Gut Microbes 3:2, 145–167; March/April 2012; © 2012 Landes Bioscience

Models for the study of *Clostridium difficile* infection

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Keywords: *Clostridium difficile* infection, model, hamster, in vitro, mouse

Models of *Clostridium difficile* infection (*C. difficile*) have been used extensively for *Clostridium difficile* (*C. difficile*) research. The hamster model of *C. difficile* infection has been most extensively employed for the study of *C. difficile* and this has been used in many different areas of research, including the induction of *C. difficile*, the testing of new treatments, population dynamics and characterization of virulence. Investigations using in vitro models for *C. difficile* introduced the concept of colonization resistance, evaluated the role of antibiotics in *C. difficile* development, explored population dynamics and have been useful in the evaluation of *C. difficile* treatments. Experiments using models have major advantages over clinical studies and have been indispensible in furthering *C. difficile* research. It is important for future study programs to carefully consider the approach to use and therefore be better placed to inform the design and interpretation of clinical studies.

C. difficile Infection

Clostridium difficile (*C. difficile*) was first described in 1935 by Hall and O'Toole,[1] but it was 40 years before it was identified as the primary cause of pseudomembranous colitis, and shown to be the main organism isolated from the feces of patients with diarrhea undergoing clindamycin treatment.[1-4] These early studies and experimental animal model observations revealed *C. difficile* as a potential emerging pathogen capable of causing severe gastrointestinal disease in individuals undergoing antibiotic therapy. *C. difficile* infection (CDI) has increasingly become a problem in hospitals with outbreaks of infection which can be difficult to control.[5] CDI is a toxin-mediated intestinal disease, and extraintestinal manifestations are rare. *C. difficile* strains can produce two major toxins, termed A and B, which crucially mediate the intestinal inflammation and pathology characteristic of CDI. Some strains are non-toxigenic, i.e., do not produce either toxins A or B, while occasional strains produce toxin B but not toxin A. The latter appear capable of causing CDI. Strains that produce toxin A but not toxin B have not been convincingly described. *C. difficile* infection is thought to arise as a result of antimicrobial-mediated depletion of the normal gut flora allowing *C. difficile* proliferation and toxin production.[6] Clinical outcomes can range from asymptomatic colonization, through mild self-limiting diarrhea to more severe disease syndromes including abdominal pain, fever and leucocytosis. Fulminant or severe complicated CDI is characterized by inflammatory lesions and the formation of pseudomembranes in the colon. This can cause life threatening pseudomembranous colitis (PMC) which can result in toxic megacolon, bowel perforation sepsis shock and death.[5,6] Those at greatest risk for *C. difficile* tend to be those who have received antibiotic therapy, particularly the hospitalized elderly, those with serious underlying disease and patients undergoing surgery.[6,7] All antibiotics have been associated with *C. difficile*, but some carry a higher risk than others including clindamycin, cephalosporins and fluorquinolones,[7] while others (piperacillin-tazobactam, tigecycline) are rarely implicated in the disease. Symptomatic recurrences are a major problem in CDI patients:[8] either relapse due the original infecting strain, or re-infection with *C. difficile* spores.[8,9]

Despite the discovery of *C. difficile* as the etiological agent of PMC in the late 1970s, antimicrobial treatments for the disase have changed little in the intervening period. Antimicrobial treatments for CDI remain limited to oral metronidazole (400–500 mg three-times daily) or vancomycin (125 mg four-times daily).[10] Until recently, metronidazole was the preferred option for reasons of cost, and lower risk of vancomycin resistance selection. Early studies demonstrated little difference between metronidazole and vancomycin in terms of response or recurrence rates,[11,12] although response time was faster with the latter.[13] More recent reports of poor metronidaozle efficacy, particularly for disease attributable to apparently hypervirulent *C. difficile* PCR ribotype 027 (NAP1/BI),[14] and reports of reduced metronidazole susceptibility[15] have led to doubts over the efficacy of this drug. The need for new treatments for CDI, and the search for the mechanisms behind its pathogenesis mean that a number of experimental model approaches have been employed.

Models and *C. difficile*

Experimental animal models for *Clostridium difficile* have existed for over 30 years, with a number of studies using experimental clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in humans.[16-20] In vitro models have been also been used since the late 1970s and have developed in the intervening years from small test-tube fecal emulsions to

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Submitted: 10/14/11; Revised: 12/21/11; Accepted: 01/30/12
http://dx.doi.org/10.4161/gmic..19526

multi-stage chemostat models. Experimental studies using models have major advantages over clinical studies. In animal models these include the availability of study subjects, standardization of disease severity, the ability to perform invasive tests, extensive tissue sampling and the possibility to test prophylactic and novel treatment strategies. A wide range of animal models of *C. difficile* have been developed and these have been indispensable in providing insight into disease pathophysiology, including population dynamics, colonization and environmental factors and potential treatment options.[16,21-25] In particular, early studies of *C. difficile* using a hamster model enabled the link to be made between antibiotic associated colitis and the *C. difficile* toxins.[20,21,26,27] More recent work has substantiated the evidence in relation to the action of *C. difficile* major toxins, and further information on immune responses and associated pathology.[28-30]

Animal Models of *Clostridium difficile* Infection

Many different animal models have been used to study the mechanisms of *C*DI, including small animals such as hamsters, mice, rats, rabbits, hares, guinea pigs, prairie dogs and quails.[30,32-42] There have also been a number of limited descriptions of the use of larger animals including foals, piglets and monkeys.[43-45] Zebrafish embryos have also recently been used to examine the action of *C. difficile* toxins.[46] The hamster model is the most well described having been used for the greatest variety of studies including testing of new potential antibacterials, the effects of *C. difficile* on the immune system, prophylactic treatments and population dynamics.[16,30,47-50]

Hamster Models

Hamster models, in particular Syrian hamsters, have been most extensively employed for the study of *C*DI[2-4,16-21,26,27,29,30,32,51]. *C. difficile* in hamsters shares some of the recognized features of *C. difficile* isolated from humans, especially with respect to the susceptibility of the animal to infection following the administration of antimicrobial agents, but there are also key differences. In the hamster model, disease is induced by the administration of antibiotics which disrupt the normal gut flora, following infection with *C. difficile*, hamsters display many of the pathophysiological features seen in humans.[19] In addition to overall deterioration of the animal, there are changes in the appearance of the gastrointestinal tract in particular in the colon and cecum. Redness, inflammation, fluid accumulation and enlargement of the colon are usually seen, accompanied by a decrease in gut motility. If left untreated the disease is rapidly fatal in hamsters, and therefore, the endpoint of any experiment is the survival rate in days; actually, animal welfare legislation in some countries now means that animal euthanasia must occur before death caused by CDI. This rapidly and uniformly fatal disease pattern is not characteristic of human *C. difficile*, and a key drawback of the model is that hamsters do not typically develop diarrhea. They may occasionally develop "wet tail", in which the hamster displays symptoms of watery diarrhea, lethargy, irritability and refuses food, but invariably, this leads to death.[49,51] Thus, in the context of *C. difficile* treatment experiments, the hamster model is actually a prevention of death model.

The first hamster model was described by Small in 1968,[52] in which lincomycin hydrochloride was reported to cause fatal enterocolitis. Early studies with hamster models aimed to detect and characterize what was referred to as the "transmissible agent" in clindamycin-associated enterocolitis. A number of studies reported this transmissible agent to be a *Clostridium* species.[3,4,16,53] Bartlett et al.[27] reported that clindamycin-associated colitis in hamsters was due to a clindamycin-resistant, toxin producing clostridial strain; however, they conceived that this may not be the only agent or toxin responsible. These early *C. difficile* models enabled elucidation of the etiological mechanism for pseudomembranous colitis[20] and then the identification of specific therapies. **Table 1** shows the main uses of hamster models, which have included toxin and strain characterization, tests for new treatment strategies, studies into the population dynamics and colonization capacity of *C. difficile* in the intestinal environment, investigations into virulence determinants, the effects of different diets and characterization of immune responses.

Colonization Studies in the Hamster Model

Colonization and maturation of the infant gut flora following birth have been well studied using the hamster model.[54-57] It was hoped that the hamster model may explain the mechanism whereby *C. difficile* colonizes the intestinal tract of human infants in both the presence and absence of disease.[55] *C. difficile* colonizes around 50% of humans (depending on the extent of neonatal transmission) during the first few months of life,[58] but these infants remain asymptomatic despite large quantities of *C. difficile* toxin being present. Toxin production by *C. difficile* can be demonstrated in both healthy and symptomatic human infants and has been shown to be present in infant feces at a concentration similar to that found in the feces of adults with pseudomembranous colitis.[55] As the microflora within the infant intestine matures with infant age, *C. difficile* numbers decline, and by the age of two the gut flora resemble that of an adult without the presence of *C. difficile*.[59] A number of studies have used animal models to confirm the presence or absence of specific receptors for the *C. difficile* toxins. Work by Rolfe et al.[56] in hamster models showed that there was a specific receptor for toxin A in the adult and hamster brush border membrane, but they were unable to demonstrate a similar receptor for toxin B. They were also unable to demonstrate any differences in the binding of toxin A in the infant and adult hamster model. Eglow et al.[60] used a rabbit model and reported that newborn rabbit illeal brush border membranes do not possess specific binding sites for toxin A but noted that the number of receptors increased as the rabbit matured. In a further study by Rolfe et al.[61] it was confirmed that the receptors were present in the human infant intestine but that it was the presence of both unbound non-immunoglobulin and bound immunoglobulin fractions of human milk that inhibited the binding of toxin A to the purified receptor.

Role of *C. difficile* Toxins in the Hamster Model

Many papers describing the use of hamster models have investigated the action and role of *C. difficile* toxins.[29,50,62-64] Initial studies to characterize *C. difficile* toxins were performed using hamster models, in which it was demonstrated that the role of *C. difficile* toxins in causing disease was similar in hamsters and humans. Taylor et al.[65] reported the isolation of two toxins from *C. difficile* with physical and biological differences using models of hamsters, rabbits and guinea pigs. These findings suggested that the second toxin identified (toxin B) may have an important role in the clinical and pathological symptoms ascribed to *C. difficile.*

There has been considerable debate regarding the relative importance of each toxin; it was originally believed that toxin A was more important than toxin B.[66-69] These studies involved the administration of toxin A alone to hamsters which resulted in symptoms typical of *C. difficile*. In contrast, administration of toxin B did not elicit the same effect, unless intestinal damage was present or the toxin was co-administered with toxin A. This suggested there may be a synergistic effect, or that toxin B only had an effect following prior tissue damage by toxin A.

In 2009 Lyras et al.[29] and Carter et al.[64] provided evidence that toxin B was potentially the more important toxin and was essential for virulence. Lyras et al. used four independently derived toxin A or B mutants of *C. difficile*, which were characterized using a hamster model. The results demonstrated that toxin B was an essential virulence factor since disruption of the *C. difficile B* gene led to a significantly attenuated virulence phenotype. Those isolates which produced toxin B maintained a wild type phenotype, confirming the importance of toxin B in pathogenesis. Even the presence of toxin A at higher levels than toxin B was not lethal in the hamster model. Toxin A may still play an important part in the disease process, but appears from this study not to be an essential virulence determinant. This claim has major implications for future work on *C. difficile*. The authors suggested that it is important that studies are not solely performed on purified toxin and that the importance of the toxins in the context of the natural infection process is crucial to determining the role of toxins in disease.[29] Notably, however, a study by Kuehne et al.[70] reasserts the importance of both toxins A and B. In this study isogenic mutants of *C. difficile* producing either toxin A or B alone were shown to be able to cause fulminant disease in a hamster model of infection. Additionally, they also constructed a first ever double-mutant strain of *C. diffiicile*, in which both genes were inactivated; this completely attenuated bacterial virulence.[70] The conundrum surrounding the respective virulence of toxins A and B remains and may vary according to the host species. It should be noted that optimized treatment of human *C. difficile* using monoclonal anti-toxin antibodies may require the presence of high-affinity antibodies against both toxins.[71]

Animal models are particularly useful in the study of immunological aspects of *C. difficile* and imuno-modifying treatment. However, as the following studies highlight, animal models may show variation in host response, and there may be considerable differences between different species. The hamster model has been used to study the efficacy of various immunization strategies including inactivated toxin, antibodies to recombinant toxin A and B, and surface layer proteins (SLP).[28,72-74] Kink and Williams described production of antibodies to recombinant toxin A and B proteins and their oral administration to clindamycin-treated hamsters (1 mg/100 g body weight), which were then exposed to *C. difficile*. They found that antibodies to both toxins A and B were required to protect against *C. difficile*. They also noted that hamsters treated with antitoxin, in contrast to vancomycin, did not suffer relapse and were refractory to reinfection.[28] Giannasca et al.[72] used a series of passive and active dosaging regimens, including routes of administration, to evaluate the efficacy of toxoid vaccine preparations in a clindmycin-induced (0.5 mg) *C. difficile* hamster model. They concluded that rectal administration, in conjunction with intramuscular vaccination conferred full protection against *C. difficile*, whereas other mucosal routes did not. Passive immunization was also successful, in a dose-dependant manner and the authors underlined the importance of circulating antibodies in protection against *C. difficile*.[72] This confirmed earlier work investigating a formalin-inactivated toxoid vaccine which also indicated that a combination of mucosal and parenteral admininistration routes conferred the greatest protection against disease.[75] However, with the exception of rechallenge experiments by Kink and Williams, which ran for up to 70 d, the time courses of these experiments was fairly short (14–20 d). This means that conclusions about the protection afforded by these treatments in the long-term cannot be drawn.

O'Brien et al. showed significantly prolonged survival in clindamycin-induced *C. difficile* model hamsters treated with anti-surface layer protein (SLP) antibodies compared with control hamsters (given rabbit anti-maltose binding protein, serum or left untreated) and the authors linked this to enhanced phagocytosis. However, despite prolonged survival (median 157 h) all hamsters in all groups succumbed to infection within 2–5 d post-*C. difficile* challenge, with no difference in time to first symptoms.[73] This group subsequently examined SLP as a component of a vaccine administered to clindamycin-treated hamsters according to several different immunization regimens. Hamsters were challenged with 10^5 cfu *C. difficile* 14 d after the final immunization dose. All hamsters died within 48 h of *C. difficile* exposure, regardless of immunization route, presence of adjuvant and serum SLP IgG titer. A non-challenge BALB/c mouse model showed stronger antibody responses. The authors reported considerable variation in responses among both hamsters and mice.[74]

Induction Studies in the Hamster Model

Studies into the induction of *C. difficile* in hamsters have been performed by several groups.[17,26,48,76-79] Toshniwal et al. tested the effects of the orogastric administration of tetracyclines and observed that this caused diarrhea and death with evidence of hemorrhagic typhlitis.[17] They were able to culture tetracycline resistant *C. difficile* from the stools of the hamsters and reported that the lesions resembled those induced by clindamycin that have been attributed to *C. difficile* toxin.[26,27,80] Erbight et al.[78] tested the effects of 8 cephalosporins in a hamster model. The study concluded that poorly absorbed cephalosporins were

effective in preventing clindamycin-induced cecitis in hamsters as their minimum inhibitory concentrations for *C. difficile* were higher than those of metronizadole and vancomycin. However they did not consider them to be more effective antibiotics for treating cases of *C. difficile* associated disease.[78] They also observed that cepaholosporins induced *C. difficile* in hamsters, as in humans. It is noteworthy that they did not describe the administration of *C. difficile*, and this was perhaps left to chance rather than a controlled administration. Likewise, Onderdonk et al. did not describe how *C. difficile* was administered in their comparative study of the effects of clindamycin and its metabolites in a hamster model of antibiotic associated colitis.[81] They reported no association between antimicrobial potency and $AACD_{50}$ values, but it is possible that the likely uncontrolled *C. difficile* exposure could have influenced these results. The authors observed the reduction of de-*N*-methylclindamycin sulphoxide to the considerably more active de-*N*-methylclindamycin in hamsters. This had not previously been observed in rats and underscores some of the difficulties in comparing data between different animal models and crucially when extrapolating results to human *C. difficile*.

Larson and Borriello[77] compared various antibiotics for their propensity to induce *C. difficile* in hamsters. Infection occurred after very small doses of ampicillin, clindamycin, flucloxacillin or cefuroxime were administered and little difference in the degree of susceptibility they induced was seen. This was measured by

Table 1. Summary of animal *C. difficile* model studies

Aspect	Detail	Animal model	References
Development of model	Establishment of hamster as a model Initial toxin A and B observations and characterization experiments. Refinements in the hamster model	Golden Syrian Hamster	16, 26, 27 48 105 174
	Establishment of mouse model Refinements in the mouse model	C57Bl/6 mouse Mouse	36 115
	Evaluation of prairie dog model	Praire dogs	42
	Evaluation of piglet model	Germ-free piglets	43
	Evaluation of foal model	Pony foals	45
Toxin characterization	Reproduction of disease in hares	Gnotobiotic hares	43
	Comparison of two toxins produced by *C. difficile*	Golden Syrian Hamster	66 69 67
	Characterization of *C. difficile* toxins	Rat ileal loops Rabbit ileal loops	116 132
	Study to show toxin B essential for *C. difficile* virulence	Golden Syrian Hamster Chimeric mouse model	20 64 70 117
	Study to elucidate role of binary toxin CDT in rabbit and hamster models.	Golden Syrian Hamster; rabbit	177
	Elucidation of toxin activity	Guinea pig intestine	40
	Study to show toxin b essential for virulence of *C. difficile*	Hamster	29
	Clindamycin and tetracycline associated pseudomembranous colits	Golden Syrian Hamster	26, 27 80 81
	action of toxins	Infant Rhesus monkey zebrafish	44 46
Induction studies	cephalosporins	Golden Syrian Hamster	78
	Ampicillin, clindamycin flucloxacillin, cefuroxin	Golden Syrian Hamster	77
	Ampicillin, clindamycin	Golden Syrian Hamster	32
	Vancomycin, clindamycin, oritavancin	Golden Syrian Hamster	83
	Tigecycline	Mouse	147
	Tetracycline cepaholsporins	Golden Syrian Hamster	18 78
	aztreonam, cefoperazone, latamoxef and ceftazidime.	Golden Syrian Hamster	178
	Metronidazole, vancomycin, penicillin, ampicillin, tetracycline, cephalosporins, trimethoprim, sulfamethoxazole, clindamycin, erythromycin, aminoglycosides	Golden Syrian Hamster	19, 48

Table 1. Summary of animal *C. difficile* model studies (cont.)

Aspect	Detail	Animal model	References
Treatment tests	*Sacchromycees boulardii*	Golden Syrian Hamster Rat ileal loop Human microbiota-associated mouse	101 102 104 119 120
	Evaluation of Rifalazil vs. vancomcyin	Golden Syrian Hamster	93
	Rifamixin vs. vancomycin	Golden Syrian Hamster	39
	Human monoclonal antibodies directed against Toxins A and B to prevent *C. difficile* induced mortality in hamsters	Golden Syrian Hamster	63
	Eremomycin in treatment studies	Golden Syrian Hamster	179
	Treatment of *C. difficile* colitis in hamsters with lipopeptide (LY146032) daptomycin vs. vancomycin Daptomycin vs. teicoplanin	Golden Syrian Hamster	22 180
	Nitazoxanide vs. metronidazole and vancomycin	Golden Syrian Hamster	25
	Ramoplanin and vs. vancomycin	Golden Syrian Hamster	94
	tolevamer vs. metronidazole and cholestyramine	Golden Syrian Hamster	88, 89
	REP3123 vs. vancomycin	Golden Syrian Hamster	99
	Oritavancin vs. vancomyin	Golden Syrian Hamster	83
	Passive and active immunization strategies	Golden Syrian Hamster Golden Syrian Hamster and BALB/c mouse	28, 72 73 74
	DNA vaccine	BALB/c mouse	130
	cholestyramine, corticosteroids and atropine-diphenoxylate (lomotil)	Golden Syrian Hamster	19
	Anti-diarrheal properties of a novel sigma ligand	Male DBA 2 or NMRI mice	181
	APAZA inhibition of acute colitis	Rats	182
	Effect of bismuth subsalicylate	Hamsters	183
Colonization resistance	Cecal flora	Golden Syrian Hamster	82
	Bacterial translocation correlation with morphological changes of the mucosa	Golden Syrian Hamster and mouse	108
	Protection by *E. coli* or *Bifidobacterium bifidum*	mouse	121
	Role of *C. difficile* in NEC and protective effect of bifidobacteria	Gnotobiotic quails	34
	Suppression of *C. difficile* by hamster fecal flora Studies on interaction of hamster flora and *C. difficile*	Gnotobiotic mouse	122 184
	Colonization resistance	Axenic mouse	124
	Molecular characterization	Mouse	127
***C. difficile* spores**	Transmission and *C. difficile* host interactions	female wild-type, Igh6$^{-/-}$, or Myd88$^{-/-}$ mice with a C57BL/6 genetic background	126
	Evaluation of health care disinfection regimes	Mouse	125
	Population dynamics Colonization studies on different strains.	Golden Syrian Hamster	122 47, 51
	Involvement of bile salts in in vivo spore germination	Mouse	128
Population dynamics	Evaluation of toxigenic and non toxigenic *C. difficile* strains. Comparison of historical and current epidemic strains Studied interplay between toxigenic and non toxigenic *C. difficile* strains.	Golden Syrian Hamster	47, 110, 112 51, 49 109, 111
Relative virulence of *C. difficile* strains	Evaluation of several *C. difficile* strains Testing of strains from different sources	Golden Syrian Hamster	21
	C. difficile 630 vs. B1 (not NAP1/BI/027)	Golden Syrian Hamster	114
	High fat	Golden Syrian Hamster	33, 41
Diet effects	Soy Fiber	Golden Syrian Hamster	24
	Bacterial translocation correlation with morphological changes of the mucosa	Mouse	108
Immune response	Aged host response to infection with *C. difficile* NAP1/BI/027	C57BL/6 aged mouse	129

calculating 50% lethal doses in CFU for hamsters following antibiotic treatment administered at various intervals. Clindamycin administration induced a longer period of susceptibility to *C. difficile*. The authors suggested while the results could not be applied to humans, they provided further lines of investigation into how antibiotics may differ in terms of *C. difficile* risk. This was also the first study to attempt to control hamster exposure to *C. difficile*, which had previously largely been left to chance. By standardising the *C. difficile* strain and inoculum, and providing a sterile environment, the inherent problem of re-infection via contaminated environments was tackled. Larson and Welch drew on this work to examine colonization resistance in vivo and in vitro following clindamycin- or ampicilin-induced toxin production.[32] They demonstrated inhibition of *C. difficile* growth in hamsters and in hamster cecal contents in vitro by normal (non-antibiotic exposed) cecal contents. These inhibitory properties were lost upon filtering, freezing/thawing and dilution. The authors also reported longer inhibition in cecal contents of clindamycin- vs. ampicillin-treated hamsters, in agreement with the previous study. In a study demonstrating prevention of antibiotic-induced cecitis by administration of normal cecal homogenates, Wilson et al. used vancomycin (200 mg/kg) to successfully induce *C. difficile* in hamsters. Extrapolation from the drug elimination curve suggested that deaths from cecitis would have occurred when vancomcyin levels fell below the MIC of *C. difficile*, on days 6–10 after administration.[82] Similarly, in a recent comparative study of the propensities of oritavancin (50 mg/kg), clindamycin (100 mg/kg) and vancomycin (50 mg/kg) to induce *C. difficile* in the hamster model[83] vancomcyin and clindamycin-treated hamsters died within 6 d of treatment, demonstrating the potential of vancomycin to induce *C. difficile*. Only oritavancin-treated hamsters survived until the experimental end-point (day 20).

New Potential Antibiotics and Therapies for Treatment of *C. difficile* Infection Using the Hamster Model

The identification of new potential therapies for *C. difficile* is a research priority, and many studies have reported on the use of the hamster model for this aim (**Table 2**). Notable studies include the following. The macrolide antibiotics tiacumicins B and C were tested in vivo in a hamster model of antibiotic associated colitis. Administration of tiacumicins at 0.2, 1 or 5 mg/kg hamster body weight protected all clindamycin-treated hamsters (100 mg of clindamycin per kg of body weight intraperitoneally) exposed to *C. difficile*,[84] while vancomycin was only effective at higher doses. More recently tiacumicin B, now known as fidaxomicin (OPT-80 or PAR-101),[85,86] has shown good results in a phase III trial in comparison with vancomycin for the treatment of *C. difficile* infection. Recurrent *C. difficile* was significantly less frequent in fidaxomicin recipients (13.3% vs. 24%).[85]

The efficacy of rifaximin a novel non absorbed antibiotic was tested in a hamster model.[87] Hamsters were given subcutaneous clindamycin (10 mg/kg), 24 h later challenged with *C. difficile*, and then given either vancomycin (50 mg/kg) or rifaximin (100, 50 or 25 mg/kg) for 5 d. Rifaximin was reported to be as effective as vancomycin in the prevention and treatment of colitis caused by the two strains tested, and no relapse occurred with this agent. Vancomycin was associated with significantly more recurrences than rifaximin in *C. difficile* due to strain VPI 10463

Table 2. Aspects investigated using mouse models

Aspect	Detail	References
***C. difficile* infection cycle**	Mice were used to study the spore-mediated transmission and interactions between *C. difficile*, the host and microbiota. Molecular characterization of *C. difficile* spores Evaluation of healthcare disinfection regimes. Investigation of host immune response to *C. difficile* infection in an aged germfree mouse model.	126 127 125 129
***C. difficile* environmental interactions**	Gnotobiotic mice fed hamster fecal flora which suppressed infection with *C. difficile*. Protective effect of pre and probiotics. Interaction between the entire fecal flora of hamster and *C. difficile* investigated in a mouse model. Colonization resistance in an axenic mouse	122 108 184 121
Model development	Establishment of a mouse model of *C. difficile* disease.	36
Anti -diarrheal studies	Investigation of the properties of a novel sigma ligand which when given orally exerted a potent anti diarrhea effect on toxigenic diarrhea in mice.	181
Toxin studies	Investigation into the protection from *C. difficile* infection when mice were previously inoculated with *E. coli* or *Bifodobacterium bifidum*.	121
Treatment Tests	Testing of the hypothesis that tigecylcine has a low propensity to promote colonization and toxin production by *C. difficile* due to inhibitory activity in the colon. Inhibition of adenosine deaminase prevented *C. difficile* toxin A induced enteritis in mice	147 185

(75% vs. 0%, $p < 0.01$). Rifaximin has been used to treat *C. difficile* in humans although it is not licensed for this indication; there have been encouraging case reports notably in patients who have experienced multiple prior recurrences of *C. difficile*, although resistance emergence may be an Achilles heel.[87]

Tolevamer is a novel toxin binding polymer and was investigated for the treatment of *C. difficile* in a hamster model.[88,89] Studies involved challenging hamsters with *C. difficile*, giving clindamycin subcutaneously (10 mg/kg) 24 h later followed by tolevamer after a further 24 h. Tolevamer dosages of 500, 1000 and 1500 mg/kg/day led to survival rates of 90%, 70% and 70%, respectively. Tolevamer reduced the incidence of diarrhea and led to higher survival rates. The authors noted that there was no relapse of symptoms after discontinuation of dosing, compared with hamsters given metronidazole that died within 72 h of treatment cessation. The drug was also compared with an additional toxin binding compound cholestyramine; 80% of tolevamer treated hamsters survived compared with only 10% of those given cholestyramine.[88,89] The tissue histology of the ceca of hamsters treated with tolevamer appeared normal, compared with saline treated controls. These results were consistent with the non-antibacterial activity of the compound. Despite the promising hamster data, the clinical development of tolevamer was halted in 2008 due to the failure to demonstrate non-inferiority in comparison with metronidazole or vancomycin in phase III studies.[90,91]

Rifalazil a new benzoxazinorifamycin with activity against *Mycobacterium tuberculosis* and Gram-positive bacteria was compared with vancomycin for the prevention and treatment of clindamycin-induced cecitis in a hamster model of *C. difficile*.[92,93] Hamsters were injected subcutaneously with clindamycin phosphate (10 mg/kg), 24 h later challenged with *C. difficile*, and then either vancomycin (50 mg/kg) or rifalazil (20 mg/kg). No animals given vancomycin or rifalazil developed illness after 7 d. Within the ceca of rifalazil treated animals there was no demonstrable epithelial cell damage and significantly less edema and neutrophil infiltration when compared with vancomycin treated animals; hamsters relapsed following discontinuation of vancomycin but not rifalazil treatment. The authors suggested that once daily rifalazil may be effective for the treatment of *C. difficile*.[93]

Freeman et al.[94] tested the efficiency of ramoplanin (a novel glycolipodepsipeptide) and vancomycin in a hamster model and in an in vitro gut model (see section on in vitro models). Hamsters were given 10^4 cfu *C. difficile* by mouth and housed in germ-free conditions with HEPA filtered air. Symptomatic disease in the hamster was initiated by administration of 100 mg/kg clindamycin after 24 h. Ramoplanin and vancomycin were administered after a further 24 h and the ceca analyzed between 1 and 10 d following the clindamycin challenge. *C. difficile* symptoms (watery feces) were present in 33% of both ramoplanin- and vancomycin-treated hamsters; control hamsters were uniformly symptomatic (wet tail, watery or hemorrhagic cecal contents). All animals in both treatment groups were asymptomatic by day 4 and thereafter. On day 4, *C. difficile* total counts and spores were quantifiable in 75 and 100% of vancomycin-treated hamsters, respectively, but *C. difficile* was undetectable in all ramoplanin-treated animals. The spore persistence results were consistent with those generated in an in vitro gut model of human *C. difficile*. Ramoplanin was suggested to be superior at killing/inhibiting spores and thus may be more effective at preventing *C. difficile* occurrence due to spore recrudescence. Ramoplanin has completed a phase II clinical study in which 25/29 patients with *C. difficile* responded[95]; it remains unclear whether the hamster and gut model data showing spore suppression will translate into superior clinical efficacy.

Nitazoxanide (NTZ) is a thiazolide derivative, was initially developed as an antiparasitic drug, but is active against a variety of enteric parasitic pathogens including protozoa in humans and animals.[96-98] In vitro studies showed inhibition of *C. difficile* strains.[98] In vivo experiments were performed using a hamster model. Hamsters pre-treated with clindamycin but then only saline died within 60–70 h after challenge with 10^5 *C. difficile*; conversely, NTZ (15, 7.5 or 3 mg/100 g body weight), vancomycin (5 mg/100 g body weight) or metronizadole (15 mg/100 g body weight) prevented the rapid onset of disease. Most of the hamsters died within two weeks of stopping the treatment and it was unclear whether this was due to reinfection with an environmental strain of *C. difficile*, or possible relapse due to a failure to eradicate the existing infection. As a result of these experiments vancomycin was reported to be the most effective antibiotic, based on 3 out of 10 hamsters surviving, compared with none of those receiving MTZ.[25] These results are similar to those found in a study by Fekety et al.[48] comparing the effects of several other antibiotics to vancomycin and metronizadole. In this study similar contrasting efficiencies for vancomycin and metronizadole were reported, most likely as a result of vancomycin reaching higher concentrations in the gut than metronizadole.

Dong et al.[22] tested the lipopeptide antibiotic daptomycin (LY146032) in hamsters. Animals were administered 10^4 cfu *C. difficile*, then 5 d later they were given 1 mg clindamycin, followed by 5 d of treatment with either daptomycin or vancomycin (both 0.05 mg/day). The authors made no reference to the conditions under which the hamsters were kept. Administration of daptomycin delayed death in a hamster model and required a lower dose than that required for equivalent protection by vancomycin. The authors were unable to confirm a reason for the improved outcome, but suggested that it may be due to longer persistence of daptomycin in the gut or improved adherence to mucosal cells. Additionally, they suggested that there may be differential effects on intestinal flora, but substantive data are not available.

REP3123, a synthetic methionyl-tRNA synthtase inhibitor that inhibits toxin production and sporulation in vitro, was tested in a *C. difficile* hamster model[99] After a subcutaneous dose of clindamycin (50 mg/kg), followed 24 h later by *C. difficile* (3.2×10^7cfu/ml), REP3123 or vancomycin (at dosages of 5 and 0.5 mg/kg bd) was administered for 5 d commencing after a further 24 h. At both dosages REP3123 was more effective than vancomycin in terms of hamster survival up to the end of the study (day 33), although a maximum of only 75% of animals

survived.[99] Further study would be required to determine optimum dosage and whether these results could translate into effective treatment of human *C. difficile*.

Oritavancin is a lipoglycopeptide with good in vitro anti-*C. difficile* activity. Hamster and gut-model derived data both indicate anti-spore activity, and reduced risk of spore recrudescence/recurrence with oritavancin vs. vancomcyin. Marquis et al. described a comparative study of *C. difficile* induction by oritavancin (50 mg/kg) vancomycin (50 mg/kg) and clindamycin (100 mg/kg) in hamsters. All received 10^5 cfu *C. difficile* spores.[83] The study was controlled by including a vehicle-only (polyethylene glycol 400) group and hamsters given clindamycin (100 mg/kg) but no spores. The authors reported 100% survival of oritavancin-treated hamsters at the experimental endpoint compared with no survival by day 5 and 6 in vancomycin- and clindamycin-treated groups, respectively. Microbiological analysis of cecal contents found detectable levels of *C. difficile* toxin, spores and vegetative cells in only the clindamycin- (and spore inoculated) and vancomycin-treated hamsters. Investigations in a human gut model had previously indicated that oritavancin reduced spores to undetectable levels following clindamycin-induced germination and toxin production. In contrast, although total viable counts were reduced by vancomycin administration, spores remained.[100] The anti-spore activity of oritavancin is promising and further study of this compound continues.

Other Treatment Strategies Investigated Using the Hamster Model

The probiotic yeast *Saccharomyces boulardii* has been investigated as a potential exogenously administered agent for maintaining colonization resistance during antibiotic therapy.[101] *S. boulardii* decreased *C. difficile* associated cecitis and death in hamsters[101,102] and antibiotic associated diarrhea in humans.[103] A hamster model of recurrent *C. difficile* was described by Elmer et al.[38] The authors suggested that *S. boulardii* administration may help prevent extensive overgrowth of *C. difficile* and could be used to reduce the high incidence of relapse after vancomycin treatment in humans. In a more recent clinical trial, *S. boulardii* was administered to patients in combination with standard antibacterials for treatment of *C. difficile*;[104] the authors concluded that this regimen was successful for recurrent *C. difficile* disease but had no effect on an initial *C. difficile* infection. Of some concern, examples of cross infection have been reported (i.e., from a *S. boulardii* treated patient) highlighting the potential virulence of this yeast in humans. Furthermore, *S. boulardii* isolates obtained from varied commercial sources were found to differ in virulence in a mouse model, suggesting a lack of uniformity of yeast strains in such preparations.[105]

Diet alteration to modify intestinal flora has been investigated as a means of protecting against *C. difficile*. Frankel et al.[24] studied the role of a high fiber diet in the hamster model and found it delayed disease onset and prolonged survival from *C. difficle* ileocecitis. Increased intake of fiber by hamsters normalized the gut structure and improved absorption. Toxin positivity and stool liquidity were significantly reduced and survival times were improved by 34%. It is unclear if these findings are relevant to human *C. difficile*. A fiber rich diet may be beneficial for a patient at risk of *C. difficile*, although if this necessitated tube feeding, there may actually be an increased chance of infection.[106,107] In a similar study, Blankenship-Paris et al.[33,41] used a hamster model to investigate the effects of a high-fat diet, based on previous observations that hamsters fed a this type of diet were more susceptible to *C. difficile* enterocolitis. Hamsters were fed either a high fat or a control diet and then challenged with a toxigenic *C. difficile* strain. There was an increased rate of morbidity (80% vs. 11%, $p \leq 0.05$) in hamsters fed the high fat diet. *C. difficile* was recovered from the ceca of affected hamsters and the presence of toxins A and B could be demonstrated. In vitro tests showed that cecal flora from both high-fat treated and control hamsters inhibited *C. difficile* growth, suggesting that impaired colonization resistance was not a factor in disease development in this study.[41]

Naaber et al.[108] used a hamster model (and mouse model) to investigate bacterial translocation, intestinal microflora and the morphological changes within the intestinal mucosa as a result of *C. difficile*. The quantitative composition of luminal and mucosal microflora was evaluated as well as inflammatory changes of the mucosa and bacterial translocation into the blood, liver, spleen and mesenteric lymph nodes. They concluded that in mild *C. difficile* cases the extent of disturbance of intestinal microflora appeared to be more important in translocation than inflammatory activity in the mucosa. In fatal cases translocation was frequent with facultative species and *C. difficile* was present. The authors also investigated the protective effect of both probiotic (lactobacilli) and a prebiotic (xylitol) and reported that these were protective against *C. difficile* in these models.[108]

Population Dynamics and Colonization Studies in the Hamster Model

Hamster models have also been used in studies on population dynamics and colonization. Wilson et al. compared the transit of *C. difficile* spores and vegetative cells to a ^{51}Cr tracer in hamsters.[50] The majority of spores germinated within 1 h of inoculation (intragastric). Two independent forms of inhibition were seen: initial death of cells, which was unaffected by antimicrobial treatment, and a further inhibition of germination and proliferation, which was affected by antimicrobial treatment. Wilson et al. calculated dilution rates in hamsters and highlighted the similarity in dynamics between the hamster cecum and continuous culture systems. Wilson and Sheagren[109] studied the interplay between toxigenic and non toxigenic *C. difficile* strains. They induced colitis with clindamycin in a hamster model and found that administration of a non-toxigenic strain after 24 h was protective against a subsequent toxigenic strain administered 48 h later. Borriello and Barclay reported that prior colonization of clindamycin-treated hamsters (5 mg) with a non-toxigenic *C. difficile* strain provided protection against colonization by a toxigenic strain. Hamsters were housed individually and in a clean environment (autoclaved cages, food, water bottles and bedding). *C. difficile* inocula were administered orally and were

derived from overnight cultures of 4 *C. difficile* strains of varying toxigenicity. *C. difficile* inocula were not standardized (range 3×10^6–3×10^9 cfu) and spores were not enumerated. All hamsters given toxigenic *C. difficile* alone developed disease and died within 48h. In contrast, 13 of 18 hamsters receiving both toxigenic and non-toxigenic *C. difficile* survived to day 25. The authors drew attention to the fact that colonization by the non-toxigenic *C. difficile* strain did not confer full protection over the longer term, but highlighted the potential use of non-toxigenic strains.[110] Szczesny et al.[111] studied the interactions between toxin deficient strains of *C. difficile* and a toxigenic strain. They observed that toxin A or B deficient strains did not cause disease in the hamster, but when hamsters were subsequently infected with a toxigenic strain, clinical disease and death resulted. However on macroscopic and microscopic observation, histopathological changes were less evident following re-infection with the toxigenic strains.

Sambol et al.[47] also examined the use of non-toxigenic *C. difficile* strains to protect against *C. difficile* in hamsters. Animals were given clindamycin to induce colitis and then exposed to a non-toxigenic *C. difficile* strain 48 h later. This study involved higher initial dosing of clindamycin (30 mg/kg), compared with 7.5 mg/kg in the study by Wilson and Sheagren and an additional 24 h before the administration of a non-toxigenic strain. Also, hamsters were housed individually and more comprehensive testing was performed; toxigenic strains were administered after 5 and 40 d. Merrigan et al.[112] examined the protective effect of colonization with non-toxigenic strains during daily ampicillin or ceftriaxone administration. Hamsters were housed individually in isolator cages and were given antibiotics, followed by oral administration of 1×10^6 non-toxigenic cfu *C. difficile* spores 3 h later. All animals were colonized by day 3 (determined by culture of feces), and correlated with survival after challenge with toxigenic *C. difficile*. Data showed that the relative antibiotic resistance of the administered non-toxigenic strain (i.e., to the *C. difficile* inducing agent) affected the likelihood of successful colonization. Such issues will need to be overcome if this approach is to be translated into a successful prophylactic strategy against human *C. difficile* in patients receiving antibiotic therapy.

Characterization of Relative *C. difficile* Virulence in the Hamster Model

Hamster models have been used to characterize virulence determinants. Borriello et al.[21] studied a number of different toxigenic strains of *C. difficile* in hamsters to determine differences in virulence and try to correlate this to production of toxins A and B. They concluded that the high virulence of *C. difficile* was determined by efficient disease-inducing colonization of the gut, and the ability to rapidly generate high levels of toxin A in vivo. They postulated a number of reasons for the difference in virulence of different strains, including ability of different strains to associate with the gut mucosa. Alternatively, there may be an affect on the rate of repair of the gut epithelium, and that in order to cause disease the rate of toxin production must be more than a certain minimum such that the rate of tissue damage exceeds that of tissue repair. Human data are unavailable to confirm or refute this theory.

Sambol et al.[51] compared the virulence in hamsters of five different toxigenic strains of known human epidemiological importance. They demonstrated pathogenicity differences between toxin variant strains and standard strains but no observable differences among standard strains. They concluded that human epidemic *C. difficile* strains predictably cause disease in the hamster model, but suggest that that there may be host factors affecting the strain virulence, such as pre-existing antibodies to toxins (as shown by Kyne et al.[113]), but which are not present in the hamster model. This work was published prior to the emergence of *C. difficile* NAP1/BI/027 as strain associated with increased morbidity and mortality. Subsequent work by this group investigated historical and current epidemic BI (NAO1/BI/027) strains against toxinotype 0 isolates. The recent epidemic strain BI6 showed more rapid time to death from colonization, but there was little strain difference in time to death from challenge. The authors also noted high mortality with no prior signs of morbidity among hamsters infected with BI strains, consistent with clinical observations.[49]

Goulding et al. investigated the profiles of infection and pathology of *C. difficile* strains 630 and B1 (as distinct from 027/BI/NAP1), highlighting the usefulness of the hamster model in terms of ability to study host reactions during the disease process.[114] Study hamsters were of similar weight, and the environment highly controlled throughout the experiment. In addition to sterile cages, food, water and bedding, hamsters were re-housed in a sterile environment following infection with *C. difficile* spores and every 24 h thereafter. Environmental contamination was monitored using hamsters treated with clindamycin alone, alongside infected animals. Detailed histological analysis and electron microscopy of hamster gut, alongside quantitation of *C. difficile*, showed clear differences in disease pathology and time course between the two *C. difficile* strains. The B1 strain caused more severe pathology and shorter time to death, and larger numbers of bacteria were present at the mucosal surface and within non-phagocytic cells; strain 630 was more often found in the crypt regions.

Mice and Rat Models

In a similar manner to the hamster model, mouse models have been used extensively to study the pathogenic process of *C. difficile* infection as well as toxin production (**Table 2**). Mice have also been used to determine the role of environmental conditions in modulating infection. Models have included the conventional mouse model, gnotobiotic mice, monoaxenic mice and the human micro biota-associated mouse model.[115] There has been considerable study of specific toxin-mediated and immunological events in tissue explants or ileal loops originating in mice, rats and rabbits. Some important studies relating to the elucidation of relative toxin A and B activity are considered here, but many studies are highly specific and beyond the scope of this review. Rat ileal loop models have provided useful insights into the actions of toxins. Characterization of the differing actions of

C. difficile toxins A, B and an additional compound (which the authors called toxin C) were studied in the small intestine and colons of rats.[116] Fluid secretion in the small intestine was caused by all toxins, but only toxins A and C caused shedding of epithelial cells from the villi without visible damage to the crypt cells. In the colon, toxin A caused secretion and shedding of epithelial cells. The authors concluded that the differences in action of the various toxins may explain the differences in the broad spectrum of disease and intestinal disorders caused by *C. difficile*.[116]

Savidge et al. transplanted human intestinal xenografts into immunodeficient mice to create a chimeric animal model which was used to examine the actions of *C. difficile* toxins on human intestinal tissue.[117] This study questioned the traditional view that toxin B acts as a cytotoxin and toxin A as an enterotoxin. They were able to show that toxin B, as well toxin A induced intestinal epithelial cell damage, increased mucosal permeability and caused an acute inflammatory response. They concluded that *C. difficile* toxin B is a potent inflammatory enterotoxin for the human intestine, and suggest that in future, therapeutic or vaccine strategies for *C. difficile* infection need to target both toxins. An explanation for the differences in responses to toxin B seen in earlier rodent models and the humanized mouse model is the absence of toxin B specific receptors in the rodent intestine. Riegler et al. 1995[69] found a strong correlation between the sensitivity of cells toward the action of the *C. difficile* toxins and the presence of toxin-specific receptors. It appears that the mechanisms employed by toxins A and B require specific receptors to which the toxins bind as an initial step in the intoxication process. While the detail of the receptors is not known, toxin A binds to receptors on the apical surface of enterocytes and toxin B binds to receptors on the basolateral surface.[118] Tissue damage that disrupts the intestinal mucosa must first occur to allow access to the basolateral receptors. This may explain why purified toxin B, which does not bind to the apical surface, did not elicit symptoms in the hamster model unless prior intestinal damage was present. If toxin A compromises the epithelial barrier at the apical surface, this may explain why sub-lethal concentrations of toxin A potentiate the activity of toxin B. This work suggested that if mutants of toxin A are as virulent as the wild type, then possibly another protein or compound produced by *C. difficile* may be responsible for priming the intestinal mucosa.[64]

A rat ileal loop model was used to test the protective effect of a protease produced by the yeast *S. boulardii* for the prevention of *C. difficile* disease in humans.[119] The authors concluded that the protective effect was attributable to a yeast serine protease that hydrolized toxin A and inhibited binding of this toxin to its brush border glycoprotein receptor. This prevented the diarrhea and other intestinal effects of the toxin in the model. The relevance to the mechanism for possible prevention of *C. difficile* colonization in humans was not discussed. Further investigations into the influence of *S. boulardii* on fecal microbiota were made in a human-microbiota-associated mouse model.[120] Molecular analysis of the fecal flora using oligonucleotide probes demonstrated more rapid recovery from the deleterious effects of amoxicillin-clavulanate treatment to baseline levels in mice that had received *S. boulardii*.

A study by Corthier et al.[121] showed that it was possible to protect mice from inoculation with a toxigenic strain of *C. difficile* when they were previously inoculated with a strain of *E. coli* or *Bifdiobacterium bifidum*, although no mechanisms for this modulation were proposed. In a study by Wilson and Freter[122] the interactions between the entire fecal flora of hamsters and *C. difficile* (and *E. coli*) in gnotobiotic mice was investigated. The same interaction was also studied in a continuous–flow culture system. In each case the hamster flora suppressed the potential pathogens, the *C. difficile* population decreased, and the addition of the continuous culture contents decreased the size of the germfree mouse ceca back to normal size. It was suggested that an extract of the fecal flora contained a substance important for the colonization of the normal ceca flora. Results of a continuous culture model and the mouse mode concurred. Wilson and Perini[123] did not use a mouse model but studied the effects of using the entire cecal flora from mice for elimination of *C. difficile* from the mouse cecum. They suggested that as yet unidentified commensal organisms competed more efficiently than *C. difficile* for the available nutrients, and that substances produced by indigenous flora may decrease the growth rate even when a nutritionally rich medium was available. Studies have shown that *C. difficile* loses viability when introduced into hamster cecal flora or human feces,[31,123] suggesting nutrient depletion is not solely responsible for the effect. Boureau et al.[124] was able to isolate anaerobic strains from hamster flora which exhibited colonization resistance to *C. difficile*. These strains were unable to colonize the axenic mouse either alone or in combination, but were able to colonize a *Bifidobacterium bifidium* monoxenic mouse. They concluded that the contribution of enzymes from various strains were important for establishment of the colonization resistance effect.

A recent mouse model was described by Chen et al.[36] to overcome some of the problems associated with the current hamster models. They exposed C57Bl/6 mice to a mixture of antibiotics for 3 d. Clindamycin was administered 48 h later and mice were subsequently challenged with *C. difficile* of varying doses. They reported that the disease closely resembled human *C. difficile*, with typical histological features and would be valuable for testing of new treatment strategies and for future studies of pathogenesis. Disease in the mouse model varied in severity in accordance with the challenge dose. The mouse model offers potential benefits over hamsters as mouse specific reagents are more readily available and genetically modified animals are also more easily obtainable. Some more recent studies incorporating mouse models have extensively characterized *C. difficile* spores and the effect in the environment in relation to health care disinfection regimes.[125] Mice models were also used to investigate transmission of spores between immunocompetent and immunocompromised mice, the results of which may guide infection control strategies in the future. In this study Lawley et al.[126] showed that virulent *C. difficile* asymptomatically colonised the intestines of immunocompetent mice, and established a persistent carrier state. The authors demonstrated that high level *C. difficile*

shedding following induction ("supershedder" state) promoted efficient transmission in contrast to the carrier state, which did not. Infected immunocompetent mice suffered mucosal inflammation, but infection of immunocompromised mice led to severe fatal disease, underlining the involvement of host factors in the *C. difficile* disease process. The authors concluded that the mouse models provided a useful tool for studying the transmission of spores in relation to *C. difficile*. In a further study by the same authors, a mouse model was used to characterize the nature of the *C. difficile* strain 630 spores.[127] They established the highly resistant nature of spores and also demonstrated the low infectious dose required to establish infection in a mouse model ($\leq$ 7 spores per cm^2).

Spore germination was also investigated in the mouse model by Giel et al.[128] Spores were able to germinate to a greater degree in antibiotic-treated mice, but this was attenuated by treatment with cholestyramine, which can bind bile salts. The authors suggested that results of this study, which also showed that populations of cecal bacteria from antibiotic-treated mice were less able to modify taurocholate, a spore germinant, were further evidence for the role of bile salts in in vivo germination of *C. difficile*. An aged mouse model has also recently been used to study disease progression following infection with a *C. difficile* NAP1/BI/027 strain.[129] The authors described the production of previously undocumented cecal and colonic cytokines, highlighting the role of the host response in disease development. This study provides a novel model for investigation of the host immune response and its relation to aging.

Investigations of the administration route and efficacy of *C. difficile* toxoid vaccines were performed in both hamster[28,72,73] and BALB/c mouse models.[74] Ghose et al. also investigated transcutaneous immunization (TCI) of a toxoid A vaccine in Swiss Webster mice. As TCI is needle-free, it is a potentially attractive option for vaccine delivery. Good serum IgG levels, mucosal serum and stool anti-*C. difficile* toxin IgA levels and neutralizing antibody responses were seen following TCI with a toxoid A vaccine and C adjuvant.[130] Gardiner et al. vaccinated BALB/c mice with a DNA vaccine targeted to the receptor-binding region of toxin A and subjected them to intraperitoneal injection of toxin A over 2 weeks. The vaccine was immunogenic and protected mice against the effects of toxin challenge. The authors highlighted variation in immunogenicity on the basis of mouse strain and recommended further testing in other animal models.[131] Such observations highlight one of the drawbacks of the proliferation of new *C. difficile* animal models. Inter-model result variability raises uncertainly about which models are most predictive of human *C. difficile*.

Other Animal Models

Xia et al.[40] used a guinea pig model to study the effects of *C. difficile* toxin A to determine if stimulation of secretion and motility and the associated inflammatory response have a neurally mediated component. While a guinea pig model was described, the method involved removing segments of the small intestine, which were mounted into recording chambers where the effect of the toxin was measured.

Dabard et al.[37] showed that it was possible to reproduce disease experimentally in young axenic hares. They described the first proliferation of *C. difficile* disease in the digestive tract not induced by the ingestion of antibiotics; the significance of this observation remains uinclear. They concluded that *C. difficile* is the causal agent of neonatal diarrhea in conventional and gnotobiotic young hares, and that other clostridia enhanced its pathogenic effect. Additionally, they observed that *C. difficile* alone or associated with *C. perfringens* or *C. tertium* did not play any pathogenic role in young mice, rats or rabbits.

Triadafilopoulas et al.[132] used a rabbit ileal loop model and performed a number of experiments to study the effect of purified toxins A and B on intestinal secretion, epithelial permeability and morphology. After toxin A or B exposure intestinal permeability was measured by assessing the clearance of [3H] mannitol from the blood to the lumen. Toxin A (dose 5–100 mg/10cm illeal loop) caused a 3–5-fold increase in [3H] manitol permeability when compared with toxin B. Additionally more neutrophils were seen in the toxin A treated loops compared with the toxin B ones. Toxin A caused severe epithelial cell necrosis including destruction of villi and polymorphonuclear infiltration. By contrast, similar tests using toxin A on in vitro illeal explants revealed no effect on epithelial cell permeability, protein synthesis, release of alkaline phosphatase or morphology.

Interest in swine related *C. difficile* has increased markedly, noting the overlap with strains seen in humans. *C. difficile* has become the most commonly diagnosed cause of enteritis in neonatal pigs.[133-135] In a study by Steele et al.,[43] germ-free piglets were consistently and extensively colonized after oral challenge with *C. difficile* PCR ribotpye 027. Piglets inoculated with a non-toxigenic *C. difficile* strain showed no signs of disease. By altering the dose and piglet age, it was possible to induce either acute and severe systemic infection or milder chronic disease. Gastrointestinal and systemic symptoms were seen along with characteristic mucosal lesions of the large bowel. Additionally, *C. difficile* toxins (as measured by a cytotoxicity assay) were detected in feces, body fluids and serum, along with increased serum interleukin 8 levels, in piglets with severe disease. The piglets infected with *C. difficile* appeared to mimic many of the key characteristics observed in humans, and thus this model could potentially be exploited to investigate why some strains are associated with more severe *C. difficile*.

Prairie dogs have been described as potentially useful models in studies of gallstone formation and billary tract motility.[136] As their bile composition and extrahepetic billary anatomy are similar to that in the human, they have been described as a potentially useful model for *Clostridium difficile* induced colitis.[42] In the study the sample size was small (6 test and 6 control animals), but the results showed that the dogs developed pseudomembranous cecitis following a single dose of antibiotic, and survived for up to 4 weeks.[42] The practical issues surrounding the use of large numbers of dogs appear to have precluded further use of this approach. There is limited information on the utility

Table 3. Comparison of hamster experiments performed in testing new potential treatment

Treatment Test	Hamster housing / specific conditions	Pre-treatment	*C. difficile* administration	Dosing	Observations
REP3123 99	Individually, filtered, water and food available	(Day -1) clindamycin phosphate subcutaneously (50 mg/kg)	(Day 0) Oral gavage 0.5ml suspension ($3.2x10^7$ cfu/ml) ATCC 43596. Clindamycin resistant toxinogenic reference strain. (vegetative cells with 1% spores)	(Day 1) 5 and 0.5 mg/kg Twice a day for 5 d Vanocmycin or REP3123 given	Daily observations and necropsy at end of study (33 d)
Ramoplanin 94	Individually in cages with raised floors (to prevent coprophagia). HEPA filtered with food and water.	(Day 0) clindamycin administered (100 mg/kg)	(Day -1)10^4 in 1 ml *C. difficile* strain 421 given by mouth. Susceptible to vancomycin and ramoplanin (vegetative cells)	(Day 1) 50 mg/kg/day for 5 d Vancomycin or ramoplanin given	Serial sacrifices days -1, 1, 2, 4, 6, 8, 9, 10
Daptomycin (LY146032) 22	Caged in groups of 5	(Day 0) clindamycin administered (1 mg) intraperitoneally.	(Day-5) 10^4 *C. difficile* in 0.1 ml by orogastric intubation (vegetative cells)	(Day 1) 5, 0.5 or 0.05 mg/day vancomycin or daptomycin in 0.1 ml volume for 5 d.	Feces collected and toxin tests performed for 14 d.
Nitazoxanide (NTX) 25	Housed individually with food and water	(Day -1) 3 mg clindamycin	(Day 0) single dose 10^5 *C. difficile* strain 614. (vegetative cells)	(Day 1) 15, 7.5 or 3 mg/100 gbw NTZ (vancomycin or metronizadole) daily for 6 d.	Monitored daily for signs of disease. 35 d necroscopy performed.
Tolevamer 88	No mention	(Day 0) 10 mg/kg clindamycin subcutaneously	(Day-1) single dose of *C. difficile* (vegetative cells)	(Days -2 to 4) 1000 mg/kg/day tolevamer or cholestyramine	Observed for morbidity and mortality until day 7.
Rifaximin 39	Groups of two hamsters housed with food and water.	(Day 0) subcutaneous injection of clindamycin (10 mg/kg)	(Day 1) gavage with 10^5 CFU of reference toxinogenic *C. difficile* strain ATCC 43255 or hypervirulent epidemic strain toxinotype III (B117–6443). (vegetative cells)	(Days 2–6) Daily doses vancomycin (50 mg/kg) or rifaximin (100, 50 and 25 mg/kg).	Observed on day 7 or day 27 (or at death) the cecum was collected for histological evaluation.
Rifalazil 93	Groups of two hamsters housed with food and water.	(Day -1 subcutaneous injection of clindamycin (10 mg/kg)	Day 0 gavage with 10^5 *C. difficile* (preparation not stated)	DMSO (0.8%), oral vancomycin (50 mg/kg/day), or oral rifalazil (20 mg/kg/day) for 5 d	Observed three times daily. Moribund animals (according to pre-defined criteria) euthanized. Histological and microbiological analysis

of foals as a model for *C. difficile*. Arroyo et al.[45] described colonization of the gastrointestinal tract by *C. difficile* spores and vegetative cells, subsequent production of toxin and onset of clinical signs. Despite fulfilling Koch's postulates for *C. difficile* in foals, practical limitations have also limited further use of this model. In a study by Arnon et al.[44] infant rhesus monkeys were given *C. difficile* toxins A or B to investigate whether *C. difficile* causes a systemic illness, and sometimes death in infants, similar to that caused by *Clostridium botulinum* toxin. The animals showed no abnormalities for several hours but then developed lethargy, hypotonia, hypothermia, with subsequent breathing cessation. Post-mortem findings were insufficient to explain the cause of death, and no further *C. difficile* studies in monkeys have been reported.

Zebrafish embryos have been described as suitable models for investigation of damage to organs by *C. difficile* toxins. Zebrafish possess many of the major organs present in humans, and due to the transparency of the embryo, organ damage can easily be visualized and followed up by light microscopy (unlike in higher order animals). A study by Hamm et al.[46] found that the toxin B functioned as a potent cardiotoxin. These findings could be considered in the context of human disease, as patients with severe *C. difficile* may experience multi-organ failure, including impaired cardiac function.

Non-Animal Models

In vitro models of *C. difficile* infection. In vitro models of *C. difficile* have been in use since the late 1970s,[137] and have evolved into a practicable alternative to animal models in many respects. They offer the advantages of a greater control, greater numbers of replicates (in some cases), easier manipulation and elimination of the ethical issues surrounding animal models. In vitro models circumvent some of the practical and ethical issues inherent in animal models and also allow a high degree of experimental control. Investigations using in vitro models introduced the concept of "colonization resistance," evaluated the role of antibiotics in *C. difficile* development, explored population dynamics and most recently have been useful in the evaluation of *C. difficile* treatments (**Table 4**).[31,32,94,100,123,137,138] However, the interaction of host-related factors such as secretory or immunological events in the disease process cannot be represented.

Fecal Emulsions

Borriello and Barclay devised an in vitro model to study *C. difficile* behavior,[31] following the success of fecal enemas in treatment of recurrent *C. difficile*, and the antagonism of *C. difficile* growth by fecal emulsions from healthy adults.[31,139-141] Borriello and Barclay investigated *C. difficile* growth in fecal emulsions from healthy subjects, patients receiving antimicrobial treatment but without diarrhea, patients not receiving antimicrobial treatment but with diarrhea, and patients with AAD and with proven *C. difficile*. *C. difficile* growth and cytotoxin production occurred in fecal emulsions from *C. difficile* patients, but less so in fecal emulsions derived from other patient groups (infants, children and geriatric patients); there was no growth in fecal emulsions from healthy individuals. This inhibition was removed from fecal emulsions by heating or filter sterilization. They attributed this to viable components within the fecal emulsion, known as "colonization resistance." underlining the possible involvement of the human gut microbiota in preventing *C. difficile*.

The credibility of this model was scrutinized by comparing its predictive value against that of the hamster model.[77,142] In these experiments, hamsters were treated with various antibiotics, and then challenged with *C. difficile*. A parallel set of antibiotic-treated hamsters were killed and their cecal contents used in the in vitro colonization model. There was a close correlation between in vivo and in vitro results. All the antibiotics tested (clindamycin, ampicillin, flucloxacillin and cefuroxime) rendered hamsters susceptible to small doses of *C. difficile*. However, the window of susceptibility varied greatly between antibiotics and was not necessarily dependant on the presence of detectable antibiotic. Larson and Welch investigated *C. difficile* toxin production in cecal suspensions from untreated and clindamycin-treated hamsters. They demonstrated high level toxin production in cecal contents of clindamycin-treated hamsters in vitro, but not in untreated hamster cecal contents. Toxin production was not seen in a 1:1 mixture of treated and untreated cecal contents.[32] Following reports of *C. difficile* due to an emerging *C. difficile* strain showing increased fluoroquinolone resistance, Adams et al. used the cecal contents of a female CF-1 mouse model to examine epidemic and non epidemic *C. difficile* strain growth and toxin in response to fluoroquinolone exposure.[143] The authors based the model on the earlier fecal emulsion model described by Borriello et al.[31] Levofloxacin, moxifloxacin, gatifloxacin, ciprofloxacin, ceftriaxone and clindamycin were administered subcutaneously in doses equivalent (in mg/g body weight) to that given to humans over a 24 h period. Cecal contents were harvested after animal sacrifice and challenged with 10^4 cfu/mL *C. difficile* prepared from an overnight culture. They reported that emergence of epidemic fluoroquinolone resistant *C. difficile* strains may be promoted by fluoroquinolones in this model. Pultz and Donskey[144] showed that clindamycin, piperacillin-tazobactam and ceftriaxone promoted growth and toxin production by *C. difficile* strains in mouse cecal contents, while levofloxacin, cefepime and aztreonam did not. They were able to demonstrate detectable concentrations of the former three antimicrobials 3 d post-treatment, while the latter were undetectable by this time. These observations only partly support the colonization resistance theory in humans; for example, piperacillin-tazobactam (in contrast to clindamycin and ceftriaxone) has been shown to have a lower risk of *C. difficile* in humans,[145] despite causing significant disruption to fecal flora.[146] A similar study reported that tigecycline did not promote toxin production or colonization of mouse cecal contents, alone or in combination with clindamycin.[147] This is in line with previous results from an in vitro gut model reporting a lack of germination and toxin production following tigecycline dosing,[148] and other clinical evidence of a low *C. difficile* risk.[149] This group also used the same model to show that cecal contents from mice treated with parenteral ampicillin or piperacillin developed

Table 4. Comparison of in vitro models of *C. difficile* infection

	Test tube fecal emulsion 31	Test tube animal cecal emulsion 32,77,142-144,147,150	Batch fermentation 151	Continuous flow culture model 122,123	Continuous flow culture model 137	Continuous flow culture model—infant feces 165	Triple stage chemostat gut model 94, 100, 138, 146, 148, 151, 157–164	Single stage chemostat gut model 170
Fecal inoculum	Healthy adults, healthy children, geriatric patients; breast fed infants; bottle fed infants; AAD patients; non AAD patients; hamster cecal	Cecal suspensions from Clindamycin treated hamsters or mice	Feces (source not stated)	Extract of germ-free mouse fecal pellet + hamster cecal flora	BHI	Single frozen feces from healthy infants < 3yrs age	Pooled feces from healthy elderly volunteers	Pooled from 2 donors (aged 25 and 60yrs)
tableNature of system	batch	batch	batch	Single stage continuous	Single stage continuous	Single stage continuous	Triple stage continuous	Single stage continuous
Volume	10 ml	250 ul	230 mL	7 ml	Not stated but probably 300ml	100 mL	280 + 280 + 300	200 mL
Fecal dilution factor	1:20 in pre-reduced sterile distilled water	1:5 in sterile phosphate buffered water	1:10 in pre reduced phosphate buffered saline	Equal volume of balanced salt solution added to washed fecal pellet	N/A	1:5 in Gifu Anaerobic Medium (GAM) broth	1:10 in pre-reduced sterile phosphate buffered saline	1:5
C. difficile inoculum	10^8 cfu/mL total count 10^2 cfu/mL spores	$10^{4\text{-}6}$ cfu/mL	$2x10^6$ cfu from overnight culture	Not stated	Not stated, but probably 10^8 cfu/mL total count	10^5 cfu/mL total count	10^7 cfu/mL *C. difficile* spores	10^6 cfu/mL *C. difficile* total count
Duration of experiment	72 h	48 h	48 h	2 weeks	72 h	17 days	6–8 weeks	24 h
Sampling frequency	24 h	once	0,6,24,36,48	Not stated	0,2,4,6,8,24,48,72	0, 10, 17d	24 h	4 h
Antimicrobials/ compounds/ conditions evaluated	Stools from antibiotic treated patients or hamsters used	clindamycin, ampicillin, flucloxacillin, cefuroxime, levofloxacin, moxifloxacin, gatifloxacin, ciprofloxacin, piperacillin ± tazobactam, ceftriaxone, cefipime, aztreonam, tigecycline, β-lactamase - treated hamsters	Bacteriophage ΦCD27	supression of *C. difficile* by fecal/cecal flora; nutrient competition	vancomycin, penicillin, clindamycin, temperature, Eh	Amino acid utilization and volatile fatty acid production by fecal flora and *C. difficile* inhibition	clindamycin, cefotaxime, piperacillin-tazobactam, ciprofloxacin, levofloxacin, moxifloxacin, tigecycline, metronidazole, vancomycin, ramoplanin, tolevamer, mecillinam, NVB302, linezolid population dynamics, resistance development	vancomycin, metronidazole, lacticin 3147, thuricin C. difficile
Dilution rate	No dilution	No dilution	No dilution	0.157 h^{-1}	0.085 h^{-1}	0.05 h^{-1}	0.015 h^{-1}	Not stated

C. difficile overgrowth and toxin production following challenge with the organism, in contrast to cecal contents of mice given parenteral ampicillin or pipercillin with oral tazobactam-inactivated β-lactamase. This treatment strategy was relatively sparing of gut flora, but the authors noted that the reality of polypharmacy in many patients could diminish the efficacy of this approach in humans.[150]

Batch culture systems have, to some extent, been replaced by continuous culture models which are more gut-reflective. However, Meader et al. recently used a 48 h batch fermentation model in which to evaluate the effects of bacteriophage treatment upon *C. difficile*.[151] The authors used fresh fecal slurries, diluted in growth media and inoculated with an overnight culture of *C. difficile*. A *C. difficile* growth control was compared with a remedial treatment (phage introduced 6 h into the experiment and also at 24 and 36 h) and a prophylactic treatment (phage introduced at 0, 6, 24 and 36 h). The authors reported a significant decrease in mean *C. difficile* total counts ($p = < 0.0001$); however, it should be noted that in real terms this difference was, in fact less than 1 log cfu/mL. Prophylactic phage administration resulted in a more marked decrease in recoverable *C. difficile* [151].

Continuous Culture Models

Fecal and cecal suspension/emulsion models are batch culture systems, whereas conditions within the gut are more analogous to continuous culture systems.[50] Batch culture bacterial growth dynamics differ significantly to those found in continuous culture. Simple studies by several investigators into the effects of antimicrobials upon *C. difficile* toxin production in batch (broth) culture have produced conflicting results[152-156] and therefore researchers turned increasingly to more complex continuous culture systems to more accurately represent the conditions within the gut. Although Borriello and Barclay described their in vitro test tube fecal emulsion model of *C. difficile* in the mid 1980s, researchers were, in fact, already examining the behavior of *C. difficile* under continuous culture conditions as early as 1979.[137] Continuous culture systems not only allow the study of the organism in a more reflective environment but also enable controlled manipulation and monitoring of that environment over considerably longer time periods than in batch culture experiments. They have therefore been used to evaluate several possible factors in *C. difficile* development, including environmental stresses, antimicrobial agents and commensal microflora competition [32,94,100,122,123,137,157–165]. Onderdonk et al. examined the effects of environmental stress on *C. difficile* toxin levels in continuous culture.[137] They showed that changes in Eh and temperature caused increased levels of *C. difficile* toxin during continuous culture, and also demonstrated increased toxin in response to sub-inhibitory concentrations of vancomycin or penicillin. Onderdonk et al. suggested that release of toxin by *C. difficile* was affected by changes in environmental conditions (temperature, Eh or sub-inhibitory antibiotics), and indicated the potential contribution of continuous culture systems to the field of study.

Hamster model studies had indicated that a stable, normal gut flora forms a natural barrier to *C. difficile* infection.[31,109,141] The few continuous culture studies of *C. difficile* therefore focused on further attempts to elucidate those components of the fecal/cecal flora responsible for colonization resistance.[122,123,166] Wilson et al. reported that hamster flora grown in continuous culture or in germfree mice suppressed *C. difficile* growth in germfree mice. Moreover, they also showed the ability of components of hamster cecal flora to suppress *C. difficile* in both murine and continuous flow culture experiments.[122] A further study by this group investigated the role of nutrient competition in *C. difficile* using the continuous culture model, and found that *C. difficile* growth was slowed in the absence of glucose, *N*-acetylglucosamine or *N*-acetylneuraminic acid. The authors suggested that competition for nutrients by the colonic microflora could suppress *C. difficile* growth. However, the study did not encompass any characterization of fecal flora components, and was performed using hamster rather than human-derived floras.[123] These issues were addressed to a certain extent by Yamamoto-Osaki et al.[165] who expanded on earlier work[122] that examined the effect of infant fecal flora on *C. difficile* growth. They compared *C. difficile* positive and negative feces for their ability to inhibit *C. difficile* growth and noted that *C. difficile* counts decreased in the presence of flora from *C. difficile* negative feces. Contrastingly, when *C. difficile* was re-introduced to flora from *C. difficile* positive feces, counts of the organism increased. The authors also suggested that inhibition of growth of *C. difficile* may be due to competition for amino acids by the normal flora. Amino acid analysis of the continuous flow cultures with intestinal strains (as opposed to whole microfloras) showed depletion of several amino acids, and replacement of these elicited *C. difficile* growth. However, it should also be noted that this system was not pH controlled and experienced considerable decreases in pH which were also associated with inhibition of *C. difficile* growth. In common with later researchers examining the diversity of fecal flora among human volunteers[166], they noted a greater diversity of bacterial species in *C. difficile* negative vs. positive feces.

Triple Stage Human Gut Model

A more recent series of studies has used a triple-stage chemostat model of the human gut to investigate the relationship between antibiotics and *C. difficile* in a series of experiments [94,100,157–164]. The model consists of three anaerobically-maintained and pH-controlled vessels in a weir cascade series and top fed with growth media at a specific flow rate (**Fig. 1**). The system was first described by MacFarlane et al. in 1998 and was designed to reproduce the spatial, temporal, nutritional and physicochemical characteristics of the proximal to distal bowel. The model was validated against intestinal contents of sudden death victims.[167] Gut bacterial populations are introduced via a slurry comprising pooled feces from healthy volunteers, which are allowed to equilibrate over a 2 week period, in common with the earlier continuous culture systems of Wilson and Perini [122,123]. This study series includes experiments running for 6–8 weeks, with daily

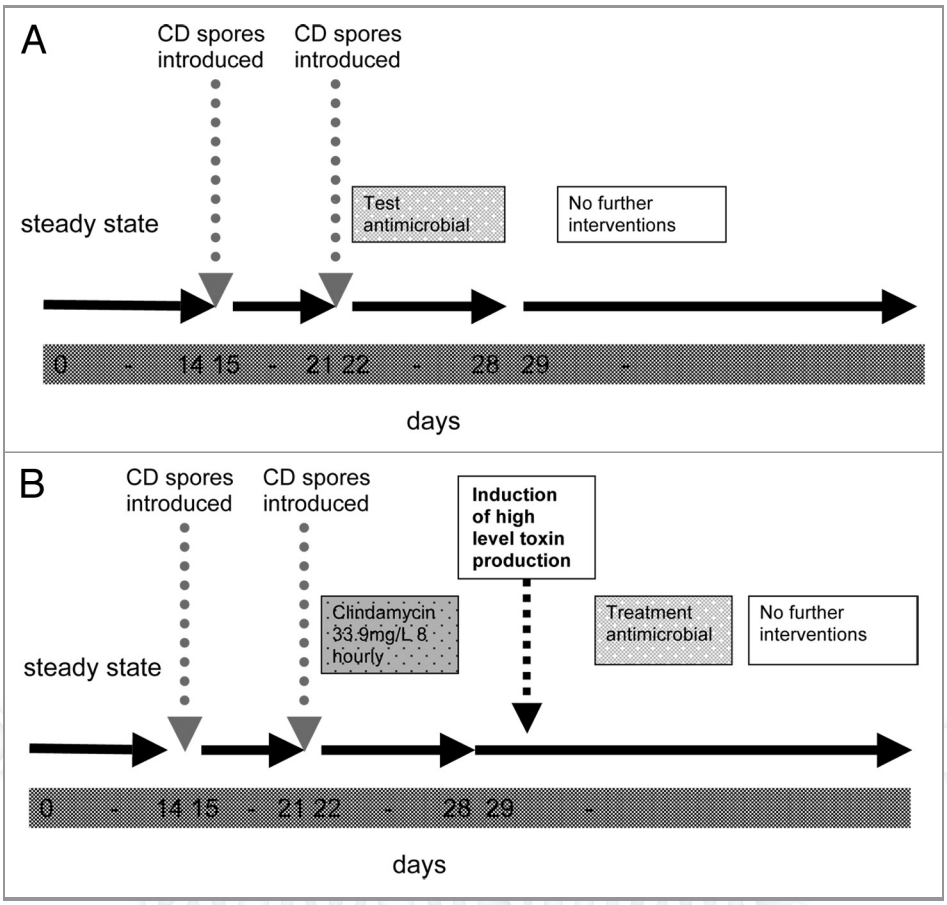

Figure 1. Schematic representation of triple stage gut model experiments. (A) Approach to assessing potential predisposition to *C. difficile*. (B) Approach to assess *C. difficile* treatment efficacy

monitoring of *C. difficile* vegetative cells, spores, toxin and gut flora components in all vessels, as well as determination of antimicrobial concentrations. Two main experimental designs have been used, one for evaluating potential predisposition to *C. difficile*, and the other to assess *C. difficile* treatment efficacy (**Fig. 1A and 1B**).

Previous studies had only examined single doses of antimicrobials and at concentrations derived from the MIC relative to test *C. difficile* strains. This is not necessarily reflective of the environment surrounding *C. difficile* in the gut. Contrastingly, in the triple-stage gut model experiments, antimicrobials are instilled into the model at fecal or biliary levels and according to a clinically reflective dosing regimen. The gut model has been used to examine antimicrobials for their propensity to induce *C. difficile*[146,148,157,161] their efficacy as *C. difficile* treatments,[94,100,138,158,159,162,163] to examine emergence of resistance,[164] and relative *C. difficile* strain fitness.[160,162]

C. difficile Induction Studies in the Gut Model

Control experiment data showed that *C. difficile* spores remain in their quiescent state in the absence of antimicrobial pressure. Studies examining the propensity of antimicrobials associated with clinical *C. difficile* (cefotaxime, clindamycin, fluoroquinolones) demonstrated that drug exposure was followed by sustained *C. difficile* germination and high level toxin production.[94,138,158,162,164] Conversely, antimicrobial exposure to piperacillin-tazobactam, tigecyline or mecillinam did not precipitate *C. difficile* germination or high level toxin production;[146,148,157] these results are consistent with clinical data showing a lower risk of *C. difficile* with these antibiotics. Sampling for antimicrobial levels within an in vitro system is much simpler and can be performed more frequently than in animal models. Antimicrobial bioassays were therefore performed for many of these studies.[138,146,148,157-159,162-164] They showed that *C. difficile* remained in its quiescent spore form until antimicrobial concentrations fell below the MIC of the organism, germinating and producing toxin thereafter. This interesting observation extends similar results in both hamsters[78,82] and in vitro continuous culture[137] that suggest antimicrobial stimulation of *C. difficile*.

C. difficile Treatment Studies in the Gut Model

This model has also been used for the evaluation of existing and novel treatments for *C. difficile*. In these experiments clindamycin was used to elicit *C. difficile* germination and

high level toxin production prior to treatment with the test compound, dosed according to a clinically reflective regimen. Metronidazole and vancomycin were both evaluated against epidemic *C. difficile* strains.[158,162] Comparison with the results of a previous gut model study showed that vancomycin was more effective in reducing *C. difficile* PCR ribotype 027 numbers and cytotoxin titers than metronidazole.[158] Evaluation of metronidazole treatment of simulated *C. difficile* demonstrated unexpectedly low drug concentrations in distal vessels of the gut model; it was postulated that low drug concentrations may have been caused by fecal inactivation of metronidazole, possibly mediated by enterococci.[162]

Novel treatments for *C. difficile* which have been evaluated in the gut model include ramoplanin, oritavancin, linezolid, tolevamer and other investigational compounds.[94,100,138,159,163] In the case of ramoplanin, the results of the in vitro gut model experiments were concordant with in vivo hamster experiments performed as part of the evaluation. Both ramoplanin and vancomycin treatment reduced cytotoxin production in the gut *C. difficile* model and resolved symptoms in the hamster model. Notably, however, vancomycin was associated with greater persistence of *C. difficile* spores in both the hamster and gut models. *C. difficile* spores were recovered significantly more often from the cecal contents of vancomyin-treated (n = 19/23) compared with ramoplanin-treated (n = 6/23) hamsters ($p < 0.05$).[94] The results indicate that ramoplanin is more effective than vancomycin at inhibiting spores and thus preventing spore recrudescence. The novel lipoglycopeptide oritavancin was also effective in treating clindamycin-induced *C. difficile* in a human gut model; the comparator, vancomycin, was associated with *C. difficile* recurrence (repeat germination and toxin production). Oritavancin demonstrated greater inhibition of spores, and again may prevent recrudescence of *C. difficile* spores.[100] Data from a recent study of oritavancin, vancomycin and clindamycin in hamsters also support these observations.[83]

Tolevamer is a novel non-antimicrobial styrene derivative that binds to and neutralizes the toxic effects *C. difficile* toxins A and B in vitro. Early in vitro, hamster model and phase II clinical trial data were encouraging. However, evaluation of tolevamer as a treatment for simulated *C. difficile* in the gut model showed that neither the duration nor magnitude of cytotoxin activity was reduced by the agent.[138] These data supported Phase III clinical trial results in which tolevamer failed to meet its primary endpoint of non-inferiority to vancomycin.[91] An evaluation of linezolid for treatment of simulated *C. difficile* in the gut model concluded that linezolid may reduce toxin levels, but not viable counts and was associated with spore recrudescence in one model.[159] Examination of the efficacy of the novel antibiotic NVB302 in treatment of simulated *C. difficile* in the gut model demonstrated non-inferiority of NVB302 to vancomycin in terms of reducing cytotoxin levels and total *C. difficile*, with no evidence of spore recrudescence.[163]

Rea et al. recently evaluated the bacteriocin thuricin C for its effects on *C. difficile* using a single vessel distal colon model. Their approach had several drawbacks in contrast to that of the triple stage model antimicrobial evaluations. Unlike previous continuous culture *C. difficile* models, there was no steady-state period during which fecal bacterial could equilibrate. Additionally, the short study duration (24 h) meant that it was not possible to draw clear conclusions concerning antimicrobial metabolism, effects on gut flora components or the potential for recurrence.[168]

Indigenous Gut Microflora Changes

The data obtained from gut model experiments also allow the comparison of gut flora changes in response to antimicrobials. Neither gut flora components nor antimicrobial effects upon them had been extensively evaluated in previous in vitro models, despite the establishment of the colonization resistance theory. Interestingly, gross depletion of gut flora components (total facultative aerobes, total anaerobes, lactose fermenters, enterococci, clostridia, bacteroides spp, bifidobacteria and lactobacilli), for example as seen with piperacillin-tazobactam and tigecycline, did not lead to *C. difficile* germination.[146,148] In contrast, cefotaxime instillation produced modest decreases in bifidobacteria and bacteroides populations but was nevertheless accompanied by *C. difficile* germination and high level toxin production.[161] Similarly, although clindamycin had a markedly deleterious effect upon gut flora, numbers invariably re-established to pre-drug levels before *C. difficile* germination and toxin production occurred.[94] This suggests that either those components responsible for colonization resistance were not assayed for, or that the role of the gut flora in moderating *C. difficile* is more complicated than previously thought.

C. difficile Population Dynamics and Resistance Development

C. difficile PCR ribotype 027 remained in a vegetative form and producing toxin for substantially longer than *C. difficile* PCR ribotype 001 in human gut model experiments. Such data are consistent with the presence of an 18 bp deletion in the putative negative regulator for toxin production in *C. difficile* PCR ribotype 027 and may explain the increased severity of symptoms associated with this *C. difficile* strain.[162] These observations part-contradicted and extended those of Warny et al. who reported higher peak median toxin production by NAP1/BI strains in vitro than median toxin production of a group of 12 other PFGE types.[169] Gut model continuous culture techniques showed that there was neither an increase in rate of toxin production by *C. difficile* PCR ribotype 027, nor in the titer of toxin present in the gut model at any given time; instead the duration of toxin production by *C. difficile* 027 was markedly longer than that of *C. difficile* PCR ribotype 001. These discrepancies highlight the drawbacks of using simple batch culture techniques to study *C. difficile* behavior.

Ciprofloxacin, levofloxacin and moxifloxacin all induced *C. difficile* germination and toxin production in the gut model.[164] Interestingly, fluoroquinolone-resistant isolates were detected during the experiments, implying the potential for in vivo selection. Moxifloxacin was also associated with early toxin production (pre-detectable germination and proliferation) in *C. difficile* PCR

ribotype 027, suggesting the existence of strain-specific differences in response to fluoroquinolones. This may be particularly relevant in the light of controversies regarding the relative risk of *C. difficile* associated with fluoroquinolones. Reports of metronidazole failure in *C. difficile* cases,[170,171] and the emergence of reduced susceptibility among *C. difficile* epidemic ribotypes[172,173] prompted an investigation into the relative fitness of strains with differing metronidazole and clindamycin susceptibilities in the gut model.[160] Neither resistance to clindamycin nor reduced susceptibility to metronidazole elicited a fitness cost when no selective advantage for either strain was present, and there was no antagonism observed between strains. These studies highlight the value of using an in vitro gut model to evaluate potential resistance development and heterogeneous *C. difficile* populations.

Discussion

An animal model of *C. difficile* should ideally be standardized, produce reproducible results, and as far as possible resemble the etiology, pathophysiology and disease course seen in humans. The hamster model has been widely recognized as one of the most useful and predictive in vivo models of *C. difficile*. Hamster model studies initially focused on elucidating the pathogenesis of *C. difficile* and more recently exploring therapeutic and preventative strategies. Despite its recognition as a useful animal model, the hamster model is not fully standardized, has distinct pathophysiological differences compared with human disease, is problematic to establish, prone to contamination and suffers from reproducibility issues. As a result of these issues and ethical considerations surrounding animal models in general, the hamster model is only available in a few centers worldwide.

While hamsters are the most extensively used *C. difficile* animal models, particularly for the testing of novel therapeutic agents, there is a great deal of variation in the experimental methods employed. Such issues affect the reproducibility and interpretation of results. Many of the problems associated with hamster model experiments are also applicable to other small animal models. A key issue, exacerbated in hamsters given their exquisite susceptibility to *C. difficile* after antibiotic priming, is the potential for cross-contamination. This can occur via the local environment, food, water or by the animals themselves. In addition coprophagia may lead to further *C. difficile* exposure, compromising experimental validity. Animal environments have differed considerably between studies, with some hamsters housed individually and some in groups of up to five per cage.[22] In some cases HEPA filtered air, sterile food and water were used,[94] while in other reports these factors are not detailed. Douce and Goulding recommend individual housing in sterile cages with sterile food, water and bedding and transfer to a newly sterile environment every 24 h post-infection to minimize additional *C. difficile* exposure.[174]

While vancomycin was used to induce *C. difficile* in early hamster model studies, clindamycin has become the inducing agent of choice. There has however been little standardization of either the dose or route of administration of either agent, both of which may potentially affect the timing and magnitude of *C. difficile* germination and toxin production. The dosing of clindamycin has varied 100-fold (1–100 mg/kg), and similarly in experiments to evaluate *C. difficile* treatments, marked differences in (apparently effective) dosages of the comparator antibiotic, which is usually vancomycin, also make inter-study comparison difficult. Although 50 mg/kg vancomycin administered orogastrically was used in the majority of studies, doses have varied between 0.05 mg/day[22] to 200 mg/kg.[82] Vancomycin treatment regimens have ranged from single dose therapy[82] to daily administration over 5 d.[93,94]

Other potentially important confounding factors include qualitative and quantitative variations in *C. difficile* inocula. Most early studies did not deliberately expose hamsters to controlled amounts of *C. difficile*, instead relying on the exquisite susceptibility of the animal to *C. difficile* following antibiotic priming. Several studies did not determine relative populations of spore and vegetative cells; while others administered only vegetative populations, which may not survive the acid barrier of the stomach. *C. difficile* is thought to be ingested primarily as spores, and this has been considered in more recent animal model work, with researchers using controlled inocula of *C. difficile* spores.[47,49,114] Choice of *C. difficile* strain may also influence experimental outcome, since strains differ widely in their "virulence" properties, including toxigenicity, antimicrobial resistance, germination and sporulation capacities and colonization efficacy. Some studies stated whether known toxigenic and hypervirulent strains were administered,[39,49] while others provided no information at all. Phenotypic properties can also affect the timing of *C. difficile* induction by clindamycin; clindamycin resistance in *C. difficile* strains has been associated with delayed disease onset in hamsters.[51] Such issues may be amplified according to the choice of model endpoint and duration of study. In hamster models the endpoint has invariably been animal death (or euthanasia), whereas some studies have included histological and/or microbiological analysis of gut mucosa and contents, while others did not. Duration of study is particularly relevant when evaluating treatment efficacy, and especially the likelihood of *C. difficile* recurrence; the hamster model has generally not been useful for measuring *C. difficile* recurrence, given the natural history of *C. difficile* in these animals.

The use of more complex in vitro models to examine the behavior of an organism, necessarily introduces more variables, and therefore it is difficult to compare experiments directly. Likewise, these systems have often evolved according to the advances in knowledge and technology made several decades. Such development may overcome many of the problems first encountered, but later models have often become more complex and expensive in the process. In vitro disease models also cannot mimic secretory or immunological events, which are important aspects of *C. difficile* disease. In vitro models of *C. difficile* have progressed from simple test-tube fecal emulsions to complex triple-stage chemostat models (**Table 3**). Batch fermentation and test-tube models carry the advantages of short experimental duration and high numbers of replicates. However, they poorly simulate the behavior of *C. difficile* or the gut microflora in vivo. Bacterial growth dynamics are significantly different under

continuous vs. batch culture, and the human gut environment is more akin to the former. Moreover, continuous culture affords the researcher further flexibility and a longer experimental time-course, allowing a profile of *C. difficile* and gut microflora behaviors and responses to environmental changes to be determined. A number of authors have compared hamster and either gut model[94] or alternative continuous flow culture systems[122] and found results to be generally concordant.

With the exception of studies in the triple stage gut model, most feces based *C. difficile* experiments have been performed as stand-alone investigations. This inevitably means wide variations in total operating volume, duration of experiment, sources of fecal inoculum, fecal dilution factor and, importantly, the size and nature of the *C. difficile* inoculum. In addition, until later studies, the effects of antimicrobials on *C. difficile* was investigated using feces from antibiotic-treated patients or hamsters, and so were prone to variation in antimicrobial concentrations and thus difficult to compare. Continuous culture models have also differed in their volumes and retention times, all of which may influence the environment surrounding *C. difficile* and potentially its behavior. The series of experiments performed in the triple stage gut model have been well-standardized in terms of equipment, experimental protocol and microbiological and antimicrobial monitoring. The drawbacks of this approach are the labor-intensive nature of the model, additional expense and longer experimental duration.

Conclusions

It is clear that the choice of model may substantially affect the results obtained in *C. difficile* research. Hamster models have been considered the definitive option for *C. difficile* testing, especially for the evaluation of novel therapeutic agents. Experimental refinements in recent years have addressed some of the associated ethical and practical issues associated with animal testing, but standardization of methods remains a key issue. Recent advances in mouse models offer new opportunities and possibly improved reproducibility. However, the relevance of results obtained via animal models to human *C. difficile* remains a crucial issue. The development of standardized in vitro models additionally allows data comparison according to a range of variables, which in turn can provide confidence about the robustness of results. Appropriate model choice and standardization of experimental procedures are essential to generate clear and reproducible data. Animal and in vitro models of *C. difficile* both have pros and cons, and comprehensive study programs should ideally incorporate both approaches, taking care to understand key concordant and discrepant results. Such approaches can better inform the design and interpretation of clinical studies.

Future Directions

There appears to have been a reduction in use of animal models in *C. difficile* research over the past 30 years probably as a result of the ethical and practical drawbacks associated with animal testing. Crucial inter-species differences with respect to susceptibility to *C. difficile* and disease severity mean that extrapolation of results obtained from one animal model to another, and particularly to humans, may not be applicable. Such inter species variability is likely influenced by multiple factors, including presence of toxin receptors, and gut anatomy and flora.[175,176] There has been only very limited use of larger laboratory animals (e.g., foals, piglets, monkeys) for the study of *C. difficile*, and hamsters and mice models remain the most practicable options. The use of germ-free, gnotobiotic mice and axenic mice indicates that these may be more reliable models for future studies.[36] The potential availability of specific genetic mouse breeds is both an advantage in terms of flexibility but also a disadvantage considering the desirables of standardization and reproducibility.

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