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Animal models for dissecting *Vibrio cholerae* intestinal pathogenesis and immunity

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Abstract

The human diarrheal disease cholera is caused by the bacterium *Vibrio cholerae*. Efforts to develop animal models that closely mimic cholera to study the pathogenesis of this disease began >125 years ago. Here, we review currently used non-surgical, oral inoculation-based animal models for investigation of *V. cholerae* intestinal colonization and disease and highlight recent discoveries that have illuminated mechanisms of cholera pathogenesis and immunity, particularly in the area of how *V. cholerae* interacts with the gut microbiome to influence infection. The emergence of high-throughput tools for studies of pathogen-host interactions, along with continued advances in host genetic engineering and manipulation in animal models of *V. cholerae* will deepen understanding of cholera pathogenesis, uncovering knowledge important for control of this globally important bacterial pathogen.

Keywords

Cholera; Vibrio cholerae; animal models

Introduction

The Gram-negative bacterium *Vibrio cholerae* causes cholera, a severe human diarrheal illness that remains endemic in >50 countries [1]. The ongoing 7th cholera pandemic is estimated to cause >3 million cases annually and is caused by 'variant' El Tor *V. cholerae*

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Declaration of interests

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O1 strains that are continually evolving [2]. The secretory diarrhea that is the hallmark of cholera is largely explained by the actions of cholera toxin (CT) [1], an AB_5-type toxin secreted by *V. cholerae* in the small intestine (SI), the site where the pathogen primarily colonizes the human intestine and causes disease [3]. While many pathogen factors facilitate SI colonization, a bacterial surface appendage - toxin-coregulated pilus (TCP) - is *V. cholerae's* chief colonization factor [3]. The mechanisms by which TCP enables colonization are incompletely understood.

The self-limiting nature of cholera has prompted the development of a controlled human infection model (CHIM), where *V. cholerae* is orally administered to volunteers [4]. CHIM studies have revealed the requirements for CT for disease, TCP's critical role in intestinal colonization, enabled investigations of immunity to *V. cholerae*, and demonstrated the relative efficacy of killed and live oral cholera vaccines (OCVs) [4,5]. However, CHIM work is limited by several logistical and ethical considerations. Many mechanistic questions regarding *V. cholerae*-host interactions in the SI cannot be readily answered in this human model. CHIM studies also cannot currently be conducted in cholera-endemic countries, potentially limiting the applicability of conclusions from volunteer studies in non-endemic regions [6]. Instead, efforts to understand *V. cholerae* pathogenesis have led to the development of over a dozen different animal models for this pathogen. Historically, many different medium and large mammalian species ranging from guinea pigs to non-human primates have been used, but these suffer some of the same limitations as the CHIM (reviewed in [7]). The field is now largely driven by small mammal and non-mammalian/invertebrate models.

The utility of any given animal model can be gauged by the extent to which it recapitulates human disease. In our view, it is useful to stratify currently used models (Table 1) by how well they reflect two critical aspects of human cholera pathogenesis - TCP-dependent SI colonization and CT-dependent diarrheal illness. Although models that lack either or both criteria can yield important information, the direct applicability of this knowledge to understanding cholera is unclear. In this review, we use this framework to present and discuss current oral, non-surgical infection models of *V. cholerae.* We illustrate how new discoveries from the last several years across different models illuminate themes in *V. cholerae* research and identify open areas of investigation for deepening understanding of cholera pathogenesis and immunity.

Insights into *V. cholerae* pathogenesis from animal models

The acuity of cholera is well-captured by short-term (~1 day) animal infection models. Conventionally reared, specific-pathogen free (SPF) adult mice (*Mus musculus*) are commonly used to study bacterial pathogens but are resistant to intestinal colonization by orally-inoculated *V. cholerae,* likely because of colonization resistance from the resident murine gut microbiota. Streptomycin-treated adult mice can be colonized by *V. cholerae*, but the pathogen is found almost exclusively in the colon and not the SI; moreover, intestinal colonization appears to be TCP-independent, and the animals do not develop diarrhea [8,9]. Alternate adult mouse models for oral *V. cholerae* infection (clindamycin or ketamine

pretreatment) that may surmount these obstacles have been reported, but have yet to be widely adopted [10,11].

The dominant model of *V. cholerae* pathogenesis is the 3–5-day-old infant mouse, where oral gavage of *V. cholerae* leads to TCP-dependent SI (but not colon) colonization and in some variations, CT-dependent diarrheal illness within ~16 hours [3,12]. Infant mice are a logistically simple system for addressing many targeted questions about the pathogen. This model has enabled limited-throughput studies of within-host virulence regulation, such as the use of engineered *V. cholerae* to identify in vivo-activated and -repressed loci, and the use of clinical *V. cholerae* isolates to understand how ongoing genetic variation in 7th pandemic strains impacts pathogenicity [12–16]. Advanced tissue imaging of the infant mouse SI has revealed the sub-intestinal localization patterns of *V. cholerae* during infection, and spatiotemporal control of virulence gene regulation [18,19]. The most common use of this model has been to validate suspected colonization factors with competitive index studies, where a mutant strain is competed against a WT strain in vivo [3]. Competition studies have recently highlighted the contributions of fatty acid and carbon metabolism, cell wall maintenance and *V. cholerae* LPS modifications to intestinal colonization [9,20–23].

A key limitation of infant mice is the relative scarcity of biological material for high-throughput, hypothesis-generating studies such as transcriptomics and whole-genome functional genetic screens. The infant rabbit (*Oryctolagus cuniculus*) model of cholera, which exhibits rapid CT-dependent lethal diarrheal illness along with TCP-dependent SI colonization, has filled this niche in *V. cholerae* animal studies [24]. Up to 10^{10} in vivo *V. cholerae* can be rapidly collected from the ~1 mL of diarrheal fluid that accumulates in the infant rabbit cecum, providing a valuable reagent for high-throughput analyses. The chemical composition of this fluid is highly similar to that of human choleric diarrhea [24]. Infant rabbit studies have revealed the genetic landscape of colonization factors in *V. cholerae* through transposon-insertion sequencing screens, which are largely limited by bottlenecks in infant mice [25–28]. Infant rabbits have enabled the acquisition of high-resolution in vivo *V. cholerae* RNA-seq, metabolomic, and proteomic datasets, as well as insights into *V. cholerae* population dynamics during infection [29–31]. A recent transcriptomic study in infant rabbits revealed novel roles for CT in the shaping of the pathogen's nutritional microenvironment [32]. This and other studies have illustrated the value of combining animal models for complementary purposes - discovery experiments in infant rabbits and targeted competition assays in infant mice. Combined approaches offer considerable practical advantages, as infant rabbits are relatively expensive and large-scale experiments with these animals are technically challenging.

The most commonly used oral non-mammalian species for studying *V. cholerae* are fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*), and nematodes (*Caenorhabditis elegans*). Although flies can exhibit CT-dependent mortality, infection and host killing in these models is otherwise neither CT- nor TCP-dependent [33–35]. Despite these important limitations, these models offer increased throughput, lowered cost, and ease of host genetic manipulation relative to infant mice. As the *Drosophila* intestinal immune system bears similarity to that of mammals, flies have been used to investigate host responses to *V. cholerae,* including pathogen manipulation of host lipid and carbon metabolism and how

V. cholerae quorum sensing controls host outcomes [36–39]. Studies in *C. elegans* have identified putative accessory *V. cholerae* virulence factors [35,40], and work in *D. rerio* aided by tissue imaging has been informative regarding *V. cholerae*'s interaction with gut symbionts and the intestinal epithelium [41–43]. However, as most non-mammalian studies remain to be validated in either human or small mammal cholera models, their applicability to our understanding of cholera is limited.

An emerging theme in animal-based investigations of *V. cholerae* is the pathogen's relationship with the intestinal microbiome. One of *V. cholerae*'s primary modes of interaction with other microbes is its type 6 secretion system (T6SS), an apparatus that mediates interbacterial antagonism [44]. Recent studies across several models using defined intestinal microbial communities have demonstrated the importance of the T6SS in mediating intestinal colonization, and dissected its role in creating colonizable niches for *V. cholerae* [25,41,42,45–47]. Investigations in infant and adult murine models with altered gut microbiota have been complemented by efforts to identify microbiome changes in humans exposed to *V. cholerae* [48–50]. Intestinal microbes that can promote or inhibit *V. cholerae* intestinal colonization in mice have been identified, and reconstitution of germ-free (GF) mice with these microbes offers an in vivo system in which to investigate interbacterial interactions (recently reviewed in [51]). The mechanisms underlying these phenotypes remain to be extensively characterized, but several appear to involve regulation of *V. cholerae* virulence programs by commensal-derived metabolites like secondary bile acids [10,50,52]. An important caveat to these findings is that *V. cholerae* intestinal colonization occurs primarily in the colon in GF mice. *V. cholerae*'s intricate relationship with intestinal microbes has also been hinted at by studies in infant rabbits that demonstrated *V. cholerae* is conjugation-competent in the intestine, and that pre-colonization with an avirulent *V. cholerae* live vaccine strain or *V. cholerae*-targeting phages can limit subsequent infection [28,47,53].

Insights into immunity to *V. cholerae* from animal models

Samples from cholera patients and volunteers in CHIM studies have been instrumental in the investigation of immune responses to *V. cholerae.* Observations that cholera bestows protection against future infection inspired development of currently used killed OCVs. However, there are significant limitations associated with killed OCVs, including their limited efficacy in young children; furthermore, many gaps remain in our understanding of the scope and mechanisms of protective immunity to *V. cholerae* [54]. Most questions in OCV development and immunity to cholera concern long-term adaptive immunity, but infant mammals and invertebrates have immature (or absent) adaptive immune systems. As such, adult mice have become the model of choice for studies of adaptive immunity to *V. cholerae*. Although adult mice have well-characterized and easily manipulated immune systems, mimicking OCV vaccination or natural infection by the oral route in this model is constrained by several critical limitations described above. These models exhibit no discernable illness, TCP-independent colonization, and colonic-biased pathogen localization, and GF mice are additionally confounded by sustained (months-years) *V. cholerae* colonization [8,55]. CHIM studies have shown that TCP-dependent intestinal colonization is critical for immunogenicity of live *V. cholerae* [5], and interpretation of

immune responses observed in adult mice is complicated since the colon lacks immune features of the SI such as Peyer's patches. Despite these shortcomings, viable alternatives to adult mice for studies of adaptive immune responses to *V. cholerae* are currently lacking.

Orally-infected adult mice can be used to profile diverse immune responses to *V. cholerae*. Many assays used in humans that report on known correlates of protection against cholera, such as antigen-specific ELISAs for CT and TCP, and the vibriocidal antibody titer assay, can be directly applied to murine samples. Immunological metrics can be read out from serum and fecal samples over time from the same mouse, enabling kinetics of immunity to be dissected [56]. Genetically modified adult mice could be used to explore the necessity and sufficiency of host factors in the development of immunity to *V. cholerae,* but these investigations have been rare [57].

Sustained *V. cholerae* colonization in GF adult mice precludes their rechallenge with virulent *V. cholerae,* a critical measure of vaccine efficacy. However, since colonization is transient in antibiotic-treated mice, rechallenge after clearance is possible [8,58]. While pulmonary inoculation of *V. cholerae* to circumvent obstacles associated with oral infection has been described, the translational significance of observations with this route of infection are unclear [59]. It is instead possible to use infant mice as proxy hosts to interrogate immunity in vaccinated dams, as well as to use *V. cholerae* pre-mixed with serum from immunized mice as an infant mouse challenge inoculum. These approaches, combined with readouts of immunogenicity, have collectively proven useful to test the protective efficacy of cutting-edge cholera vaccine candidates, including new live OCV strains, outer membrane vesicle-based formulations, and transcutaneous conjugate vaccines [23,56,60,61]. These studies underscore the importance of combining appropriate models (adult immunization with infant challenge) in the investigation of cholera vaccines and immunity. Standardization and consolidation of immunization schema and efficacy measures will aid these efforts [23].

The throughput and ease of host manipulation in mouse models makes them well-suited for targeted investigations of hypotheses derived from observations of human immune responses. For example, clinical investigations have suggested that immune responses to the *V. cholerae* O-antigen are the protective factor against reinfection [54]. Infant mice have been used by multiple groups to test and ultimately support this hypothesis with a variety of strategies, including direct isolation and in vivo testing of human-derived monoclonal antibodies [62,63]. A transgenic mouse line expressing a *V. cholerae* core/lipid A-targeted monoclonal antibody was developed to evaluate the sufficiency of this target for protection from intestinal colonization [64]. These examples demonstrate how hypotheses from human studies can be mechanistically evaluated using engineered animals. Similarly, the emerging concept that the intestinal microbiome shapes human immune responses to *V. cholerae* [65] is starting to be addressed using defined human microbial communities in GF mice. For example, prebiotic and microbial treatments in a GF model of CT-based vaccination were recently shown to enhance immune responses to this *V. cholerae* antigen [66]. Insights from mouse models may not only provide explanations for observations regarding human immunity but offer useful platforms to test interventions for future translation back to the clinic.

Conclusions

Despite the long history of studies of cholera pathogenesis, many questions regarding V. cholerae-host interactions remain (Table 2). Metchnikoff recognized the importance of 'favorizing microbes' in promoting experimental cholera in 1894 [67], but elucidation of the tripartite host-pathogen-commensal interaction axis during infection is just beginning. Surprisingly, despite the use of infant and adult mice to study *V. cholerae* for decades, experiments with genetically modified hosts are almost entirely lacking in the study of both pathogenesis and immunity. Although human loci conferring susceptibility to cholera have been identified [68,69], there are relatively few studies that use genetically engineered mice to investigate host innate defense against *V. cholerae* [70–72]. Such studies will be valuable because cholera can be one of the most rapidly fatal acute infections and innate rather than adaptive responses may determine outcomes. Defining the innate immune pathways that are modulated by *V. cholerae* infection, and whether these responses are beneficial or detrimental to the host, should be feasible in engineered newborn mice despite the immaturity of their adaptive immune systems.

Another key open area of investigation is *V. cholerae* host-to-host transmission. There is no controlled *V. cholerae* animal model of fecal-oral transmission. Approximate models of transmission such as transfer of infected intestinal homogenates or diarrheal fluid gavage have revealed a transient host-priming ("hyperinfectious") phenotype of host-derived *V. cholerae* [73,74]. The mechanism(s) underlying this phenomenon, which could be critical to understanding the explosive kinetics of cholera outbreaks, remains unclear. Adaptation of new barcoding frameworks in future studies of transmission in animals will facilitate understanding of how particular regions of the intestine, including the colon, contribute to host-priming [75]. It is likely that studies involving host-, pathogen-, commensal-, and environment-targeted permutations across differing animal models will be needed to understand the biology of cholera transmission.

The power of animal models for understanding *V. cholerae* is heightened by the broad and accessible genetic toolkit for this bacterium, including multiplex genetic engineering and numerous reporter systems. The continued integration of data from different models and implementation of cutting-edge genome-scale techniques from other fields, such as single-cell sequencing and transcriptomics, will be crucial to construct a holistic view of cholera pathogenesis and to reveal novel therapeutic opportunities against *V. cholerae* and other bacterial pathogens.

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Highlights

- Mammalian, non-mammalian, and invertebrate species are currently used to model *Vibrio cholerae* pathogenicity.
- Models vary considerably in their recapitulation of *V. cholerae*-host interactions in the human intestine.
- Recent work in animals has revealed molecular details of the host-*V. cholerae*-microbiome axis.
- Open areas for investigation include intestinal innate defense against *V. cholerae* and determinants of cholera transmission.

Table 1.

Major non-surgical oral infection animal models for *V. cholerae*.

	TCP-dependent colonization	CT-dependent diarrhea	ST colonization**	Genetic host manipulation	Throughput/ accessibility	Application(s)***
Mammalian						
M. musculus (infant mice)	+	+	+	++	++	Investigation of putative colonization factors
M. musculus (germfree/streptomycin-treated adult mice)	–	–	–	++	++	Adaptive immunity/ vaccination, commensal-pathogen interactions
O. cuniculus (infant rabbits)	+	+	+	+ (rare)	+	High-throughput and "omics" approaches
H. sapiens (human volunteers)	+	+	+	–	–	Clinical-stage investigations
Non-mammalian and Invertebrate						
D. melanogaster (fruit flies)	–	–*	N/A	+++	+++	Investigations of host defense and metabolism
C. elegans (nematodes)	–	–	N/A	+++	+++	Identification of putative accessory *V. cholerae* toxins
D. rerio (zebrafish)	–	–	N/A	+++	+++	Tissue imaging and commensal- pathogen interactions

* CT-dependent mortality

** SI as the primary site of pathogen colonization

*** Applications are not unique to models and indicate a representative use of the species

Scale: − (lowest) to +++ (highest) (columns with only +/− indicate presence or absence/unknown)

Table 2.

Future questions for animal models in the study of *V. cholerae.*

Pathogenesis and host response	• Why is *V. cholerae* primarily a small intestinal pathogen? • Why is TCP required for *V. cholerae* pathogenesis? • How does CT modulate host responses beyond diarrhoeagenesis? • How does *V. cholerae* intestinal colonization modify intestinal epithelial function? • What are the molecular pathways that underlie the host -induced 'hyperinfectious' transmission state?
Microbiome- *V. cholerae*	• How do the murine and human gut microbiomes prevent *V. cholerae* intestinal colonization? • Why does *V. cholerae* display colonic tropism in mice lacking intestinal microbes? • How does the gut microbiome control immune responses to *V. cholerae* and OCVs?
Innate Immunity	• What pathways of innate immunity contribute to protection against or resolution of cholera? • What is the role of the inflammasome and/or pyroptosis at the intestinal epithelial surface in defense against cholera? • What is the function of suspected innate immune cholera susceptibility loci such as *LPLUNC1*?
Adaptive Immunity	• Can a TCP-dependent animal model of adaptive immunity be developed? • What are the pathways of adaptive immunity and cellular populations that contribute to protection against cholera? Do they differ in naturally-infected versus vaccinated hosts? • Why do young children have poor memory responses to OCV immunization? • Can non-mucosal immunization routes confer long term immunity to cholera?