CRISPR/CAS9 – THE ULTIMATE WEAPON TO BATTLE INFECTIOUS DISEASES?

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ABSTRACT

Infectious diseases are a leading cause of death worldwide. Novel therapeutics are urgently required to treat multi-drug resistant organisms such as *Mycobacterium tuberculosis* (Mtb) and to mitigate morbidity and mortality caused by acute infections such as malaria and dengue fever virus as well as chronic infections such as human immunodeficiency virus -1 (HIV-1) and hepatitis B virus (HBV).

The CRISPR/Cas9 system, which has revolutionized biomedical research, holds great promise for the identification and validation of novel drug targets. Since its discovery as an adaptive immune system in prokaryotes, the CRISPR/Cas9 system has been developed into a multi-faceted genetic modification tool, which can now be used to induce gene deletions or specific gene insertions, such as conditional alleles or endogenous reporters in virtually any organism. The generation of CRISPR/Cas9 libraries that can be used to perform phenotypic whole genome screens, provides an important new tool that will aid in the identification of critical host factors involved in the pathogenesis of infectious diseases.

In this review, we will discuss the development and recent applications of the CRISPR/Cas9 system used to identify novel regulators, which might become important in the fight against infectious diseases.

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INTRODUCTION

Despite decades of intense research, infectious diseases caused by bacteria, viruses and parasites still account for a quarter of deaths worldwide. They are ranked as the top cause of death in developing countries as well as the number one killer of children under the age of five years globally (WHO, 2012). Rising hospitalization rates for cancer, diabetes and aged care are associated with an increased risk of acquiring infections particularly with multidrug-resistant organisms. This underscores the economic burden of infectious diseases on the public health systems throughout the world (Spellberg et al., 2008). Frequent epidemic outbreaks and the challenge of the rapid spread of infectious agents around the globe, facilitated by higher travel frequencies, indicate that we are a long way away from winning the battle against infectious disease.

The recent development of the CRISPR/Cas9 system as a genome-editing technique has significantly facilitated gene modifications in both pathogen as well as host cells and enables profound analysis of the molecular mechanisms that are involved in pathogenesis of infection. CRISPR/Cas9 can be utilized in whole genome screens in host and pathogen to identify novel regulator proteins that are central for manifestation of infection and thus present novel therapeutic targets. Furthermore, the CRISPR/Cas9 system has great potential in the development of novel antimicrobials and vaccines and therefore has the ability to open new avenues both for treatment and prevention of infectious diseases. Although research has already revealed the tremendous assets of CRISPR/Cas9, there is still a lot we have to learn about the system itself in order to fully utilise its potential as a powerful tool in the fight against infectious diseases.

THE CRISPR/CAS9 SYSTEM –A UNIVERSAL GENE EDITING TOOL

20 years after the discovery of CRISPR (clustered, regularly interspaced short palindromic repeats) in bacteria, Horvath and colleagues provided the first experimental evidence for its role in adaptive bacterial immunity (Barrangou *et al.*, 2007). In the following years, intense research helped to elucidate the molecular mechanisms of CRISPR-mediated immunity (Brouns *et al.*, 2008, Garneau *et al.*, 2010, Deltcheva *et al.*, 2011, Sapranauskas *et al.*, 2011) and the type II CRISPR system from *Streptococcus pyogenes* was then adapted for genome editing in mammalian cells (Jinek *et al.*, 2012, Cong *et al.*, 2013, Mali *et al.*, 2013). Following its discovery and adaptation, CRISPR-mediated genome-editing has had tremendous impact on biological research, as it allows for the fast and easy generation of mutant cells and organisms. The CRISPR system is classified into two distinct classes with different types and subtypes. Whereas class I CRISPR systems utilize multiple Cas proteins to degrade foreign nucleic acids, class II systems use a single large Cas endonuclease (Makarova *et al.*, 2015). The type II CRISPR/Cas9 system only requires two components, namely the Cas9 DNA endonuclease and a small guide RNA (sgRNA) and this simplicity permits its application to almost every organism. This includes not only mammalian cells, but also various pathogens such as viruses, bacteria and fungi (Mali *et al.*, 2013, Vercoe *et al.*, 2013, Kennedy *et al.*, 2015, Vyas *et al.*, 2015). Mechanistically the CRISPR/Cas9 works through the action of the Cas9 endonuclease, which is targeted to a particular locus in the genome by a sequence specific sgRNA. Once a DNA double strand break (DSB) is induced, repair processes in the cell are triggered to seal the break by a highly error prone process called non-homologous end joining (NHEJ). During this process bases are additionally inserted or deleted into the genomic DNA at the break, causing so called Insertion/Deletion mutants (InDels), which often result in frameshift mutations and therefore knockout of the gene of interest (Sander *et al.*, 2014). However, the basic CRISPR/Cas9 system has been further advanced over the recent years, allowing the introduction of specific mutations and

spatiotemporal insertions of small and even large pieces of DNA. These new developments have been extensively and comprehensively reviewed elsewhere (Lopes *et al.*, 2016, Xue *et al.*, 2016).

CRISPR/CAS9 IN FUNCTIONAL GENOMIC SCREENING

Functional genomic screens provide a powerful tool for the identification of critical genes in different cellular pathways. Previous technologies, such as retroviral insertional mutagenesis or RNAi libraries, although promising, did have inherent limitations such as incomplete inactivation or off-target effects (Shalem *et al.*, 2015).

In contrast, the CRISPR/Cas9 gene editing system facilitates highly efficient and robust screening that can reliably identify critical gene products. For example we, and others have shown that the typical validation rate of novel targets identified in whole genome CRISPR/Cas9 screens ranges around and above 30% (Herold MJ *et al*., unpublished and Parnas *et al*., 2015). CRISPR/Cas9 loss-of-function screens, CRISPR/Cas9-mediated 'activation' (CRISPRa) and 'repression' (CRISPRi) screens can be performed with minimal off-target effects and allow targeting of non-transcribed genomic regions such as enhancers and promotors, providing a new level of functional genomic screening (Shalem *et al.*, 2015). Using viral vectors, the sgRNAs are delivered into cells in a pooled library format targeting the whole genome. Alternatively, specific pools of gene subsets can be targeted. Pooled functional screens are very useful in positive selecting assays, e.g. survival screens. It is also possible to use pooled libraries in negative selecting assays such as an assay in which the deletion of a gene leads to cell death. In the latter case, the complexity of the pool (number of genes being targeted) has to be much smaller and higher number of sgRNAs are required per gene (Shalem *et al.*, 2015). A better option for performing whole genome negative selection screens is the use of arrayed sgRNA libraries. In an arrayed library screen sgRNAs targeting individual genes are introduced into the cell populations across thousands of wells to cover the whole genome. This setup not only allows negative

selecting screens, but many other phenotypic screens (e.g. migration screen, morphology screen). Thus far no one has reported on an arrayed whole genome CRISPR screen, but partial genomes have been targeted (Schmidt *et al.*, 2015). Pooled libraries are less costly and less labor intensive compared to arrayed libraries. Additionally, the use of arrayed libraries is restricted by diverse factors associated with assay development and readout (Agrotis *et al.*, 2015). However, with growing interest and investment from academia and industry and with the expanding applicability of CRISPR-based genomic screens, arrayed libraries have the potential to develop into valuable genomic tools.

In a conventional loss-of-function (LOF) CRISPR pooled screen, the gRNA library is transduced into cells that either stably express Cas9 or receive the endonuclease simultaneously on the same viral vector encoding the sgRNAs. The specific cellular phenotype caused by the genetic perturbation is then selected either by positive selection such as mutations that confer resistance to drug treatment or pathogen infection. In negative selection screens genes essential for cell survival or proliferation that lead to the depletion of cells can be identified (Shalem *et al.*, 2015). After the selection process, genomic DNA is extracted from the remaining cell population and the sgRNA-encoding regions are amplified by PCR and subjected to 'Next Generation Sequencing'. Representation of the enriched or depleted sgRNAs, identified by sequencing the sgRNAs, can then be analyzed and correlated with the observed phenotype obtained after the experimental treatment / intervention (Shalem *et al.*, 2015). The first applications of pooled CRISPR loss-of-function screens - providing proof of principle - identified known resistance genes in human cancer cell lines and mouse embryonic stem cells (Koike-Yusa *et al.*, 2014, Shalem *et al.*, 2014, Wang *et al.*, 2014b). In the last two years, modified loss-of-function CRISPR screens gained popularity and, amongst others, revealed functional regulatory networks in primary immune cells in response to activation by LPS (Parnas *et al.*, 2015), led to the identification of novel cancer drug targets (Shi *et al.*, 2015a) and were expanded to *in vivo* screens identifying mutations that drive tumour growth and metastasis to the lung (Chen *et al.*, 2015).

As outlined above, the development of variable Cas9 nuclease genomic screens allow us to investigate genetic regulation beyond the coding genome providing a novel way to answer questions about epigenetics and transcriptional regulation. Gilbert *et al* utilized CRISPR-mediated repression (CRISPRi) and activation (CRISPRa) tools for functional genomic screening by fusing transcriptional activators or suppressors to enzymatically inactive/dead dCas9. These screens identified genes essential for cell survival, tumour suppression as well as sensitivity to toxins (Gilbert *et al.*, 2014). In a dCas9/scRNA-mediated activation screen, Zhang and colleagues recruited transcriptional activators such as p65 via scRNAs and identified activation mutants responsible for resistance to the antimelanoma agent Vemurafenib (Konermann *et al.*, 2015). A very recent study elegantly expanded the utility of CRISPR/Cas9 to elucidate the functions of the noncoding genome: Korkmaz *et al* systematically identified noncoding regulatory elements in enhancer regions of TP53 and ESR1 regulated genes (Korkmaz *et al.*, 2016).

The above studies demonstrate the great potential of the CRISPR/Cas9 technology as a screening tool to elucidate cellular pathways, which can further be utilized to dissect factors involved in manifestation and pathophysiology of infectious diseases, both in the pathogen as well as in the host. In the last two years, several groups have already successfully applied CRISPR screens to a variety of pathogenic bacteria, viruses and parasites (see Table 1).

[TABLE 1 – CRISPR screens in infectious diseases]

UTILIZING CRISPR/CAS9 TO FIGHT INFECTIOUS DISEASE

Bacterial infections - beat bacteria at their own game?

Originally identified as adaptive immune system of bacteria against invading viruses and foreign plasmid DNA, the CRISPR/Cas9 system can now elegantly be reprogrammed in order to fight bacterial pathogens 'with their own weapons'. It can be utilized to characterize the function of genes and study potential targets for antibiotics in bacteria providing useful information for novel therapeutic intervention strategies. A prominent example is *Mycobacterium tuberculosis*, which despite many decades of intense research is still considered to be 'the world's most successful pathogen' with 10 million cases and 1.5 million deaths per year globally (Zumla *et al.*, 2013). The emergence of an increasing number of multi-drug resistant strains underscores an urgent need for novel treatment options such as chemotherapeutic agents, as well as improved vaccines. Choudhary *et al* and Singh *et al* succeeded in utilizing CRISPR interference (CRISPRi) in *Mycobacterium tuberculosis* infection models, allowing efficient transcriptional repression of target genes. This presents a valuable tool in identifying and characterizing essential bacterial virulence genes as well as verifying potential new targets for small molecule inhibition (Choudhary *et al.*, 2015, Singh *et al.*, 2016).

Interestingly, the CRISPR/Cas9 system in the gram-negative intracellular pathogen *Francisella novicida* has been reported to be itself involved in pathogenesis. *Francisella* represses the production of an immunogenic membrane protein via an antisense RNA-based silencing mechanism that uses two different RNA molecules and the Cas9 protein (Sampson *et al.*, 2013). Further studies revealed that this function is critical during infection and mediates antibiotic resistance and inflammasome

evasion (Sampson *et al.*, 2014). Similar findings were recently observed in *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli* where the type I CRISPR/Cas9 system modulates the endogenous quorum-sensing regulator protein LasR to dampen host immunity and inflammatory responses by evading TLR4 recognition. This potentially suggested a common strategy employed by bacteria to evade host immune function by "self-priming" through endogenous target regulation (Wu *et al.*, 2016). Taken together, these findings represent a paradigm shift in our understanding of the function of CRISPR/Cas9 systems as regulators of bacterial physiology. Therefore, investigating the roles of the CRISPR/Cas9 system of certain pathogens could lead to a more detailed understanding of virulence and possibly unfold novel strategies for therapeutic interventions.

Based on its high selectivity, CRISPR can also be utilized to specifically target the genome of pathogenic bacteria. Based on early work demonstrating that CRISPR/Cas9 systems can cleave bacterial plasmid DNA (Garneau *et al.*, 2010), CRISPR was subsequently used to immunize bacteria against the spread of multi-drug resistant plasmids (Vercoe *et al.*, 2013). Seminal work by Marraffini and colleagues suggests that CRISPR/Cas9 systems could be used for the sequence-specific killing of bacteria (Bikard *et al.*, 2012). Moreover, Gomaa *et al* provided insights of repurposing CRISPR to develop "smart" antibiotics that circumvent multidrug resistance and differentiate between pathogenic and beneficial microorganisms (Gomaa *et al.*, 2014). However, the delivery of the exogenous CRISPR/Cas9 system into the host bacteria was very inefficient. Citorik *et al* and Bikard *et al* have greatly improved delivery by using phagemids and thus exploited the ability of bacteriophages to inject their genetic material into the host bacterium (Bikard *et al.*, 2014, Citorik *et al.*, 2014). Encouragingly, the surviving pathogenic bacteria did not evolve resistance but rather received a defective or lacked a CRISPR-Cas system. Furthermore, both groups were able to show moderate, albeit significant reduction of infection using *in vivo* models of *Staphylococcus aureus* and enterohemorrhagic *Escherichia coli* (EHEC), respectively. In order to circumvent the obstacles of

phage administration into infected cells as well as to prevent antibiotic-resistance escape mutants, Yosef *et al* recently modified this conventional phage therapy by using lytic phages to sensitize pathogenic bacteria while concomitantly enriching for these sensitized populations. Their proposed approach does not aim to directly kill pathogens, but rather sensitize them to conventional antibiotics. This is achieved by linking a trait that is either beneficial to the bacteria, such as spacers conferring protection from lytic phage or a trait that reverses drug resistance such as spacers targeting resistance genes (Yosef *et al.*, 2015).

Taken together, the CRISPR/Cas9 system has highest potential to be a valuable tool providing tailored antimicrobials for the treatment of multidrug-resistant bacterial infections, thereby targeting pathogenic bacteria 'with their own weapons'.

The CRISPR/Cas9 system has also been used as a screening platform to advance our understanding of essential bacterial genes that confer survival and chemical vulnerabilities. In a comprehensive CRISPRi-mediated knock-down screen in *Bacillus subtilis*, Peters *et al* revealed whole-genome interaction networks and identified complex phenotypes using the technique. The authors propose that this systematic and unbiased approach could be extended to other bacterial species, including pathogenic strains, and be beneficial for the design of novel antibiotic strategies (Peters *et al.*, 2016).

CRISPR-based screening systems have also been successfully applied to decipher host-pathogen interactions by targeting the host cell genome. One of the first published genetic screens using CRISPR/Cas9 identified host genes in mouse cells conferring resistance to *Clostridium septicum* α-toxin (Heikel Yusa *et al.*, 2014). Shortly thereafter, Zhou *et al* utilized human knockout library screens and identified the host genes essential for the intoxication of cells by anthrax and diphtheria toxins (Zhou *et al*, 2014). In a recent study, Virreira Winter *et al* applied a genome-wide loss-of-function CRISPR screen and revealed novel host factors required for *Staphylococcus aureus* virulence, a very

common multi-drug resistant bacteria that causes major problems in hospitals. Their results suggest that the *Staphylococcus aureus* pore-forming toxin alpha-hemolysin (αHL) utilizes membrane lipid rafts on the host cell for attachment and cytotoxicity (Virreira Winter *et al.*, 2016). Another recent study using CRISPR technology deciphered host processes essential for bacterial virulence that are mediated by injection of bacterial effector proteins via type 3 secretion systems (T3SS), an important pathogenic mechanism for many gram-negative bacteria. The study investigated toxicity of *Vibrio parahaemolyticus* and revealed the importance of ubiquitous surface modifications of the host cell such as sulfation and glycan fucosylation for bacterial adhesion, membrane insertion and downstream T3SS-associated cytotoxicity (Blondel *et al.*, 2016). Very recently, a whole genome CRISPR screen identified members of the Wnt receptor frizzled family (FZDs) as receptors responsible for the severe pathophysiology caused by *Clostridium difficile* toxin B (Tao *et al.*, 2016).

Sepsis remains a significant problem in hospitals around the world. In recent years, accumulating evidence suggests that targeting the host immune system rather than the systemic underlying infection might hold the key for successful sepsis therapy (Hotchkiss *et al.*, 2009). In line with this, we recently showed in a clinically relevant 'two-hit' model of sepsis that ER-stress-mediated lymphocytopenia could be targeted to improve survival of immunosuppressed mice upon pneumonia challenge (Doerflinger *et al.*, 2016). We are now utilizing CRISPR/Cas9 whole genome screens to identify unknown host proteins involved in sepsis-induced ER-stress-mediated lymphocyte cell death. To uncover the regulation of inflammatory caspases during endotoxic shock, Shao and colleagues have used whole genome CRISPR/Cas9 screens and identified gasdermin D as a mediator of inflammasome-mediated pyroptotic cell death (Shi *et al.*, 2015b).

The findings described above, demonstrate the robustness and utility of CRISPR/Cas9 genetic screens in identifying pathogen and host factors that participate in the pathogenesis of infectious disease. Therefore, functional genomics using the CRISPR/Cas9 system show great potential to identify novel

and possibly targetable host-pathogen interactions that could translate to therapeutics for bacterial infections.

Viral infections – turning CRISPR 'back to the roots'

Treatment of viral infections presents a particular therapeutic challenge, as viruses do not have their own cellular metabolism and rely on host proteins for their replication. Many pathogenic viruses manifest as persistent or latent infections which further complicates treatment (White *et al.*, 2015). Given that the CRISPR/Cas9 system originally evolved in bacteria to specifically target invading viruses and foreign DNA, it seems self-evident that this gene-editing tool might be used as a therapeutic tool against viral infections.

Vaccination has been one of the success stories of infectious disease research and this single intervention protects millions of people from viral and bacterial infections every year. However, high vaccine manufacturing costs, access to primary health facilities and logistical issues that impede distribution and maintenance of a "cold chain" are significant obstacles that hinder universal vaccination. Furthermore, antigenic diversity and variation remains a major roadblock in the development of vaccines against many viruses (White *et al.*, 2015).

Yuan *et al* reported the use of CRISPR/Cas9 to efficiently edit vaccinia virus to improve its vector function in vaccine development for infectious diseases and immunotherapies for cancer (Yuan *et al.*, 2015). Recently, Tang *et al* reported the development of live attenuated pseudorabies virus, providing proof that CRISPR/Cas9 can be readily used to attenuate novel strains for vaccine production (Tang *et al.*, 2016). Doran and colleagues proposed to use CRISPR/Cas9 to edit genes that result in hypoallergenic chicken eggs used for vaccine production – the reason why routine vaccinations cannot be received by 2% of children worldwide - and appropriately called their approach CRISPi chicken (Dhanapala *et al.*, 2015). Moreover, to address the problem of high costs involved in vaccine generation, van der Sanden *et al* utilized CRISPR technology to engineer vaccine manufacturing cell lines with dramatically increased virus vaccine production (van der Sanden *et al.*, 2016).

Infections with the human immunodeficiency virus (HIV) remain a major global public health problem. Despite more than 30 years of intense research, HIV-1 infection remains incurable due to the integration of the virus into the host genome, facilitating viral latency and the risk of viral reactivation (Maartens *et al.*, 2014). Several studies have successfully used the CRISPR/Cas9 system to target either essential HIV genes or the viral long terminal repeat (LTR) of the HIV-1 DNA and achieved suppression of virus production and infection in CD4+ primary T cells and T cell lines (Ebina *et al.*, 2013, Hu *et al.*, 2014, Liao *et al.*, 2015, Kaminski *et al.*, 2016). Importantly, one of the major hurdles for HIV treatment is to overcome dormancy of HIV provirus in latently infected cellular reservoirs. Using CRISPR/Cas9-derived activator systems as targeted approaches to induce activation of dormant HIV-1 proviral DNA, Bialek *et al* were able to antagonize HIV latency, which then led to production of infective viral particles, targetable for efficient antiviral therapy (Bialek *et al.*, 2016). However, recent work by Wang *et al* and Yoder *et al* revealed that CRISPR/Cas9 might actually be a double-edged sword when used for HIV infection therapy, as the DNA double-strand repair by NHEJ initiated upon Cas9 cleavage of HIV-1 genomic regions generates a variety of mutations at the cleavage site which facilitated virus resistance (Wang *et al.*, 2016b, Yoder *et al.*, 2016). Recognition of the limitations of the CRISPR/Cas9 system will drive strategies to overcome viral escape mechanisms. Targeted engineering of new Cas9 variants or concurrent suppression of NHEJ as repair mechanism will advance its potential for future application in HIV research. Another strategy to battle HIV, as in other infectious diseases, is targeting the host cell mechanism rather than the pathogen. Hou *et al* have provided proof that genetic manipulation of the cell surface receptor CXCR4, which mediates viral entry into the host cell, confers resistance to HIV-1 infection (Hou *et al.*, 2015). Another study has recently generated CCR5 triploid human embryo mutations as a means to hinder HIV infection through this receptor (Kang *et al.*, 2016). CRISPR/Cas9 whole genome screens could be a very helpful tool to further elucidate the host-pathogen interactions that underlie

HIV infection, persistence and reactivation, which would have the potential to reveal novel therapeutic targets.

Hepatitis B virus infection affects more than 2 billion people worldwide and although vaccination and current HBV antivirals are highly effective in preventing infection and suppressing viral replication, current existing therapies cannot provide a functional cure for infection. While the host immune system can effectively control the acute phase of HBV infection, a proportion of HBV carriers develop chronic infection due to a persistent viral minichromosome that takes the form of a covalently closed circular DNA (cccDNA) template in hepatocyte reservoirs. The resulting chronic inflammation predisposes infected individuals to cirrhosis and hepatocellular carcinoma (Ebert *et al.*, 2016). The promise of CRISPR/Cas9 as a novel therapeutic strategy for specific disruption of viral cccDNA has produced a considerable amount of publications in the last two years that provide proof of principle *in vitro* and *in vivo* that this approach has the potential to contribute to a functional cure for chronic HBV infection (Lin *et al.*, 2014, Dong *et al.*, 2015, Kennedy *et al.*, 2015, Liu *et al.*, 2015, Wang *et al.*, 2015, Zhen *et al.*, 2015). In our own studies (Ebert *et al.*, 2015a, Ebert *et al.*, 2015b), we were able to eliminate HBV infection in preclinical models using a novel approach of inducing death of HBV-infected hepatocytes. Therefore, like in other infections such as HIV or tuberculosis, it is of great interest to utilize CRISPR/Cas9 to further decipher host-pathogen interactions that allow manifestation of chronic persistence. Ren *et al* have already successfully applied CRISPR/Cas9 library screening for Hepatitis C and identified essential genes for both the cell-free entry and the cell-to-cell transmission of HCV (Ren *et al.*, 2015).

CRISPR/Cas9 has also been used to target a variety of other chronic human virus infections. More than 90% of adults have been infected with at least one of the eight subtypes of herpes viruses. Latent infection persists in most people within a wide range of host cells and current treatment and vaccine strategies are ineffective. Eppstein Barr virus (EBV) is associated with various malignancies

including Burkitt's lymphoma and CRISPR/Cas9 has been used to edit and disrupt genes important for genome structure, host cell transformation and infection latency (Wang et al., 2014a, Yuen et al., 2015). Van Diemen *et al* could abrogate Human Cytomegalovirus (HCMV) and Herpes simplex virus (HSV-1) replication by targeting essential viral genes and almost completely cleared Eppstein-Bar virus (EBV) from latently infected EBV-transformed human tumor cells utilizing multiple gRNAs with no off-target effects (van Diemen *et al.*, 2016). Khalili and colleagues have used CRISPR/Cas9 to specifically target DNA sequences at the N-terminal region of polyomavirus JCV large T-antigen and successfully induced gene disruption that suppressed initiation of viral gene transcription and DNA replication (Wollebo *et al.*, 2015). This offers novel therapeutic strategies for the prevention of fatal demyelinating disease of the central nervous system (CNS) caused by this human virus.

The recent outbreak of Zika virus infections in South America has drawn huge public attention to the need of discovering novel drugs that prevent the spread of acute viral infections transmitted by mosquito vectors. In 2016, published all within the same month, three groups have independently utilized CRISPR screening platforms to genetically dissect host-pathogen interactions in flaviviruses including Zika and dengue virus (Marceau *et al.*, 2016, Savidis *et al.*, 2016, Zhang *et al.*, 2016). The screens identified host factors involved in viral cell entry, endocytosis and transmembrane protein processing and showed that ER-associated multi-protein complexes are essential to cleave off the flavivirus structural proteins prM and E for secretion of mature viral particles. Moreover, Ma *et al* identified seven host genes that conferred protection against West Nile virus induced cell death in a CRISPR-based screen (Ma *et al.*, 2015). CRISPR/Cas9 mediated gene editing has also been achieved in the flavivirus vector *Aedes aegypti*, paving the way for further functional genomics related studies in this mosquito species (Dong *et al.*, 2016) with the aim to potentially restrict viral propagation and transmission between vector and human host.

Parasite and fungal infection – expanding the application of CRISPR/Cas9

About 3.2 billion people – nearly half of the world's population – are at risk of malaria infection, and more than 200 million malaria cases were reported in 2015 (White *et al.*, 2014). Approaches to eliminate malaria infections by targeting the transmitting mosquito vector with CRISPR are already under investigation. Gantz *et al* revealed a mosquito strain incorporating a synthetic system called a 'gene drive' that passes a malaria-resistance gene on to the mosquitoes' offspring (Gantz *et al.*, 2015). As malaria parasites are exclusively transmitted by female mosquitoes, Hammond *et al* utilized CRISPR to manipulate three different genes in *Anopheles gambiae* that confer a recessive female-sterility phenotype upon disruption (Hammond *et al.*, 2016). Therefore, the CRISPR/Cas9-system could be utilized to introduce 'gene drives' that suppress mosquito populations to levels that do not support malaria transmission. Studies on *Plasmodium falciparum* have shown that the malaria parasite itself can also be targeted using CRISPR/Cas9 to further understand pathogenesis and drug resistance (Ghorbal *et al.*, 2014). The closely related parasite *Toxoplasma gondii*, one of the most common parasites in the world and the cause of toxoplasmosis, was also shown to be susceptible to genetic manipulation using CRISPR/Cas9 (Sidik *et al.*, 2014, Sugi *et al.*, 2016). Recently, a genome wide CRISPR/Cas9 screen in *Toxoplasma gondii* identified essential genes during infection of human host cells and provided proof for the feasibility of CRISPR/Cas9-based screening platforms to expand the horizon of anti-parasitic interventions (Sidik *et al.*, 2016). Efficient genome editing of parasitic pathogens with CRISPR/Cas9 has also been applied to *Leishmania* (Sollelis *et al.*, 2015, Zhang *et al.*, 2015) and *Trypanosoma* (Peng *et al.*, 2015) parasites and open avenues for functional studies to speed up research on leishmaniasis and infectious myocarditis, respectively.

Systemic fungal infections caused by the filamentous fungus *Aspergillus* species and the yeast species *Cryptococcus* and *Candida* have markedly risen over the past decade, due to an increase in both, the

aged population and the number of immune-compromised individuals, as well as antifungal-resistant subspecies (Pfaller *et al.*, 2007). The CRISPR/Cas9 system has been recently applied in genome editing of *Candida albicans* (Vyas *et al.*, 2015, Min *et al.*, 2016), *Aspergillus* (Fuller *et al.*, 2015) and *Cryptococcus* (Wang *et al.*, 2016a) and has the potential to further uncover the molecular basis of fungal infection and resistance to antifungals.

[TABLE 2 – CRISPR/Cas9 as novel therapeutic and prophylactic strategy]

CONCLUSION AND FUTURE DIRECTIONS

Infectious diseases still cause significant morbidity and mortality and novel therapeutic approaches are desperately required to conquer the increasing amount of multidrug-resistant pathogens (Spellberg *et al.*, 2008). Within only a few years since its development into a genetic tool, the CRISPR/Cas9 system now holds great promise in boosting our chances of controlling and eradicating some of the major global infectious diseases. However, despite the progress made in the understanding of CRISPR function, many central aspects remain obscure. The somewhat intriguing finding that CRISPR/Cas9 in bacteria not only functions as adaptive immune system, but also appears to be important for pathogenesis, poses the question of whether CRISPRs have other unknown physiological functions and itself present a target for antimicrobial strategies. Therefore, in order to fully explore CRISPR/Cas9 in our battle against infections, we first have to gain more insight into its function and regulation.

There have been great improvements in sgRNA/Cas9 design, synthesis, selection and delivery and these discoveries continue to build on the specificity and efficiency of CRISPR/Cas9 systems. However, to progress CRISPR/Cas9 from a genetic research tool to its application in the clinic, improved safety and efficient delivery has to be achieved. In particular, potential off-target effects of the sgRNAs have to be considered and require improved design strategies to avoid unwanted side-effects. New delivery methods are being investigated using adeno-associated viral (AAV) vectors, which compared to lentiviral or adenoviral vectors, have the advantage of low immunogenicity, broad tissue tropism and minimal insertional mutagenesis (Senis *et al.*, 2014). However, the small packaging capacity of AAV vectors necessitate either modified versions of the commonly used Cas9 from *Streptococcus pyogenes* (SpCas9) or smaller orthologues such as the recently discovered Cpf1 DNA endonuclease (Zetsche *et al.*, 2015). Alternatively it has recently been described that the Cas9

protein could be delivered as a split protein (Chew *et al.*, 2016). The ongoing quest for more precise, more flexible and more efficient CRISPR/Cas9 variations will expand the scope for applications in the near future.

The development and refinement of the CRISPR/Cas9 gene-editing system also allows for more reliable and highly efficient screening strategies to perform large-scale target identification in the genome and epigenome of both host and pathogen (Xue *et al.*, 2016). This is of particular interest in order to decipher host-pathogen interactions that underlie factors such as pathogen cell entry, persistence, propagation and dissemination. Several successful screens have been conducted and shed light on how these host-pathogen interactions during bacterial, viral or parasitic infection could be targeted for therapeutic intervention (see Table 1). The advancement of arrayed CRISPR/Cas9 libraries would greatly expand the scope of cellular phenotypes. Utilizing 'high throughput screening' platforms that measure fluorescence or luminescence, multi-parametric features such as changes in cellular shape, expression of reporter genes, as well as host cell traversal or intracellular location of pathogens could be investigated.

CRISPR/Cas9 technologies also offer the potential to overcome some of our present obstacles in vaccine development and novel strategies have improved yield and efficiency of vaccine manufacturing (Yuan *et al.*, 2015, Tang *et al.*, 2016, van der Sanden *et al.*, 2016).

About a fifth of all infectious diseases are vector borne and transmitted by arthropods such as mosquitos and ticks (WHO, 2016). Therefore, utilizing CRISPR/Cas9 to understand the mechanisms and dynamics of arthropod infection by viruses, such as dengue virus, and parasites, such as *Plasmodium*, may be a powerful means to study and prevent disease in humans. Vector control by CRISPR/Cas9-mediated 'gene drive' has been proposed to interrupt the infectious cycle and would

present an alternative strategy that circumvents the need for treating human patients with antimicrobials (Hammond *et al.*, 2016).

The use of the CRISPR/Cas9 system to act as 'smart antimicrobials' in pathogenic bacteria might be an elegant answer to address the rising numbers of multi-drug resistant pathogens. CRISPR/Cas9 can be used to immunize bacteria against the spread of multi-drug resistant genes and for the sequence specific killing of bacteria 'by their own weapons', while differentiating between beneficial and pathogenic microorganisms (Bikard *et al.*, 2014, Gomaa *et al.*, 2014).

CRISPR/Cas9-mediated gene editing rendering cells vulnerable to small molecule inhibitors and thus enables validation of potential drug targets. CRISPR has been adapted successfully for the use in a broad spectrum of pathogens including bacteria *(Mycobacterium tuberculosis*), viruses (Hepatitis B virus, Hernes Simplex Virus), parasites (*Plasmodium falciparum*) and fungi (*Candida albicans*) and has the potential to uncover novel therapeutic intervention strategies.

[FIGURE 1]

However, CRISPR/Cas9 may not be the magic bullet to directly target every pathogen. New gene-editing tools are being promoted and utilized to identify curative interventions for HIV/AIDS. Early findings using CRISPR/Cas9 achieved suppression of virus production and infectivity. Enthusiasm for these outcomes was quickly blunted when it was discovered that double strand repair by non-homologous end joining initiated upon Cas9 cleavage of HIV-1 genomic regions could generate a variety of mutations at the cleavage site which further facilitated virus resistance (Yoder *et al.*, 2016). Therefore, CRISPR/Cas9 appears to be a double-edged sword in the fight against HIV and targeting the host rather than the highly mutagenic virus may hold the key for future success.

In conclusion, the rapid advance in the area of CRISPR/Cas9 as a genome-editing approach has enabled widespread application to infectious disease research and identification of preventative and therapeutic interventions (see Figure 1). Therefore the CRISPR system might be the desperately required weapon in the battle against multidrug-resistant pathogens and epidemic outbreaks that are responsible for a quarter of all deaths worldwide.

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Author contributions

MD discussed the subject matter with all co-authors and wrote the manuscript and created the tables. MJH, MP, GE and WF contributed to manuscript revision and WF created the figure.

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Figure legend:

Figure 1: Applications of CRISPR/Cas9 in infectious diseases
Within only a few years since its discovery and application as genetic tool, the CRISPR/Cas9 system has found widespread application in infectious disease research. It can be used to directly target known virulence factors of the pathogen as well as host genes conferring pathogenesis during infection or utilized in functional genomic screens to identify novel unknown mediators of infection. CRISPR/Cas9 offers great potential in vaccine production and the development of 'smart' antimicrobials and can help in drug target validation in virtually any pathogenic organism. Furthermore, CRISPR/Cas has been proposed as promising tool in vector control by introducing 'gene drives' that interrupt the infectious cycle.

Reference	Screening type	Selection target
Pathogenic bacteria		
Koike-Yusa *et al.*, 2014	whole genome library; wtCas9 loss-of-function	*Clostridium septicum* α-toxin
Zhou *et al.*, 2014	whole genome library; wtCas9 loss-of-function	Diphtheria and chimeric anthrax toxins
Winter *et al.*, 2016	whole genome library; wtCas9 loss-of-function	*Staphylococcus aureus* α-hemolysin toxin
Tao *et al.*, 2016	whole genome library; wtCas9 loss-of-function	*Clostridium difficile* toxin B
Blondel *et al.*, 2016	whole genome library; wtCas9 loss-of-function	*Vibrio parahaemolyticus* toxicity mediated by T3SSs
Gilbert *et al.*, 2014	whole genome library; dCas9 CRISPRi/a	Cholera-diphtheria toxin
Peters *et al.*, 2016	focused library; dCas9 CRISPRi	*Bacillus subtilis* essential genes and whole-genome interaction networks
Viruses		
Ren *et al.*, 2015	focused library; wtCas9 loss-of-function	Hepatitis C virus host factors
Ma *et al.*, 2015	whole genome library; wtCas9 loss-of-function	West Nile virus induced cell death
Marceau *et al.*, 2016	whole genome library; wtCas9 loss-of-function	Dengue virus and hepatitis C virus host factors
Zhang *et al.*, 2016	whole genome library; wtCas9 loss-of-function	Flaviviridae host factors
Savidis *et al.*, 2016	whole genome library; wtCas9 loss-of-function	Zika virus host factors
Parasites		
Sidik *et al.*, 2016	whole genome library; wtCas9 loss-of-function	*Toxoplasma gondii* host factors

Table 1. CRISPR/Cas9 screens used to elucidate host-pathogen interactions in infectious diseases

Purpose	Experimental CRISPR Approach	Examples + Reference
functional studies of virulence factors/ validation of drug targets	targeted pathogen genome editing	*Mycobacterium tuberculosis* - Choudhary *et al.*, 2015 *Toxosplasma gondii* - Sidik *et al.*, 2014 Hepatitis B virus - Kennedy *et al.*, 2015
functional study of host regulatory proteins	targeted host cell genome editing	HIV - Hou *et al.*, 2015
identification of bacterial essential genes	pathogen whole genome screen	*Bacillus subtilis* - Peters *et al.*, 2016
identification of novel host regulatory proteins	host cell whole genome screen	*Staphylococcus aureus* - Virreira Winter *et al.*, 2016 Hepatitis C virus - Ren *et al.*, 2015 West Nile fever virus - Ma *et al.*, 2015
vaccine development and production	engineering of vaccine vectors/cell lines/virus	vaccine cell lines - van der Sanden *et al.*, 2016 vaccine delivery vectors - Yuan *et al.*, 2015 life virus attenuation - Tang *et al.*, 2016
smart antibiotics	Sequence-specific killing of pathogenic bacteria using phagemids	*Staphylococcus aureus* - Bikard *et al.*, 2014 *E. coli* EHEC - Citorik *et al.*, 2014
vector control	Vector-specific genome editing	*Anopheles*/ Malaria mosquito - Gantz *et al.*, 2015

Table 2: Examples for CRISPR/Cas9 as novel therapeutic and prophylactic strategy in infectious disease