclude that current uses of gene editing that constitute enhancement, broadly defined, should be avoided, especially due to the current indication of public discomfort with the concept.

Reactions to the risks to individuals (e.g. off-target mutations) and the counter-reaction by the scientific community will also play a role in how CRISPR technology evolves. Currently, focus remains on CRISPR as a treatment for genetic disease (where the most research has been performed), but discussions in the public about the potential use of CRIPSR as a bioweapon or the consequences of their accidental release/application will eventually occur. Additionally, early

success or failures with gene therapy drug trials as well as news surrounding DIY biologists self-injecting CRISPR effectors will likely generate a large swing of momentum for or against the use of CRISPR technology.

One last major issue are the ethical concerns involving informed consent. The permanency of genetic changes adds a complication to the already difficult task of ensuring patients are fully informed of the potential risks of a treatment before agreeing to partake in it. When considering the panic and fast decision making that is typically required to respond to a biological outbreak, it becomes difficult to ensure the patient is properly informed. Further, due to the lack of drug trials for many of the worst agents, it is important to develop an improved ethical framework to outline what informed consent means in the context of gene editing.

Conclusion

While there are many types of threats to global health security, there are many applications for new CRISPR technologies to have a positive impact. For detection and prevention, the development of highly sensitive CRISPR-based detection platforms promises to bring rapid, field-adaptable surveillance of pathogens in the near future. Direct treatments targeting pathogens or host-cell receptors are also promising alternative to small molecule or vaccine development. Additionally, CRISPR is also making it quicker and easier to create more accurate animal models for studying new diseases or variants. If the benefits of CRISPR can be adequately balanced with the risks involved with its use, the biotechnology and medical communities will make great strides in maintaining global health security.

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