Old Herborn University Seminar Monograph 31:
Evolutionary biology of the virome, and impacts in human health and disease.
Editors: Peter J. Heidt, Pearay L. Ogra, Mark S. Riddle and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 27-32 (2017).

CO-EVOLUTIONARY DYNAMICS BETWEEN EPIDEMIC *VIBRIO CHOLERAE* AND PREDATORY PHAGE

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SUMMARY

Waterborne pathogens like *Vibrio cholerae* pose significant threats to global health. *V. cholerae* can persist in the aquatic environment, and it can emerge to cause devastating cholera outbreaks in endemic regions and in vulnerable areas where infrastructure has been compromised and populations have been displaced. The host-pathogen interactions that dictate disease outcome and cholera transmission dynamics occur in the context of a complex microbial ecosystem that includes predatory bacterial viruses (phages). Such phages are found in the aquatic environment and are co-ingested with *V. cholerae*, permitting continued phage predation of *V. cholerae* within the human intestinal tract. In our efforts to understand the interactions between phage and *V. cholerae* during human cholera infection, we interrogate the molecular mechanisms underpinning phage-bacterial co-evolution in naturally evolved microbial populations within and between cholera patients. We found that phage predation in the human intestinal tract places mutational constraints on *V. cholerae*, ensuring that phages have access to a susceptible bacterial population. However, phage resistant variants do emerge, displacing sensitive variants. Our data indicate that the key to phage resistance in successful epidemic strains of *V. cholerae* resides in mobile genetic elements. Additionally, we have identified novel genomic signatures associated with the co-evolution of phage that allow phage to overcome resistance barriers and thrive in otherwise resistant cells. Understanding *V. cholerae*-phage interactions serves as a useful platform to understand how predatory phages structure microbial communities, including those that flourish in disease states and those that comprise our microbiome.

INTRODUCTION

Illness and death caused by infectious diarrheal disease agents, like *Vibrio cholerae*, are major threats to public health and significant barriers to socio-economic development worldwide (*Havelaar* et al., 2015). Upon ingestion of food or water contaminated with pathogenic *V. cholerae* individuals can succumb to cholera, an acute diarrheal disease that leads to severe dehydration and death. The incidence of cholera worldwide is steadily increasing, the global disease burden is estimated to be 3-5 million cases resulting in more than 100,000 deaths annually (*Harris* et al., 2012). Following ingestion, *V. cholerae* must survive the gastric acid in the stomach and those bacteria that do go on to colonize the mucosal surface of the small intestine. It is here

that the organism elaborates cholera toxin, the major virulence factor for pathogenic strains (*Waldor* and *Mekalanos*, 1996). Cholera toxin binds to a receptor on enterocytes and activates adenylate cyclase leading to chloride secretion and secretory diarrhoea. Our understanding of *V. cholerae* pathogenesis has been built almost exclusively on studies to understand bacterial virulence factors and virulence gene regulation (for example: (*Finkelstein* and *LoSpalluto*, 1969; *Miller* et al., 1987; *Taylor* et al., 1987; *Merrell* et al., 2002a,b; *Mandlik* et al., 2011; *Fu* et al., 2013; *Kamp* et al., 2013). However, the role that other constituents of the microbial community, including predatory phages, play in disease outcome and the evolution and epidemiology of *V. cholerae* are not well understood.

Phages are bacterial viruses that act with exquisite specificity to kill their perpetually evolving bacterial targets. Many studies have demonstrated the presence of predatory phage co-existing with *V. cholerae* in stool when cholera victims present to the clinic with severe disease (*d'Herelle* and *Malone*, 1927; *Pasricha* et al., 1931; *Nelson* et al., 2007; *Seed* et al., 2010; 2014; *David* et al., 2015). These observations, coupled with fluctuations in environmental phage levels, have implicated predatory phages in shaping cholera outbreaks (*d'Herelle* and *Malone*, 1927; *Pasricha* et al., 1931; *Faruque* et al., 2005a,b). An interesting feature of predatory phages is that, similar to environmental factors like rainfall amounts, phage may modulate the inter-epidemic persistence of *V. cholerae* in the environment, thus impacting the occurrence of outbreaks; however, uniquely, these phages are co-ingested with *V. cholerae* into the human host and have the potential to continue to prey on their bacterial host during the course of infection. Predatory phages are also shed in appreciable amounts by infected patients where, like *V. cholerae,* they can be spread to others via faecal-oral transmission. Phages, therefore, have the potential to impact all aspects of the *V. cholerae* life cycle (including environmental persistence, transmission, infection and dissemination), on both a short and long-term evolutionary scale.

THE DOMINANT PREDATORY PHAGES

Previous work done at the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) in Dhaka, Bangladesh documented an inverse correlation between cholera disease burden and the levels of phages that specifically infect and kill *V. cholerae* in the aquatic environment (*Faruque* et al., 2005b), suggesting these viral predators have a role in shaping cholera outbreaks (*Faruque* et al., 2005a). We analysed phages isolated from cholera patient stool samples that had been collected over a decade long period at the ICDDR,B. Surprisingly, we found that the genetic diversity of predatory phages associated with *V. cholerae* in patient samples is strikingly low (*Seed* et al., 2010). We hypothesize that these particular phages have evolved unique strategies to maintain a long-term association with their bacterial host. In total, three unique and unrelated predatory phages have been identified: ICP1, ICP2 and ICP3. The continued prevalence of these phages (at least in Bangladesh) is supported by recent metagenomic data collected to understand microbial succession after cholera infection (*David* et al., 2015). Additionally, subsequent analysis of phage recovered from a Haitian cholera

patient sample revealed the presence of an ICP2 isolate that was strikingly similar, although clearly distinct from phage recovered two years earlier from a Bangladeshi patient (*Seed* et al., 2014). The limited diversity of phages associated with *V. cholerae* in human cholera patient samples allows us to simultaneously assess phage and *V. cholerae* evolution.

MECHANISTIC CHARACTERIZATION OF VIRULENCE REDUCTION IN PHAGE RESISTANT *V. CHOLERAE*

We identified the surface structure used by ICP1, the most prominent phage, to initiate infection and found that in both simulated aquatic environments and within experimental infection models phage predation can reduce bacterial loads and select for receptor mutants (*Seed* et al., 2012). In order to conclusively determine if phages influence the abundance of *V. cholerae* in the human intestinal tract, we analysed intra-patient microbial populations (*Seed* et al., 2014). We demonstrated that the predatory phage ICP2 can exert selective pressure resulting in a reduction of wild-type bacteria and the proliferation of heterogeneous resistant populations during infection in humans. We found that resistant populations had altered receptors, validating our previous observations in experimental models. We found that regardless of mechanism, receptor alterations were accompanied by decreased virulence and/or transmission potential. This observation, in part, explains how these phages maintain their long-term association: phage predation in the human intestinal tract places mutational constraints on *V. cholerae*, ensuring that phages have access to a susceptible population. Collectively, this work demonstrates that adaptations to phage predation involve trade-offs in evolutionary fitness and provides a molecular mechanism for how phage predation impacts *V. cholerae* transmission and seeding of environmental reservoirs.

IDENTIFICATION OF NOVEL GENOMIC SIGNATURES ASSOCIATED WITH CO-EVOLUTION OF PHAGE AND *V. CHOLERAE*

The pervasiveness of specific predatory phages in Bangladesh with continued cholera epidemics suggests that *V. cholerae* has strategies to limit phage predation, and that phages can evolve to overcome such defences. Our work has focused on ICP1, since it is the most prevalent phage found associated with *V. cholerae* in cholera patient samples in this region (*Seed* et al., 2010). Similar to what we discovered for ICP2 (discussed above), ICP1 uses an essential virulence factor on the *V. cholerae* surface to initiate infection. Interestingly, we have not observed surface modifications allowing escape from phage for ICP1-*V. cholerae* in the context of human infection (*Seed* et al., 2012). Instead, we discovered that novel anti-phage mobile genetic elements called PLEs are responsible for ICP1 resistance in epidemic *V. cholerae* (*O'Hara* et al., 2017). PLEs are present in epidemic *V. cholerae* isolates recovered between 1949-2011 (spanning the entire collection period for which strains were available), and from different locations including Egypt, Mozambique and Bangladesh. PLEs have no sequence similarity to other known anti-phage systems, highlighting the genetic novelty found in

studying phage-host co-evolution and, most importantly, highlighting the need to study naturally evolved bacterial and viral populations. Although the mechanistic basis for how PLE protects *V. cholerae* from ICP1 infection is not fully understood, our studies thus far have revealed a multi-faceted mode of phage interference. Perhaps the most striking finding to come from characterization of these genetic elements is that all PLEs, regardless of geographic or temporal origin, respond uniquely to ICP1. These results indicate that the molecular battle between ICP1 and *V. cholerae* has been going on for at least 60 years, and that PLEs are a key bacterial weapon in this battle.

In response to the diverse strategies that bacteria use to defend against the threat of predatory phages (*Dy* et al., 2014), phages can co-evolve to circumvent any resistance barrier that they face (*Samson* et al., 2013). In an unexpected twist, we discovered that ICP1 has co-evolved to overcome PLEs using a CRISPR-Cas adaptive immune system (*Seed* et al., 2013). We discovered that half of all ICP1 isolates recovered from cholera patients in Bangladesh encode a functional CRISPR-Cas system. CRISPR-Cas is a sequence specific adaptive immune system that is typically encoded by bacteria to defend against phage (*Barrangou* et al., 2007; *Marraffini*, 2015). The ICP1-encoded CRISPR-Cas system targets and degrades PLE to block its anti-phage activity (*Seed* et al., 2013). Collectively, we have established that the long-term interactions between *V. cholerae* and ICP1 serve as a useful platform to understand the evolution of phage-resistance and counter-resistance in the context of human disease.

CONCLUSIONS

Host-pathogen interactions are strongly affected by the complex microbial community that surrounds them. Predator-prey dynamics are notably absent between phages and their bacterial hosts in the intestinal microbiome in healthy humans (*Reyes* et al., 2010). However, disease states, which are often accompanied by significant bacterial proliferation, provide optimal conditions for rampant phage predation. Phage-mediated perturbations of microbial communities have recently been implicated in inflammatory bowel disease (*Norman* et al., 2015) and cystic fibrosis (*James* et al., 2014). Understanding the molecular consequences of phage predation on the long-term evolution of *V. cholerae* serves as a relevant platform to understand similar dynamics that are likely applicable to many bacterial diseases. In addition, there is significant interest in developing phage as biocontrol agents in human infections as well as in agriculture and food safety (*Doss* et al., 2017). Future therapies directed at using phages in therapeutic regimens will rely on a deeper understanding of the molecular mechanisms underpinning phage-host interactions in the context of human disease.

ACKNOWLEDGEMENTS

The work related to this review was supported by the National Institute of Allergy and Infectious Diseases (USA). K.S. is a Chan Zuckerberg Biohub investigator.

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