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Murine models to study *Clostridium difficile* infection and transmission

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Abstract

Clostridium difficile is the leading cause of antibiotic-associated diarrhea in healthcare facilities worldwide. *C. difficile* infections are difficult to treat because of the high rate of disease recurrence after antibiotic therapy, leaving few treatment options for patients. *C. difficile* is also difficult to contain within a healthcare setting due to a highly-transmissible, resistant spore form that challenges standard infection control measures. The recent development of murine infection models to study the interactions between *C. difficile*, the host and the microbiota are providing novel insight into the mechanisms of pathogenesis and transmission that should guide the development of therapies and intervention measures.

Keywords

Clostridium difficile; antibiotic-associated diarrhea; disease; transmission; spore; microbiota

1. Introduction

Clostridium difficile rapidly emerged in the past decade to become the leading cause of antibiotic-associated diarrhea in healthcare facilities worldwide. Much of the increase in incidence is attributable to fluoroquinolone-resistant *C. difficile* PCR-ribotype 027 variants [1, 2] although other distinct genetic variants, such as ribotypes 001, 002, 014/020, 017, 078 and 106, are endemic in many healthcare facilities and are capable of causing outbreaks [3]. The recent increase in the clinical and financial impacts of *C. difficile* infection (CDI) [4] has spurred renewed interest in studying the pathogenesis, persistence and transmission of this organism. Detailed studies of the mechanisms of pathogenesis are aided by the availability of robust animal models of infection. We will briefly review the history of the use of small animals to study CDI and discuss some of the recent work from our laboratories

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that have leveraged the availability of murine models to gain insight into the role of the pathogen, host and indigenous microbiota in disease pathogenesis and transmission.

2. History of Animal Models of *C. difficile* Infection

Koch's postulates for the role of C. difficile as the causative agent of antibiotic-associated colitis were initially fulfilled using Syrian hamsters as the susceptible host [5, 6]. The use of hamsters has been incredibly useful for understanding multiple aspects of the pathogenesis of CDI. The use of specific antibiotics targeting C. difficile and the use of microbiota transplantation was demonstrated using the hamster model [7, 8]. Hamsters have been used to demonstrate the potential utility of toxin-deficient strains of C. difficile as a preventive measure against CDI [9]. Other hosts have been used for the study of C. difficile pathogenesis. The use of these models has recently been reviewed in detail [10].

For many studies of infectious diseases and host biology the laboratory mouse has been a much-utilized platform. The use of murine models was initially investigated concurrently with the development of the hamster models. Recently however, there is a renewed interest in using murine models, spurred by our greater ability to manipulate mice as an experimental system and an extensive understanding of the immunology of these hosts. With the study of gastrointestinal infectious diseases in general and *C. difficile* infection specifically, there has been an appreciation that pathogenesis reflects the interaction between the pathogen, the host and the indigenous microbiota [11]. We will review how we have utilized murine models of CDI to study the role of each of these three interacting players in the pathogenesis of colitis due to *C. difficile*.

3. Microbial Ecology

3.1 Antibiotic effects on microbiota

Current thinking about the pathogenesis of CDI posits that hosts are intrinsically resistant to colonization and disease due to the presence of a diverse indigenous community of microbes [12]. The indigenous gut microbiota is thought to provide "colonization resistance" against *C. difficile* and other enteric pathogens through a variety of potential mechanisms. Disturbance of this intestinal community through the administration of antibiotics is thought to diminish colonization resistance leading to susceptibility to colonization and the development of clinical disease if exposure to *C. difficile* spores occurs.

We have used developments in the characterization of complex microbial communities through culture-independent, molecular surveys of sequences of the 16S rRNA-encoding gene to follow the dynamics of the gut microbiota following antibiotic administration [13]. We've observed that different antibiotics have distinct and reproducible effects on the community structure of the gut microbiome. Furthermore it appears that in some cases these changes are reversible once the drug is stopped but in other cases, persistent alterations in the microbiome are encountered. These studies in mice reflect observations made by others in human subjects and suggest that antibiotics can play a major role in shaping the structure and function of the gut microbiota [14-17].

3.2 Colonization resistance

Antibiotic-treated mice can be used to determine if specific alterations in the microbiome lead to susceptibility to see diff colonization and the development of colitis. Much of the recent interest in most models of CDI can be traced to the 2008 publication of a report from Ciaran Kelly's group in which they demonstrated that mice treated with a cocktail of five antibiotics administered in drinking water followed by a single intraperitoneal dose of clindamycin were susceptible to *C. difficile* colonization and the development of severe colitis [18]. Our groups have subsequently published reports where we have been able to confirm that this complex antibiotic regimen will lead to susceptibility to experimental CDI. However it is interesting to note that our laboratories have observed different results when we have used only clindamycin to confer susceptibility. In one report, clindamycin alone resulted in transient low level colonization by *C. difficile* which was rapidly lost within 2 to 3 days after experimental challenge [19]. Conversely, clindamycin can lead to long-term colonization and the development of high level shedding of the organism following subsequent re-challenge with the antibiotic [20]. Presumably, these differences reflect underlying baseline differences in the microbiota composition between the C57BL/6 colonies on either side of the Atlantic Ocean.

Infection of mice with epidemic *C. difficile* 027 spores in combination with clindamycin treatment leads to a persistent, chronic infection associated with a highly contagious state [21]. Such mice harbor a simplified intestinal microbiota that contains opportunistic pathogens and an altered short chain fatty acid profile. Whole fecal transplants effectively reestablished a diverse microbiota and eliminated *C. difficile* leading to the resolution of disease and contagiousness. This model provided a basis to rationally select a mixture of 6 phylogenetically diverse bacteria from the feces of healthy mice that could cure *C. difficile* disease in mice as effectively as whole-fecal transplants [21]. The mixture of 6 bacteria colonized the mice and also triggered the expansion of health-associated bacteria that were suppressed to low-levels during infection. Future experiments will focus on the exact mechanisms by which the mixture of 6 bacteria shift the balance towards a healthy, diverse microbiota to out-compete *C. difficile*.

3.3 Germ free mice and hypothesis testing

The study of antibiotic-treated mice that possess a complex intestinal microbiota have provided useful associations between specific members of the intestinal community and resistance or susceptibility to C. difficile colonization. In addition, the use of germ-free mice can provide a platform for hypothesis testing based on these results. Specifically, germ-free mice can be used to directly test interactions between *C. difficile* and specific cultivated members of the intestinal microbial community.

We recently employed germ-free mice to determine how a single bacterium isolated from the mouse intestine could interfere with subsequent *C. difficile* challenge [22]. Transfer of fecal pellets from conventional mice to germ-free animals fully restored colonization resistance, analogous to the result discussed above where fecal transplantation could eliminate the supershedder colonization state. Germ-free animals that received fecal pellets were not colonized with *C. difficile* following oral challenge. Colonization of germ-free

mice with an *E. coli* strain, which was able to establish levels of colonization up to 10^{12} colony forming units/gram of intestinal content, did not provide any protection from subsequent experimental *C. difficile* challenge. However, colonization of germ-free mice with a single member of the family Lachnospiraceae resulted in significant decreases in both *C. difficile* colonization and the severity of subsequent disease, providing evidence that this single organism could at least partially restore colonization resistance. It would be interesting to determine if the defined mixture of six murine bacterial isolates [21] would fully restore colonization resistance if transferred to germ-free mice.

4. Modeling Clinical Aspects of *C. difficile* Infection

4.1 Virulence

Human virulent variants of *C. difficile* are extremely diverse at the whole-genome level [23]. The availability of a murine infection model allows for comparison between *C. diffiicle* variants to investigate their intrinsic pathogenicity and associated host responses. We have demonstrated that antibiotic treated mice can be used to distinguish *C. difficile* strains based on the ability to colonize and cause disease. With regards to severity of disease, differences in the levels of toxin production *in vivo* appears to correlate directly with strains that produce greater levels of toxin causing worse disease. Furthermore, epidemic *C. difficile* 027 possesses enhanced transmissibility and increased persistence in mice compared to *C. difficile* 012 and 017 variants [21].

4.2 Recurrent disease

Recurrent *C. difficile* infection in humans is a severe outcome in 25-35% of patients that results in chronic diarrhea [24] linked to a pathological imbalance within the intestinal microbiota [25], termed dysbiosis. Disease recurrence occurs after cessation of antibiotic therapy to treat the initial disease episode, and can be caused by the original strain (relapse) or another strain (re-infection) [26]. Recent work has shown that mice can be used as a useful model to study the genetic and microbiological basis of recurrent disease.

Vancomycin treatment of mice shedding high levels of *C. difficile* leads to a rapid disappearance of culturable *C. difficile* in feces [21, 27]. Within 2-4 days after cessation of vancomycin treatment mice reproducibly begin to shed high-levels of the original *C. difficile* strain and the cycle of vancomycin-suppression and relapsing infection can be repeated multiple times with the same group of animals. Notably the mice experiencing relapsing disease harbored a dysbiotic microbiota that was likely the result bacterial suppression and elimination after repeated antibiotic treatments [21]. In contrast, fecal transplants can be used to restore a diverse, health-associated microbiota within mice experiencing relapsing disease and, as a result, *C. difficile* is eliminated and disease is cured [21]. These results demonstrate that *C. difficile* exploits the dysbiotic microbiota to cause recurrent disease in mice and supports the notion that treatments that restore a diverse microbiota and intestinal homeostasis are a valid option for patients with recurrent disease [28, 29].

During vancomycin treatment of *C. difficile* infected mice, *C. difficile* was not detected within the gastrointestinal tract of mice but was readily cultured from outside of the mouse (i.e. fur, paws, mouth region) and the local cage environment [27]. These observations imply

that the local environment may be an unappreciated source of *C. difficile* during relapsing disease. Further, *C. difficile spo0A* mutants that cannot make spores are unable to cause relapsing disease suggesting that after vancomycin treatment the mice are being colonized by environmental spores shed by the animals prior to treatment [27]. However, since the *C. difficile* spo0A mutants also exhibit other phenotypic defects, like elevated toxin production, further work with mutants that are defective in only spore production is needed to confirm this notion.

4.3 Spore-mediated transmission

C. difficile produces highly-transmissible, metabolically-dormant spores that are excreted from patients leading an environmental transmission reservoir [30]. The highly resistant nature of the spore promotes persistence within the healthcare system and challenges standard infection control measures. Indeed, exposure to a healthcare environment in combination with antibiotic therapy is a significant risk factor for acquiring *C. difficile* [31]. Recent work has demonstrated the murine models can be used to test various basic and applied aspects of spore-mediated transmission.

C. difficile spores can be purified from lab grown cultures to study their biology in the absence of vegetative cells [32]. Pure spores allowed for the first controlled calculation of the dose of environmental spores required to infect mice (5 spores/cm^2 during 1 hour exposure)[32]. This transmission model was the basis of evaluating the efficacy of commonly used disinfectants at blocking transmission between mice [33]. Surprisingly, many commonly used disinfectants found in a UK healthcare facility failed to inactivate pure spores and block environmental spore transmission despite the manufacturers claims to do so [33]. The results confirm many *in vitro*-based studies showing that strong oxidizing agents are superior at reducing or eliminating active environmental spores and are therefore the best candidates at containing *C. difficile* within the healthcare setting, the animal facility or laboratory.

The murine infection model has also been adapted to test various C. difficile transmission routes [27]. For example, mice infected with wild-type *C. difficile* were very effective at donors of transmission when mice were interacting (direct transmission) or were in the same cage but direct contact was blocked (airborne). *C. difficile* is extremely transmissible between mice! Interestingly, mice infected with a *C. difficile spo0A* mutant were poor donors of infection during direct transmission and were unable to transmit via airborne or environmental transmission [27]. Thus, the *C. difficile* spo0A gene is a transmission factor. Future experiments aimed at defining the downstream Spo0A signaling should provide insight into the mechanisms that *C. difficile* use to transmit between hosts and may provide targets for intervention.

5. Conclusions

C. difficile infections are challenging to treat and contain because of the limited treatment options and the persistent nature of the spore. The development and availability of reproducible, robust mouse models for *C. difficile* infection and transmission has begun to provide insight into the basic biology of this pathogen and should lead to more rapid

progress in this area. Such information should facilitate the development of pre-clinical models required to generate a deeper understanding of the pathogenesis of *C. difficile* infection to aid in the development of novel treatment and preventive strategies.

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