

## Review Article

# Mouse Models of *Escherichia coli* O157:H7 Infection and Shiga Toxin Injection

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*Escherichia coli* O157:H7 has been responsible for multiple food- and waterborne outbreaks of diarrhea and/or hemorrhagic colitis (HC) worldwide. More importantly, a portion of *E. coli* O157:H7-infected individuals, particularly young children, develop a life-threatening sequela of infection called hemolytic uremic syndrome (HUS). Shiga toxin (Stx), a potent cytotoxin, is the major virulence factor linked to the presentation of both HC and HUS. Currently, treatment of *E. coli* O157:H7 and other Stx-producing *E. coli* (STEC) infections is limited to supportive care. To facilitate development of therapeutic strategies and vaccines for humans against these agents, animal models that mimic one or more aspect of STEC infection and disease are needed. In this paper, we focus on the characteristics of various mouse models that have been developed and that can be used to monitor STEC colonization, disease, pathology, or combinations of these features as well as the impact of Stx alone.

## 1. Introduction

*Escherichia coli* O157:H7 is a member of a group of pathogenic *E. coli* (known as enterohemorrhagic *E. coli* or EHEC) that colonize the gastrointestinal tract and cause a condition known as hemorrhagic colitis (HC) or bloody diarrhea. *E. coli* O157:H7 is also a member of the larger category of Shiga toxin-producing *E. coli* (STEC). This group of *E. coli* is solely defined by its capacity to produce Shiga toxin type 1 (Stx1), Shiga toxin type 2 (Stx2), or both toxins (as well as variants of these). The capacity of STEC in general and *E. coli* O157:H7 and other EHEC in particular to produce Stxs makes them of particular concern because Stx has been linked to the development of hemolytic uremic syndrome (HUS) that can lead to kidney failure, particularly in children [1, 2].

While a number of STEC serotypes are known to cause disease worldwide, *E. coli* O157:H7 infection generally represents the largest of the STEC disease burden, both in the United States (USA) and worldwide. Although the first recorded outbreak due to organisms of this serotype in

the United States occurred in 1982, *E. coli* O157:H7 infection in both people and animals can be traced back as early as the 1970's. The prevalence of this pathogen has grown since its first description and, despite our best control measures, *E. coli* O157:H7 remains a serious health concern (Figure 1) [1, 3–8].

*E. coli* O157:H7 is responsible for an estimated 73,480 cases of illness, 2,168 hospitalizations, and 61 deaths annually in USA, according to data published by Mead et al. in 1999 [9]. The majority of such *E. coli* O157:H7 outbreaks in the USA are associated with foodborne transmission [4]. Cattle as well as other ruminants serve as a reservoir for *E. coli* O157:H7. In particular, surveys of beef and dairy cattle have demonstrated carriage rates less than 0.5 to greater than 2.0% [10]. As a result of *E. coli* O157:H7 carriage in cattle, beef, and dairy products often become contaminated and serve as the source of infection in outbreaks of *E. coli* O157:H7. Many vehicles for foodborne transmission of *E. coli* O157:H7 have been described including beef (ground beef, roast beef, steak, salami, etc.), produce (unpasteurized apple cider or juice, melons, grapes, lettuce, bean sprouts, spinach, etc.),

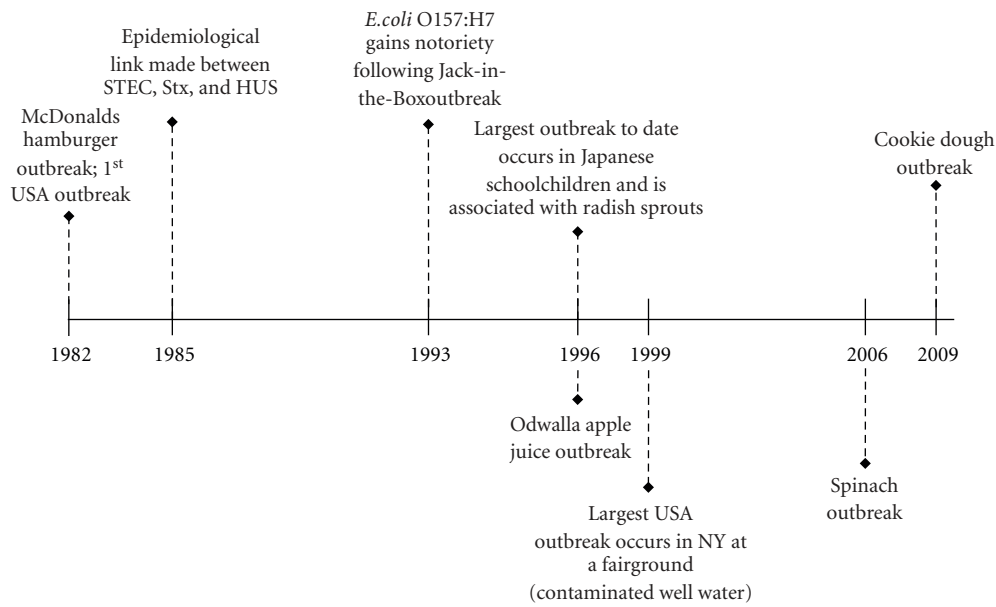


FIGURE 1: Major outbreaks of *E. coli* O157:H7 infection. Outbreaks are listed by year and in the context of key discoveries that linked Stx with development of HUS.

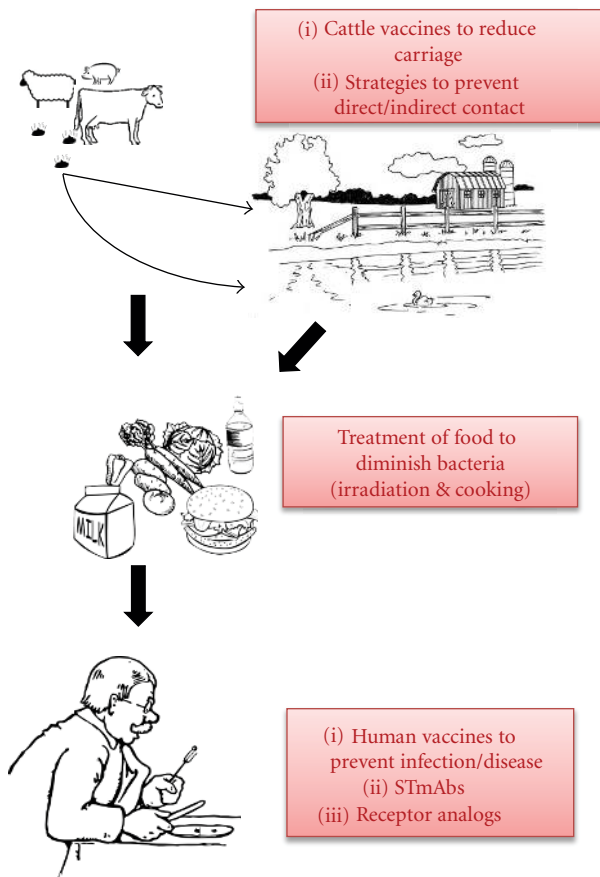


FIGURE 2: Modes of *E. coli* O157:H7 transmission to humans with emphasis on strategies for prevention/intervention. Other modes of transmission of *E. coli* O157:H7 have been reported and are described elsewhere.

and dairy products (raw milk, cheese, butter, etc.) [4]. The spread of *E. coli* O157:H7 by these various food matrices is facilitated not only by the pathogen's low infectious dose [11], but additionally by the pathogen's capacity to grow over a broad temperature range and to survive both freezing and acidic conditions [12]. In addition to transmission from contaminated food (or drink), person-to-person and waterborne transmission (both likely facilitated by the low infectious dose of *E. coli* O157:H7 [11, 13]) have also been reported (Figure 2).

*E. coli* O157:H7 infection can manifest in a variety of ways. Some individuals who are infected with the microbe remain asymptomatic, others experience diarrhea, but most develop hemorrhagic colitis, the hallmark of *E. coli* O157:H7 infection. Furthermore, children and the elderly appear to be especially susceptible to *E. coli* O157:H7-mediated disease and, for reasons that are unclear, may develop HUS (a triad of clinical manifestations including hemolytic anemia, thrombocytopenia, and renal failure [14]) and other systemic problems that include central nervous system (CNS) impairment.

A rise in both the hospitalization and HUS rates has been reported in association with more recent outbreaks of *E. coli* O157:H7. In data collected from outbreaks that occurred between 1982 and 2002, the average hospitalization rate was just over 17% and the average rate of HUS was ~4% [4]. However, in the spinach outbreak in 2006 and the cookie dough outbreak in 2009, rates of hospitalization and HUS were even higher (approximately 51% and 16%, respectively, in the spinach outbreak and approximately 44% and 13%, respectively, in the cookie dough outbreak [7, 8]). This increase in disease severity among *E. coli* O157:H7 infected persons has led to speculation that more virulent strains of the pathogen have emerged [15].

Although it has been nearly 30 years since the discovery of *E. coli* O157:H7 as an enteric pathogen and despite the recent increase in the rate of severe disease associated with infection by the organism, no treatment yet exists. In general, antibiotic therapy is contraindicated as it may promote toxin expression from the lysogenized phage that typically carries Stx genes. Additionally, antimotility agents are not recommended as they can promote the sustained presence, and consequent toxin expression, of EHEC in the gastrointestinal tract. In instances where HUS develops, supportive care is provided. A variety of treatment and prevention strategies to protect against *E. coli* O157:H7 are currently in development; these include toxin receptor analogs, passive antibody therapy, and vaccines to protect humans against the systemic effects of the toxin. Since an *E. coli* O157:H7 vaccine has not been developed and licensed for immunization of humans (two vaccines are currently in use in cattle [16–19]), the most promising prevention strategies for *E. coli* O157:H7 focus on minimizing exposure to this pathogen (Figure 2).

## 2. Pathogenesis of *E. coli* O157:H7

*E. coli* O157:H7 is well adapted to cause disease in humans. The organism has a number of virulence factors that contribute to its pathogenicity, and Shiga toxin (Stx) is certainly among them. Indeed, Stx is considered to be responsible for the severe complications of *E. coli* O157:H7 infection, including HUS [1, 2]. In addition to Shiga toxin, *E. coli* O157:H7 expresses several other important virulence factors that include intimin, translocated intimin receptor (or Tir), a type three secretion system (TTSS), and enterohemolysin (located on the pO157 plasmid) (see Figure 3). The genes for many of these factors are located on a 44 kb pathogenicity island known as the locus of enterocyte effacement, or the LEE locus.

The Stx family of AB<sub>5</sub> toxins (composed of a single A or active subunit noncovalently associated with a pentameric ring of B or binding subunits) contains two subgroups, Stx1 and Stx2. In addition to these two Shiga toxin serotypes, variants of each have been described: Stx1c, Stx1d, Stx2c, Stx2d, Stx2d-activatable, Stx2e, and Stx2f (reviewed in [20]). While Stx1 and Stx2 have the same overall structure [21], these toxins are antigenically distinct such that antibody against Stx1 will not neutralize Stx2, and vice versa [22]. Stx1 and Stx2 also have the same mode of action; however, they differ in specific activities both *in vitro* and in mice. The 50% cytotoxic dose (CD<sub>50</sub>) of Stx1 for Vero cells is lower than that for Stx2 [23], while the 50% lethal dose (LD<sub>50</sub>) of purified Stx1 for systemically inoculated adult CD-1 mice is ~125 ng compared to ~1 ng for Stx2 [24]. Moreover differences in toxicity are also evident when human renal endothelial cells are treated with purified Stx1 or Stx2; Stx2 is about 1,000-fold more toxic [25]. Finally, epidemiological data suggest a difference in Stx1 and Stx2 toxicity in people; Stx2-producing *E. coli* O157:H7 strains are more frequently associated with HUS than are strains that produce Stx1 [26–28].

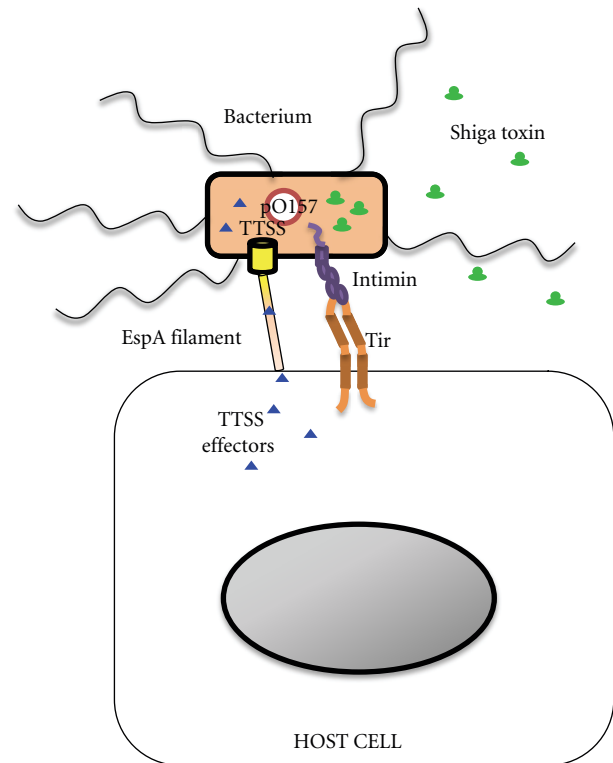


FIGURE 3: *E. coli* O157:H7 virulence factor expression and interaction with host cells. *E. coli* O157:H7 possesses a large plasmid (pO157), carries the LEE PAI (and thus is intimin positive), and expresses Shiga toxins. The LEE locus encodes a TTSS and TTSS effector proteins. One of the TSSS proteins, *E. coli* Secreted Protein (Esp) A, forms a filament that serves to translocate TTSS effector proteins from the bacterium into the host cell by way of a pore created by EspB and EspD. One of these effectors, Tir, serves as the receptor for the major adhesin, intimin, and thus allows adherence of the bacterium to the host cell.

The B subunit of Stxs forms a homopentameric ring structure as it binds the cellular toxin receptor, globotriaosylceramide (also known as Gb3 or CD77), a Pk blood group antigen found on a variety of cells [29, 30]. The A<sub>1</sub> fragment of the A subunit is translocated into the host cell cytoplasm [31], where it acts as an N-glycosidase to remove a single adenosine residue from the 28S ribosomal RNA of the 60S ribosome [32, 33]. Alteration of the 28S rRNA prevents binding of elongation factor to the ribosome so as to inhibit protein synthesis [34]. Stx-mediated inhibition of cellular protein synthesis generally results in death of the intoxicated cell by apoptosis.

Plasmid pO157 is a 93 kb plasmid (~60 MDa) found in most *E. coli* O157:H7 isolates as well as other EHEC strains [35, 36]. The plasmid encodes an enterohemolysin, an adhesin known as ToxB, and a type two secretion system [37–39]. While the plasmid is thought to be important for virulence (studies have correlated pO157 with hemolytic activity [39] and intestinal adherence [40]), its exact role in pathogenesis remains unclear (reviewed in [35]).

The locus of enterocyte effacement, or LEE, is a chromosomally encoded pathogenicity island (PAI) that encodes the pathogenic determinants intimin (an adhesin), a type three secretion system (TTSS), and TTSS effector proteins. The TTSS of *E. coli* O157:H7 is a contact-dependent system for transport of bacterial proteins, or effectors, into host cells (such as intimin and Tir). Intimin is a 97 kDa outer membrane protein that facilitates intimate adherence of *E. coli* O157:H7 to the epithelial surface [41]. Although STEC strains that lack intimin can cause human disease [42, 43], *E. coli* O157:H7 appears to require intimin to establish colonization [44–50]. Several investigators have speculated that *E. coli* O157:H7 colonization initially proceeds via the interaction of the outer membrane protein intimin with cell-surface-expressed factors such as  $\beta$ 1 integrins [51] and nucleolin [52, 53], before binding to the translocated intimin receptor. *E. coli* O157:H7 is unique among bacterial pathogens (with the exception of other LEE-encoding bacteria) in that it encodes its own receptor, Tir, which is injected into target host cells by way of the TTSS.

**2.1. Models of Pathogenesis.** Knowledge of the pathogenesis of *E. coli* O157:H7-mediated disease in humans is quite limited. The use of human subjects to investigate the steps required for *E. coli* O157:H7 to evoke intestinal pathology is considered unethical because of the possibility that a volunteer could develop HUS. Thus, numerous *in vitro* assays and animal models have been developed in an attempt to mimic various aspects of *E. coli* O157:H7 disease in humans.

*In vitro* systems such as cell monolayers, transwells, organoids, and *in vitro* organ culture (referred to as IVOC) as well as *ex vivo* cultures of biopsies are useful for the study of several aspects of *E. coli* O157:H7 pathogenesis, such as adherence of the microbe to eukaryotic cells and the impact of Stx on those cells. In addition, the type of cells used often depends on the specific aspect of infection and/or pathogenesis under investigation. For example, epithelial cells (derived from a variety of different sources) are commonly used to evaluate *E. coli* O157:H7 adherence mechanisms [47, 54], while endothelial cells (human umbilical vein endothelial cells (HUVECs) or human glomerular microvascular endothelial cells (GMVECs)) are often used to examine the effect of Stx on vascular cell integrity and cytokine response [55]. In an attempt to model not only adherence but also the early steps in *E. coli* O157:H7 pathogenesis, polarized monolayers were generated with transwell systems. A key feature of the transwell approach is that it allows for the development of polarized cell monolayers that express features more characteristic of differentiated cells, such as the formation of tight junctions and the expression of unique cellular factors [56]. Thus, researchers have explored the mechanisms of both *E. coli* O157:H7 binding to and Stx transit across the epithelium [57, 58]. In this manner, Stx1 and Stx2 have been shown to translocate, at varying rates, across CaCo-2A, T84, and HCT-8 cells, all of which are of intestinal origin [59]. Another tissue culture model used to explore *E. coli* O157:H7 adherence and subsequent host cell damage is the organoid system in which cells grown on a scaffold under microgravity conditions form pieces of

tissue-like material [60–62]. When an organoid derived from HCT-8 intestinal epithelial cells was infected with *E. coli* O157:H7, attaching and effacing (A/E) lesion formation and slight tissue damage were evident; the tissue damage was attributed to the Stx2 produced by the bacteria [63]. Finally, *in vivo* grown organ cultures of intestinal samples have been used to study adherence of *E. coli* O157:H7 to gut mucosal cells and subsequent damage to the intestinal cells [64–68].

Many animal models have been developed to facilitate study of EHEC pathogenesis *in vivo*. In general, these models exist in two varieties: those solely focused on the effects of Stx (in the absence of bacteria) and those that explore *E. coli* O157:H7 infection. Models that evaluate toxicity rely on injection of Stx (with or without LPS) and often measure mortality as the endpoint of the investigation. Such *in vivo* assays have been used to explore differences in relative toxicity among Stx toxin types [69], to assess the protective capacity of some factor [70–72], or to model the pathogenesis of HUS [73]. While each applicable model can be used to study one or more components of the steps in the pathogenesis of *E. coli* O157:H7- or other STEC-mediated disease (from initial colonization to mortality), no one animal model system that mimics the full spectrum of STEC-evoked illness in humans (to include the development of HC and HUS) has been described to date (reviewed in [74]).

Small animals that have served as models for EHEC infection and disease include mice [75–80], rats [81], and rabbits [82]. Larger animals that have been so used, albeit less frequently, include chickens [83, 84], pigs [85], cows [86], dogs [87], baboons [88], and macaques [89]. The presence of characteristic A/E lesions in the gastrointestinal tract of *E. coli* O157:H7-infected animals has been reported in gnotobiotic piglets, infant rabbits, calves, chickens, and macaques (reviewed in [90, 91]). Additionally, naturally occurring HUS-like diseases have been described in greyhounds (known as idiopathic cutaneous and renal glomerular vasculopathy of greyhounds (CRVGs) or “Alabama rot” [87, 92]) and rabbits [93].

### 3. Mouse Models of Infection

While large animal models, such as the gnotobiotic piglet, exhibit a number of features of *E. coli* O157:H7 pathogenesis, their breeding and maintenance require considerable veterinary skill, space, and financial support. Thus, small animal model systems are preferable for general use. Mouse models in particular offer a number of benefits that include the following: low relative costs for purchase and maintenance, ease of care and handling, ready availability of numerous immunological reagents, variations in genetic backgrounds among inbred mouse strains as well as access to transgenic and recombinant inbred animals, and, very importantly, the feasibility of using sufficient numbers of animals in a single study to perform meaningful statistical analyses on the resultant data. Popular mouse models of *E. coli* O157:H7 oral infection include axenic mice (no indigenous intestinal flora)

TABLE 1: Commonly used mouse models of STEC colonization and/or disease.

Model	Inoculum (CFU)	Inoculation Method	Features	Histopathology	Source
Str-treated	$10^{10}$	Feeding	Colonization, morbidity, mortality with 933cu-rev	Kidney	[79]
Str-treated (O91:H21)	$LD_{50} < 10$	Feeding	Colonization, morbidity, mortality	Kidney	[94]
MMC & str (O157:H-)	$>10^9$ (M) <sup>a</sup>	IG	Colonization, morbidity, mortality	Kidney, brain	[95]
Conventional	$10^7, 10^8$	IG	Morbidity, mortality	Kidney, intestines	[76]
Germfree	$2 \times 10^2$ (C) <sup>a</sup> , $2 \times 10^9$ (D, M) <sup>a</sup>	IG	Colonization, morbidity, mortality	Kidney, intestines, brain	[96]
PCM	$2 \times 10^5$ – $2 \times 10^7$	IG	Colonization, morbidity, mortality	Kidney, intestines, brain	[77]
Conventional	$\sim 2 \times 10^{10}$	IG	Colonization		[97]
Germfree	$5 \times 10^7$	IG	Colonization, morbidity, Mortality with hypervirulent strain	Kidney, intestines, brain	[98]
Conventional	$10^{11}$ /kg	IG	Colonization		[99]
MMC & str	$5 \times 10^3$	IG	Colonization, morbidity, mortality	Kidney, other	[78]
Germfree	$10^2$ – $10^6$	IG	Colonization, morbidity, mortality	Kidney	[75]
Conventional (weaned)	$6 \times 10^9$ /kg	IG	Morbidity and mortality	Kidney, intestines	[100]

<sup>a</sup>C: colonization, D: disease, M: mortality.

or streptomycin-treated mice (reduced normal flora) because these animals have proven amenable to EHEC colonization. A summary of the mouse models that have been used for *E. coli* O157:H7 oral infection studies is presented in Table 1.

**3.1. Development of the Streptomycin-Treated Mouse Model for *E. coli* O157:H7 Infection.** The first mouse system described for study of the pathogenesis of *E. coli* O157:H7 was the streptomycin-treated murine model developed by Wadolkowski et al. [79]. This *E. coli* O157:H7 mouse model incorporates streptomycin (str) treatment of animals via their drinking water as a means of reducing the animals' normal intestinal facultative flora so as to decrease bacterial competition for the infecting EHEC strain. This methodology was based on the work of Myhal et al. as described in 1982 [101]. The goal of the original study by Myhal and colleagues was to assess the relative colonizing capacities of different *E. coli* isolates. Prior to the report by Myhal et al., *E. coli* and several other bacteria (to include *Salmonella* and *Vibrio*) were shown to have the capacity to colonize mice if the animals had been antibiotic-treated or were axenic [102, 103]. However, Myhal et al. demonstrated that even a laboratory-adapted *E. coli* K12 given orally as a single strain challenge was capable of colonizing str-treated mice to levels equivalent to those observed for human fecal *E. coli* isolates. Thus, as the authors suggested, str-treated mice are best used to evaluate the relative colonization capacity of an *E. coli* strain if given with another isolate in a competitive infection study [101].

Myhal et al. used 5-6-week-old, male CD-1 (outbred mice, also known as ICR) mice [101]. Myhal et al. demonstrated that addition of 5 g/L of streptomycin sulfate to the animals' drinking water, for as little as one day, reduced the

number of facultative anaerobes shed (from  $10^8$  CFU/g feces prior to streptomycin treatment down to  $<10^2$  CFU/g feces) but had little or no effect on the number of obligate anaerobic bacteria present within the gastrointestinal tract ( $10^9$  CFU shed/g feces). The authors selected streptomycin as the antibiotic treatment of choice because they reasoned that a mutation that rendered the bacteria resistant to streptomycin (a presumed alteration to the ribosomes) should have little effect on the bacterial surface and thereby on colonization. In their report, str-treated mice deprived of food/water were infected with  $10^{10}$  CFU of str-resistant *E. coli* in a solution of 20% sucrose. Colony counts of feces collected daily indicated that high levels of colonization were achieved by all of the challenge strains when given alone ( $\sim 10^8$  CFU/g feces), whereas in cofeeding or competition experiments varying colonizing capacities of the strains were observed. Furthermore, the authors found that human fecal isolates heavily colonized both the cecum and the large intestines. They concluded that the large intestines, which had slightly more adherent bacteria than were observed in the cecum, were the main site of *E. coli* colonization in their str-treated model [101]. This primary colonization site was consistent with what was previously reported for *E. coli* colonization in the untreated mouse model [104].

Wadolkowski et al. followed a very similar methodology to that described by Myhal et al. for their *E. coli* O157:H7 infection studies in str-treated mice [79, 101]. Male CD-1 mice were provided 5 g/L streptomycin sulfate in their drinking water to decrease the normal flora prior to an overnight fast and inoculation with  $10^{10}$  CFU of the strain/s of interest in 20% sucrose (w/v). Food was returned after infection and animals were housed individually. Colonization levels of the infecting *E. coli* O157:H7 strain were determined

by enumeration of str-resistant *E. coli* O157:H7 in shed feces.

Wadolowski et al. tested three *E. coli* O157:H7 strains ( $n = 3$  per strain; experiments done in triplicate (at least)) in their initial experiments: WT strain (933), a pO157-cured mutant (933cu), and an additional pO157-cured mutant recovered from co-infection studies (933cu-rev) [79]. Strains 933 and 933cu colonized to similar levels ( $10^7$  CFU/g feces for 25 days) in single infection experiments with no observed disease manifestation. When co-infections were conducted, 933 outcompeted 933cu in 2/3 of the mice. In the remaining mouse, after a decline in the load of 933cu, there was a steady increase in levels of 933cu compared to 933. An isolate of 933cu recovered from the co-infected mouse was labeled 933cu-rev (“rev” meaning “revertant” to wild-type or virulent colonization levels). Although no signs of illness were apparent in the co-infected mouse with the high levels of 933-cu rev, disease manifestations were evident in mice subsequently infected with 933-cu-rev alone. The 933cu-rev-infected mice shed loose stools, were anorexic and lethargic, and died within a few days of disease presentation. Extensive necropsies (included the liver, brain, heart, stomach, small intestine, cecum, large intestines, spleen, and kidneys) of singly infected animals revealed that only the kidneys from animals infected with strain 933cu-rev demonstrated pathology of any kind. Histopathologic analysis of kidneys from 933cu-rev-infected animals indicated widespread bilateral acute renal cortical tubular necrosis despite apparently normal glomeruli with no evidence of fibrin deposits, elastic fibers, or bacteria. The authors concluded that this pathology was more indicative of insult from a toxin rather than a result of dehydration that might be expected in an anorexic animal with loose stools [79].

In this same study, Wadolowski et al. recovered strains 933, 933cu, and 933cu-rev from epithelial cells of the small intestine, cecum, and large bowel. However, 933cu-rev demonstrated an increased capacity to colonize the small intestines and the mid and distal sections of the large intestines compared to the other two strains. This finding was consistent with the increased capacity of strain 933cu-rev to multiply within mucus obtained from the different intestinal segments (note: Wadolowski et al. had previously shown that multiplication in cecal mucus was required for a human fecal isolate of *E. coli* to colonize the large intestine [105]). Wadolowski et al. went on to speculate that the increased virulence of 933cu-rev may have been attributable in part to its broader range of colonization locales and, more specifically to the increased capacity of the distal small intestines (versus the cecum or large intestines) to absorb Stxs produced at that site [79]. Furthermore, the link between site of colonization and extent of disease was also suggested by Tzipori et al. in relation to EPEC colonization [106]. Strains that caused more severe disease colonized the proximal small intestines of piglets, whereas strains that caused milder pathogenesis tended to colonize distal regions of the small intestines as well as distal portions of the large intestines.

**3.2. Extension of the Streptomycin-Treated Mouse Model for STEC Infection.** The utility of the str-treated *E. coli* O157:H7 colonization model was extended from the original report of *E. coli* O157:H7 infection and colonization to include the evaluation of non-O157 STEC strains [94]. Moreover, in 1994 Fujii et al. used the str-treated model to demonstrate the development of neurological manifestations of disease by incorporation of mitomycin C (MMC) into the treatment regimen [95]. MMC treatment results in induction of phage expression by the bacterium, which concurrently leads to increases in toxin production. Agents that induce toxin expression, to include MMC or ciprofloxacin treatment, are frequently used in mouse models of STEC infection [78, 95, 107, 108]. Alterations to the str-treated model include a reduction in the amount of bacteria fed [78, 109]; an increase in the time of streptomycin treatment prior to infection [110, 111]; the application of intragastric inoculation of the organism [112]; finally, changes in the sex, age, or strain of mouse used [110, 112–115].

**3.3. Protein-Calorie Malnutrition Mouse Model.** While the str-treated mouse model has proven particularly useful for reproducibly attaining high levels of STEC colonization, this animal system has limitations. For example, very high inocula of *E. coli* O157:H7 are required to cause morbidity or mortality in a portion of str-treated animals, even though low to moderate doses of STEC strain B2F1 (*E. coli* O91:H21) have been used to achieve colonization of str-treated mice and induce disease [94]. In an effort to reduce the inoculum of *E. coli* O157:H7 required to evoke disease in mice and to bring the mouse inoculum closer to the predicted infectious dose for humans (thought to be around 50 organisms [116]), other mouse model systems have been developed. One such system described by Kurioka et al. was based on the observation that some children who contract STEC infections subsisted on an unbalanced diet prior to infection [77]. Therefore, the authors theorized that protein calorie malnourished (PCM) mice would be more readily infected by low doses of *E. coli* O157:H7 than would conventional mice. The authors’ hypothesis proved correct in that the minimal infectious dose of *E. coli* O157:H7 in PCM mice was over 3 logs lower than that of control mice. The authors reported pathological changes in the intestinal tract of PCM mice infected with *E. coli* O157:H7 (underdevelopment of the intestinal epithelium in response to PCM, likely resulting in the animals’ predisposition to *E. coli* O157:H7 colonization) and a slight increase in TNF- $\alpha$  in the blood of PCM mice compared to controls. However, they did not observe significant renal pathology (as was seen by Isogai et al. [117]) but did note minimal degeneration of renal tubules and weak staining of the cortical tubular epithelium for Stx [77]. Kurioka et al. also postulated that retardation of intestinal development by PCM might facilitate Stx and LPS transit across the intestinal barrier. That Stx likely did cross the mucosal barrier more readily in PCM-infected animals was strongly suggested by the CNS findings in the PCM mice; cerebral hemorrhage was evident and toxin was detected in the hippocampus. Thus, this report confirmed that Stx

can affect the CNS of mice, and can cause death of the infected PCM mice within 10 days. These CNS findings in *E. coli* O157:H7-infected PCM mice are consistent with the observation noted by Kurioka et al. [77] that up to 30% of STEC-infected children display neurological manifestations of disease [118].

**3.4. Germ-Free Mouse Models.** In an attempt to extend the renal pathology evident in str-treated, STEC-infected mice to include evidence of glomerular lesions, Isogai et al. infected germ-free mice with *E. coli* O157:H7 [117]. Although these animals became colonized with *E. coli* O157:H7 following a low-dose challenge, the animals did not display signs of disease. High inocula (comparable to those reported for the str-treated model) were necessary to cause pathology (colon, kidneys, and brain), disease (lethargy, paralysis, anorexia, dehydration), and death within 7 days. However, when these mice were treated with TNF- $\alpha$  and then infected with a low dose of *E. coli* O157:H7, systemic disease, neurological manifestations, and glomerular lesions were observed [117].

Since the initial report by Isogai et al., other groups have used germ-free animals to evaluate *E. coli* O157:H7 pathogenesis. Sawamura et al. explored the effects of antibiotic treatment on *E. coli* O157:H7 infection in a germ-free mouse model they developed [119] and later used to investigate the role of bacterial internalization by epithelial cells in STEC pathogenesis [120]. Isogai and colleagues subsequently used germ-free mice to explore the effects of antibiotic and green tea extract treatment on *E. coli* O157:H7 disease [96, 121–123]. Taguchi et al. developed a variation of the germ-free mouse model in which inoculation with a hyper-toxigenic strain of *E. coli* O157:H7 caused 100% mortality among infected animals [98]. Takahashi et al. went on to use this latter model to investigate the effect of probiotics on *E. coli* O157:H7 [124]. In 2007, Jeon et al. used a germ-free mouse model to assess the virulence of a mutant strain of *E. coli* O157:H7 [125]. Furthermore, Eaton and colleagues exploited germ-free Swiss Webster mice to explore the roles of different *E. coli* O157:H7 strains and Stx types, as well as host factors such as age and gender of the mouse [75]. Eaton et al. reported that gut-adherent *E. coli* O157:H7 organisms were seen in the ileum and cecum but not the colon. The absence of detectable mucosally-adherent *E. coli* O157:H7 in the colon of germ-free mice is in contrast to the observations of Wadolkowski et al. in the str-treated model [79].

**3.5. Conventional Mouse Models.** While str-treated or axenic animals are useful as models for assessment of disease outcome, they rely on the absence of colonization resistance. The term “colonization resistance” was originally coined by van der Waaij in 1971 to explain a phenomenon whereby “a complex intestinal microflora provides protection against colonization by many pathogenic infectious agents” [107]. As a result of the partial or complete absence of a competing microbiota in antibiotic-treated or axenic animals, the inoculated microorganism has a colonization advantage. Thus, studies that apply these models are of limited utility for the assessment of the capacity of an STEC strain to colonize in

the face of the physiologically more relevant situation where normal bowel flora are present.

An alternative model that did not require the alteration of the indigenous flora of mice was described in 1997 by Karpman and colleagues [76]. These investigators administered high doses of *E. coli* O157:H7 intragastrically to C3H/HeN and C3H/HeJ mice and reported significant morbidity and mortality in the infected animals. Mice in this model developed gastrointestinal, neurological, and systemic disease manifestations. Renal pathology included both glomerular mesangial changes and tubular necrosis. Focal areas of colonic necrosis were also evident. Of note, administration of anti-Stx2 antibodies protected animals from disease symptoms and pathology [76].

Like Karpman et al., Conlan and Perry investigated conventional mice as a model for *E. coli* O157:H7 infection [97]. In their report, Conlan and Perry considered three strains of female mice: CD-1 (outbred), BALB/c (inbred) and C57BL/6 (inbred) to screen potential vaccine candidates. Following intragastric administration of  $\sim 10^{10}$  CFU *E. coli* O157:H7 to one of the three mouse strains, fecal shedding of the organism was monitored as a surrogate for colonization. Although all mouse strains were colonized after infection (generally for 1–2 weeks), no morbidity or mortality was observed. Interestingly, only BALB/c mice seemed to be relatively resistant to re-infection; they shed *E. coli* O157:H7 for a shorter duration than did other mouse strains that had also received a second challenge with the microbe. BALB/c mice produced more O157-specific IgA (both serum and fecal) in response to both primary and secondary infection despite significantly lower serum anti-O157 IgG titers following secondary infection, when compared to C57BL/6 mice. Thus, BALB/c mice can serve as models for *E. coli* O157:H7 vaccine studies, despite their reported lack of mortality and/or morbidity in the study by Conlan and Perry [97].

The reports by Conlan and Perry and Karpman et al. indicated that *E. coli* O157:H7 could colonize conventional mice [76, 97]. To extend these findings, Nagano and colleagues determined the functionality of specific pathogen free (SPF) mice as models for *E. coli* O157:H7 colonization through exploration of the susceptibility of various mouse strains to colonization after infection with *E. coli* O157:H7 [99]. The mouse strains they examined included ICR (also known as CD-1), BALB/c, C3H/HeN, C3H/HeJ, and A/J (all female SPF mice). At one week after infection, even with a high inoculum of *E. coli* O157:H7, only the ICR animals remained colonized as a group. In fact, the majority of ICR mice stayed colonized and shed *E. coli* O157:H7 in their feces for the duration of the study (28 days). Moreover, *E. coli* O157:H7 were detected in both the cecum and the colon later in infection. However, Nagano et al. concluded that the cecum was the primary site of colonization because only at that location were *E. coli* O157:H7 colonies observed adherent to epithelial cell surfaces. In spite of this persistent colonization of ICR mice by *E. coli* O157:H7, no morbidity or mortality was observed after *E. coli* O157:H7 infection of these animals [99].

Recently, Brando et al. used weaned BALB/c mice to investigate *E. coli* O157:H7 pathogenesis [100]. Weaned mice were selected over adult mice in an attempt to render the animals more susceptible to *E. coli* O157:H7 infection; age has been described to be an important factor in susceptibility of animals to EHEC disease [75]. In their model, Brando et al. showed that only mice <21 days of age demonstrated systemic manifestations of disease, which included mortality within 96 hours and increased plasma urea levels [100]. Occult blood was observed in the stools of mice that succumbed to infection. Histologic analysis revealed tubular necrosis (consistent with other STEC models) and glomerular alterations. Only a portion of infected mice had detectable bacteria by 72 hours after infection, and all survivors cleared the infection by day 7. Furthermore, only transient colonization of the small and large intestine (the cecum was not analyzed) was observed. However, histological analysis revealed damage to and inflammatory infiltrates in the intestinal epithelium. The incompletely developed intestinal epithelium in weanling mice likely contributed to the pathologic features in the intestine and the systemic absorption of toxin, as was seen in PCM-infected mice [77, 100].

**3.6. Intact Commensal Flora Model.** Other studies that used conventional mice to study STEC pathogenesis can be found in the literature, although they are less thoroughly described [111, 126–129]. However, a common feature among many of these conventional mouse model investigations of *E. coli* O157:H7 infection was that they either assessed colonization or monitored the development of disease. For example, in the studies by Nagano et al. and Conlan and Perry, colonization was followed but morbidity of infected mice was not observed [97, 99]. Conversely, in the study by Karpman and colleagues, morbidity and mortality were observed but colonization was not monitored [76]. Thus, until our report of a new conventional BALB/c mouse model of *E. coli* O157:H7 infection [80], no adult mouse studies with conventional animals that explored in detail both colonization and disease after *E. coli* O157:H7 oral infection had been reported.

We described the use of conventional BALB/c mice to model *E. coli* O157:H7 oral infection [80]. Female animals with an intact commensal flora (ICF) were inoculated with high doses ( $10^9$  CFU or greater) of *E. coli* O157:H7 strain 86-24. Thus, *E. coli* O157:H7 introduced orally by either pipette feeding or intragastric administration (gavage) were forced to compete with commensal flora to become established within the gastrointestinal tract. The use of an ICF animal model for studies of *E. coli* O157:H7 infection and pathogenesis has the advantage of reflecting a complex microbiological environment in the gut such as that typically found in an individual who ingests *E. coli* O157:H7 in contaminated food or water or through person-to-person contact. Upon infection, mice were colonized with *E. coli* O157:H7 for the seven day course of the experiments; however, high doses of *E. coli* O157:H7 were required to achieve consistent, persistent

colonization in the face of the ICF of the mouse. The primary site of *E. coli* O157:H7 colonization was demonstrated to be in the cecum of these animals. We surmised that the bacteria were shed into the cecal content where they transiently colonized or directly passed through the large intestine in the luminal contents prior to becoming encased in fecal pellets that were subsequently expelled [80].

The ICF model of *E. coli* O157:H7 infection is unique in that we were able to monitor both colonization and disease, which included ruffled fur, lethargy, weight loss, and, on average, ~30% mortality [80]. In addition, ICF mice infected with *E. coli* O157:H7 displayed evidence of renal tubular damage with increased blood urea nitrogen (BUN) and slightly increased levels of creatinine, both of which can indicate renal impairment. These blood chemistry findings are seen in patients with HUS that sometimes follows *E. coli* O157:H7 infection. Therefore, through these data and others, we inferred that Stx2 produced by *E. coli* O157:H7 at the site of infection within the gastrointestinal tract could enter the bloodstream and harm the kidneys of the mice in a manner analogous to that presumed to occur in some *E. coli* O157:H7-infected humans [80].

**3.7. *Citrobacter Rodentium* as a Surrogate for *E. coli* O157:H7.** Another mouse model system used to evaluate the virulence mechanisms of EHEC employs the natural mouse pathogen *Citrobacter rodentium* as a surrogate for *E. coli* O157:H7. *C. rodentium* is similar to both EPEC and EHEC in that it carries a homolog of the LEE pathogenicity island of EPEC and EHEC and has the capacity to evoke A/E lesions. Thus, *Citrobacter* has been used to study the molecular basis for A/E lesion formation in mice because it is the only known LEE-positive organism that is naturally pathogenic for rodents [130]. *C. rodentium* causes transmissible murine colonic hyperplasia (TMCH) [130]. While the organism is useful for assessment of the contribution of various genes/proteins to virulence, this model fails to recapitulate the pathogenesis caused by EHEC as a whole because it does not make Shiga toxin (although there was one report of a strain of *Citrobacter freundii* that produces Stx2 [131]).

**3.8. Alternative Models to Oral Infection.** In studies of mice infected with *E. coli* O157:H7, the oral route of challenge is used most frequently. Nevertheless, alternative means of inoculation with STEC, although infrequent, have been described. For instance, one of the earliest published murine models of STEC infection relied on the subcutaneous injection of large quantities ( $10^8$  CFU) of *E. coli* O157:H7 into SPF mice [132]. Intravenous injection of mice with spontaneously-derived motility mutants of *E. coli* O157:H7 was explored as a system to assess potential differences in virulence of the strains [133]. Most recently, Gao et al. evaluated the protective impact of an Stx fusion protein vaccine on intraperitoneal challenge with *E. coli* O157:H7 [134].



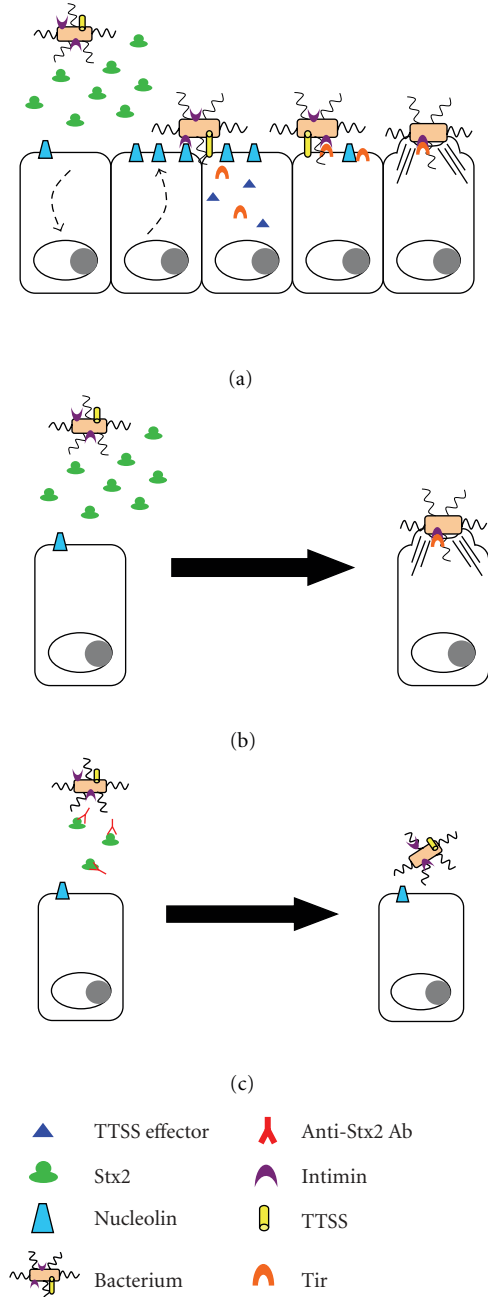


FIGURE 4: Model of the role of Stx2 in *E. coli* O157:H7 adherence and colonization. (a) *E. coli* O157:H7 elaborates Shiga toxin early during the colonization/adherence process. Stx2 exerts an effect on the host cell epithelium that leads to increased levels of cell surface-localized nucleolin. Nucleolin acts as an initial receptor for intimin, an interaction that allows *E. coli* O157:H7 to bind to the host epithelium and inject Tir and other TTSS effectors into the host cell. Intimin then engages Tir which, coupled with the cellular effects of other TTSS effectors, leads to host cell cytoskeletal rearrangement and formation of the characteristic A/E lesion. (b) Stx2, produced by the wild-type organism or provided to a *stx*<sub>2</sub> mutant, facilitates colonization of the gastrointestinal tract. (c) Neutralizing anti-Stx2 antibody present prior to and during *E. coli* O157:H7 infection results in reduced levels of *E. coli* O157:H7 colonization of the gastrointestinal tract.

#### 4. Mouse Models of Intoxication

HC and HUS, which are the most severe manifestations of disease observed after infection with *E. coli* O157:H7 in particular but with other STEC isolates as well, have been attributed to the production of Stx by the organisms. As such, mouse models are often used to explore the effects of Shiga toxin intoxication on an animal. While this can be achieved by inoculation with toxigenic strains [135–138], toxin is more commonly administered to mice by means of parenteral injection [24, 69, 135, 139–142]. Injection of purified Stx1 or Stx2 alone causes paralysis, damage to renal cortical tubule epithelial cells, and ultimately kills mice [69]. The LD<sub>50</sub> for Stx1 is ~125 ng and for Stx2 is ~1 ng [24]; these LD<sub>50</sub> data conflict with *in vitro* analyses of Stx that show that the CD<sub>50</sub> of Stx1 for Vero cells is lower than that of Stx2 [23]. However, the mouse LD<sub>50</sub>s support data that show that purified Stx2 is about 1,000-fold more toxic to human renal endothelial cells [25] as well as epidemiological findings that suggest a difference in Stx1 and Stx2 toxicity for people; Stx2-producing *E. coli* O157:H7 strains are more frequently associated with HUS than are strains that produce Stx1 [26–28]. Mice fed Stx2-producing DH5 $\alpha$  experienced mortality and exhibited renal lesions similar to those observed in STEC (*E. coli* O91:H21)-infected mice [135].

**4.1. Administration of Stx and Endotoxin.** Because injection of Stx alone initially failed to recapitulate all features of HUS, a mouse model that mimics the course of human *E. coli* O157:H7 disease was developed. In this model, mice were injected with a combination of Stx and LPS [139–141, 143, 144]. Systemic exposure to both virulence factors caused characteristic features of HUS that included neutrophilia, thrombocytopenia, red cell hemolysis, and increased BUN and creatinine levels [139]. In addition, evidence of fibrin deposition and red blood cell infiltration were observed in the glomeruli, capillaries, and intertubular spaces [139]. The renal damage was caused by Stx-induced apoptosis of the Gb3-expressing renal cortical and medullary tubular cells, which led to loss of function in the renal collecting ducts and subsequent dehydration [140]. The role of LPS in the mouse model of HUS, however, is controversial. Palermo et al. determined that mortality caused by Stx and LPS was dependent on the timing of the LPS administration relative to Stx intoxication [145]. In contrast, Suzuki et al. observed no synergistic effect of coadministration of LPS and Stx based on analysis of cytokine induction and mortality [146]. Recent studies demonstrated that repeated exposure to sublethal doses of Stx2 in the absence of LPS resulted in the development of features of HUS [73]. Extended exposure to Stx would be expected following infection with *E. coli* O157:H7 or another STEC.

**4.2. Feeding of Stx.** It has been postulated that toxin produced by *E. coli* O157:H7 and liberated into consumables may result in intoxication in the absence of infection [147]. To test this hypothesis in a mouse model, toxin was delivered to mice orally in an attempt to cause systemic

intoxication. Our lab found that mice fed large amounts of Stx2 succumbed to the effects of toxin (unpublished data). Furthermore, Rasooly and colleagues showed that mice fed Stx also died within a few days of oral intoxication [148]. In their study, toxin-mediated damage to distal organs was observed, which indicated that Stx was able to migrate out of the digestive tract in the absence of *E. coli* O157:H7 [148].

## 5. Applications of Mouse Models

**5.1. The Role of Stx.** In 2006, Robinson et al. demonstrated that Stx2 facilitated adherence of *E. coli* O157:H7 to epithelial cells *in vitro* and colonization of the intestine *in vivo* [149]. They showed that an isogenic *E. coli* O157:H7 *stx*<sub>2</sub> mutant adhered to HEp-2 cells to a lesser extent than did the wild-type *E. coli* O157:H7 strain; furthermore, this adherence deficiency could be overcome by treatment of the HEp-2 cells with purified Stx2. In single infections of conventional mice, the Stx2-producing wild-type *E. coli* O157:H7 strain colonized the mouse intestine at significantly higher levels than did its isogenic *stx*<sub>2</sub> mutant. However, in a co-infection, the *E. coli* O157:H7 wild-type and *stx*<sub>2</sub> mutant strains colonized mice similarly. These data led Robinson et al. to conclude that Stx2 produced by the wild-type complemented the *stx*<sub>2</sub> defect in the mutant *in vivo* [149]. Stx was also shown to facilitate colonization of C57BL/6 mice with *E. coli* O157:H7 [150]. Calderon-Toledo et al. reported higher *E. coli* O157:H7 counts in the feces of str-treated mice infected with a Stx2-producing strain when compared to a Stx2-nonproducing strain [150].

More recently, we confirmed the capacity of Stx2 to promote *E. coli* O157:H7 colonization in the ICF mouse model [151]. In our study, we exogenously supplied Stx2 to an *stx*<sub>2</sub> mutant *E. coli* O157:H7. This addition of Stx2 *in trans* to the *stx*<sub>2</sub> mutant restored the capacity of the mutant to colonize the intestines of mice with an ICF. Furthermore, we discovered that anti-Stx2 neutralizing antibodies administered prior to infection significantly decreased the likelihood that a mouse would become highly colonized with *E. coli* O157:H7. We also confirmed that these Stx2-neutralizing antibodies could protect *E. coli* O157:H7-infected mice against manifestations of Stx2-mediated disease such as weight loss and death. Moreover, mice repeatedly immunized with a Stx2 toxoid developed a Stx2-neutralizing fecal antibody response; when challenged with *E. coli* O157:H7, immunized mice shed fewer organisms in their feces for a shorter duration than did sham-vaccinated control mice [151].

**5.2. The Role of Gb3.** For Stx to intoxicate a host, either directly via injection or by infection with STEC, the toxin must gain entry into host cells by binding to the Stx receptor Gb3. Thus, for an animal model of Stx intoxication to be relevant, surface-expressed Gb3 must be present in the host. In addition, the degree of receptor expression by particular cells dictates the focus of toxin damage. In humans, the gene that encodes the Gb3 synthase is expressed to varying levels in the heart, kidney, spleen, liver, lung,

stomach, small intestine, and colon [152, 153]. Furthermore, Gb3-expressing cells have been identified in the colon, endothelium, kidney, and CNS [153–160].

In mice, Okuda and colleagues used Gb3 knockout mice to demonstrate that the effects of Stx injection are indeed dependent on the expression of Gb3 [161]. The concept that Gb3 may be differentially distributed in murine tissues in comparison to human tissue was supported by the work of Fujii et al., who reported that the Gb3 synthase gene is expressed most highly in the kidney and lungs, with lower expression levels in the brain, heart, gastrointestinal tract, and spleen [162]. They and others have shown that the actual glycolipid Gb3 is present in the kidney (tubules but not glomeruli, despite reports of toxin-associated glomerular damage [73, 76, 139, 144, 161]), as well as the lung, brain, and spleen [69, 161, 163, 164]. In summation, even though the relative Gb3 expression levels between humans and mice are not known, the overall tissue distribution of Gb3 between adult humans and mice appears to be similar.

**5.3. Stx and Pathogenesis.** The use of mouse models to explore the role of Stx, arguably the most important virulence factor of *E. coli* O157:H7, in colonization and disease has served to provide a better understanding of *E. coli* O157:H7 pathogenesis. A model has been postulated whereby the bacteria enter the gastrointestinal tract and then transit to the primary site of colonization (the cecum in mice [80, 99]) where they then elaborate Stx2. Data generated *in vitro* with HEp-2 cells indicate that the action of the toxin causes an increase in cell surface-localized nucleolin [149]. Nucleolin is a eukaryotic-binding partner for intimin and thus a eukaryotic receptor for intimin-expressing *E. coli* O157:H7 [48, 52]. Intimin from the *E. coli* O157:H7 and cell-surface nucleolin interact to provide an initial point of attachment for the bacterium. Once the organism associates with the host cell via the intimin/nucleolin interaction, intimin binds to Tir, the bacterially encoded intimin receptor that is translocated by the TTSS into the host cell. The intimin/Tir interaction, coupled with the effects of other TTSS effector proteins, culminates in the formation of the characteristic A/E lesion/pedestal (Figure 4(a)).

Stx2 production may occur during transit of the organism through the intestines. Stx2 was found in the cecum and at other sites where *E. coli* O157:H7 were abundant early in infection [80]. Furthermore, in our study, Stx2 was more abundant in the cecal and large intestinal luminal contents than in the corresponding tissue; these observations may indicate that toxin is absorbed into the circulation from both the cecum and the large intestine [80]. At least a portion of the toxin produced within the gastrointestinal tract enters the bloodstream because (1) renal tubular damage was evident in infected mice, and toxin was detected in the kidney of at least one infected animal [80], and (2) parenteral inoculation of antitoxin antibody protected mice from the systemic manifestations of disease [151].

Given that Stx not only acts systemically but also facilitates *E. coli* O157:H7 colonization [151], a therapeutic strategy directed against toxin has potential to be quite

beneficial. To this end, we demonstrated that toxin provided exogenously to a toxin-null mutant resulted in increased *E. coli* O157:H7 colonization [151]. Thus, toxin, produced by the wild-type or provided exogenously to the mutant, had the capacity to increase the colonization levels of *E. coli* O157:H7 in mice (Figure 4(b)). Additionally, anti-toxin antibody, either administered to animals or actively generated following vaccination, reduced the overall levels of *E. coli* O157:H7 shed by mice and protected animals from systemic manifestations of disease (Figure 4(c)).

## 6. Conclusions

As a result of the increased rate of HUS over the last several years and the lack of therapies for treatment of HUS, further research is necessary to define mechanisms involved in the pathogenesis of *E. coli* O157:H7 and to identify potential disease prevention strategies and therapeutics. The application of animal model systems is vital to achieve these goals. While no one model recapitulates all features of *E. coli* O157:H7 infection, many valuable mouse models have been developed that permit exploration of *E. coli* O157:H7 pathogenesis and that can help pinpoint the means by which *E. coli* O157:H7 infection and/or disease can be controlled or prevented.

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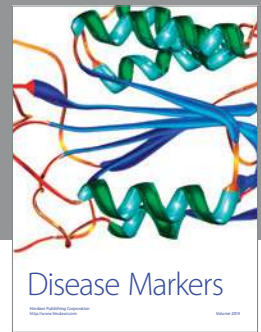
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