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MINIREVIEW

Animal Models for Salmonellosis: Applications in Vaccine Research

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Salmonellosis remains an important cause of human disease worldwide. While there are several licensed vaccines for *Salmonella enterica* serovar Typhi, these vaccines are generally ineffective against other *Salmonella* serovars. Vaccines that target paratyphoid and nontyphoidal *Salmonella* serovars are very much in need. Preclinical evaluation of candidate vaccines is highly dependent on the availability of appropriate scientific tools, particularly animal models. Many different animal models exist for various *Salmonella* serovars, from whole-animal models to smaller models, such as those recently established in insects. Here, we discuss various mouse, rat, rabbit, calf, primate, and insect models for *Salmonella* infection, all of which have their place in research. However, choosing the right model is imperative in selecting the best vaccine candidates for further clinical testing. In this minireview, we summarize the various animal models that are used to assess salmonellosis, highlight some of the advantages and disadvantages of each, and discuss their value in vaccine development.

Animal models are indispensable tools for assessing candidate vaccines, with preclinical animal safety, immunogenicity, and efficacy data being prerequisite for regulatory agencies such as the U.S. Food and Drug Administration (FDA) prior to undertaking early-stage clinical trials. However, not all animal models are created equal. While some models are highly robust and closely mimic clinical infection, others are contrived and far removed from clinical relevance. Choosing the right animal model for the vaccine under investigation is imperative in determining its safety and/or effectiveness.

Salmonella spp. are often used as model organisms to study bacterial pathogenesis and host-microbe interactions, due to the ability of certain *Salmonella* serovars to readily infect animals. As such, there are a myriad of animal models available to vaccine developers. These range from colonization or lethality models to those involving complex surgical techniques (Table 1). The choice of model is often related not only to relevance but also cost, ethics, housing requirements, and the availability of appropriate technical expertise. While most researchers utilize one model or another, integration of data from multiple animal models can provide a more complete understanding of the safety of a candidate vaccine and/or predict how a potential vaccine may perform in humans. In this minireview, we provide background on *Salmonella* clinical syndromes and pathogenesis and then summarize the various animal models that have been used for *Salmonella* vaccine research. We include a brief discussion of the technical aspects, advantages, and limitations of each model, followed by a review of the literature surrounding its use in vaccine evaluation. While there has been considerable development for veterinary *Salmonella* vaccines, here we will focus solely on vaccines and models for human salmonellosis.

SALMONELLA CLINICAL SYNDROMES

Salmonella enterica subsp. *enterica* is a Gram-negative intracellular bacterium that is the etiological agent of two clinical syndromes: enteric fever and gastroenteritis. Enteric fever is caused by three serovars, Typhi, Paratyphi A, and Paratyphi B. The clinical syndrome is characterized by fever, coughing, nausea, vomiting, and headache (1). Patients may also suffer from enlargement of the spleen and liver and bradycardia. Typhoid fever most commonly affects children, young adults up to 25 years of age, and the elderly (2). Nontyphoidal *Salmonella* (NTS), such as *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis, typically cause gastroenteritis in healthy human adults. Symptoms manifest as fever, abdominal cramping, and diarrhea (3). In the United States and other developed countries, NTS is commonly associated with large foodborne outbreaks (4). In Africa, NTS is highly associated with invasive disease and causes septicemia in young infants and HIV-infected adults and children (5, 6).

SALMONELLA PATHOGENESIS

Salmonella spp. are ingested through contaminated food and water. While typhoidal *Salmonella* serovars are human host restricted, NTS serovars have a broad host tropism and can colonize many animals, including chickens, pigs, cows, reptiles, and household pets (7). The bacterium possesses effective acid tolerance mechanisms and upon ingestion will survive passage through the low-pH conditions of the stomach. In the small intestine, the bacterium adheres to and invades the intestinal epithelial cells. The bacterium can then be transported through the mucosa, largely via microfold (M) cells, to access the submucosa and underlying lymphoid tissue (8). Macrophages within the lymphoid tissue engulf *Salmonella* cells but are unable to kill them due to the ability of the bacteria to interfere with phagosome-lysosome fusion (9). *Salmonella* then resides and proliferates in these immune cells (10). Invasive infection proceeds by dissemination from the intestinal mucosa, causing bacteremia and growth in distant organs, such as the spleen, liver, and gallbladder (1). Noninvasive NTS infections remain localized to the gastrointestinal tract, causing inflammation of the mucosa and secretory diarrhea (3, 11).

The pathogenic elements used by *Salmonella* to establish and

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TABLE 1 Examples of animal models for *Salmonella* infection

Model	*Salmonella* serovar(s)	Disease modeled	Symptoms and readouts	Vaccine safety and efficacy testing?	Reference(s)
Immunocompetent mouse	Typhimurium, Enteritidis, Dublin, Newport, Choleraesuis, Typhi, Paratyphi A, Paratyphi B	Invasive salmonellosis	Fever, malaise, wt loss, systemic spread to organs, death	Yes	Summarized in reference 36
Immunodeficient mouse	Typhimurium	Invasive salmonellosis	Fever, malaise, wt loss, systemic spread to organs, death	Yes	56, 62–65
Humanized mouse	Typhi	Typhoid fever	Colonization, systemic spread to organs, persistence	No	70, 71
Opium- treated guinea pig	Typhimurium	Invasive salmonellosis	Intestinal pathology, systemic spread to organs, death	No	73, 74
Rat	Typhimurium, Enteritidis	Invasive salmonellosis	Weight loss, malaise, systemic spread to organs, death	Yes	75
Rabbit	Enteritidis, Paratyphi A	Invasive salmonellosis	Systemic spread, fever, wt loss, death	No	81, 82
Streptomycin-treated mouse	Typhimurium, Enteritidis, Dublin, Pullorum	Gastroenteritis	Colonization, systemic spread to organs, intestinal pathology	Yes	143
Suckling mouse	Typhimurium	Gastroenteritis	Fluid accumulation	No	90
Calf	Typhimurium, Dublin	Gastroenteritis	Fever, wt loss, diarrhea, intestinal pathology, death	Yes	101
Ileal loop (calf, rabbit, pig, primate)	Typhimurium, Dublin	Gastroenteritis	Fluid accumulation, intestinal pathology	No	15, 106–108
Chimpanzee	Typhi	Typhoid fever	Fever, malaise, wt loss, diarrhea, intestinal pathology	Yes	117, 118
Rhesus macaque	Typhimurium	Gastroenteritis	Fever, malaise, wt loss, diarrhea, intestinal pathology	Yes	98, 115, 116
C. elegans	Typhimurium, Enteritidis, Dublin	Invasive salmonellosis	Persistence, death	No	124–126
G. mellonella	Typhimurium	Invasive salmonellosis	Death	No	130
Zebrafish	Typhimurium	Invasive salmonellosis	Persistence, death	No	132

propagate infection are relatively well known and are found in large part on *Salmonella* pathogenicity islands (SPIs). Twenty-three SPIs have been described to date (12), with some being found universally in *Salmonella* and others being associated with a subset of strains. Each SPI is associated with certain elements of the bacterial pathogenic process, a subset of which are described below. The ability of *Salmonella* to manipulate host cell function is accomplished by expression of type III secretion systems (TTSS), which are capable of injecting bacterial proteins directly into the host cell cytoplasm. SPI-1 encodes a TTSS that enables invasion of epithelial cells. Once the bacteria have been transported through the mucosa and are localized within macrophages, they employ the TTSS on SPI-2 to evade lysosomal fusion and set up residence in a specialized niche termed the *Salmonella*-containing vacuole (SCV). These two pathogenicity islands, therefore, encode two of the major virulence properties of these bacteria, providing the capacity for invasion and persistence. The less highly studied SPI-3 and SPI-4 are thought to be involved in intramacrophage survival, although both encode several additional genes of unknown function (13, 14). SPI-5 is involved in gastroenteritis-associated phenotypes, as it mediates an increase in intestinal fluid secretion (15). Finally, SPI-7, which is found in *Salmonella* Typhi (among others), encodes the Vi polysaccharide, which has been used in several different *Salmonella* Typhi vaccine formulations (16).

SALMONELLA VACCINES

There are currently three types of licensed vaccines for *Salmonella* Typhi, but none for the other serovars (17). The oldest of these vaccines is live attenuated *Salmonella* Typhi Ty21a, which was constructed by chemical mutagenesis and is unable to synthesize galactose due to a mutation in the *galE* gene (18). The vaccine is currently formulated as enteric-coated capsules which are taken orally on alternate days until three (in most world regions) or four (in the United States) doses have been received. This vaccine is highly efficacious against typhoid fever and shows some cross-protection against *Salmonella* Paratyphi B, but not against *Salmonella* Paratyphi A (19, 20). There are also two vaccines that target the Vi capsule polysaccharide: a polysaccharide-only vaccine and a conjugate vaccine. However, these are only effective against *Salmonella* Typhi and show no efficacy against other *Salmonella* serovars. As such, there is a need for additional vaccines to protect against paratyphoidal and nontyphoidal *Salmonella* infection.

The prevalence of *Salmonella* Paratyphi A has recently spiked in countries where it is endemic (21, 22), which has increased the interest in a bivalent typhoid-paratyphoid vaccine. Only one *Salmonella* Paratyphi A vaccine, CVD 1902, has been evaluated in a phase 1 clinical trial (registered at ClinicalTrials.gov [https://clinicaltrials.gov] under registration number NCT01129453) (K. Kotloff, personal communication), and it was found to be safe. Several conjugate vaccine approaches are also under investigation in preclinical studies (23–25).

With regard to nontyphoidal *Salmonella*, the high incidence of invasive disease occurring in sub-Saharan Africa (approximately 227 cases per 100,000 children) (26), as well as the high case fatality rate of 20 to 25% (5, 27), have spurred a number of vaccine approaches targeting these organisms. These have included novel

live attenuated vaccine strains, conjugate vaccines, protein subunit vaccines, and the more recent generalized modules for membrane antigens (GMMA) approach (25, 28, 29). There is also renewed interest in a vaccine that can prevent *Salmonella* gastroenteritis in developed countries. While morbidity and mortality are generally low for these infections, the economic burden of salmonellosis in the United States is estimated to be $3.3 billion annually (30), making a *Salmonella* gastroenteritis vaccine economically viable. In addition, elderly patients are known to be especially susceptible to *Salmonella* gastroenteritis (31). Those in long-term-care facilities are particularly vulnerable, with a case-fatality rate up to 70 times greater than the general population (32, 33). As such, a vaccine that could protect this population specifically is also desirable. All of the *Salmonella* vaccines currently in development will need to be evaluated for safety and immunogenicity in preclinical studies. The animal models that have been used to date and/or are available for future vaccines are described below.

SYSTEMIC INFECTION SMALL ANIMAL MODELS

(i) Immunocompetent mouse models. The mouse model of invasive salmonellosis has been employed as the standard in the *Salmonella* pathogenesis and vaccine development fields for decades. There are many published reviews on these models, which have listed in great detail the experimental conditions, advantages, and caveats of the various models in use (34–37). For this reason, we will touch only briefly on systemic infection mouse models in this review.

Salmonella Typhimurium (and several other serovars) can infect mice, causing a systemic disease that resembles typhoid fever. Although many *Salmonella* Typhimurium strains are virulent in mice, the disease in mice (systemic) is vastly different from the clinical syndrome these strains generally produce in humans (gastroenteric). As such, *Salmonella* Typhimurium has traditionally been used to model human *Salmonella* Typhi infections, but it cannot be used to model human NTS gastroenteritis. However, using *Salmonella* Typhimurium in mice as a model for *Salmonella* Typhi is itself imperfect, as the two strains have diverged genetically and have dissimilar pathogenic processes (38). Regardless, these mouse models have been instrumental in increasing our understanding of *Salmonella* pathogenesis and the host immune response.

There are two basic archetypes of systemic infection mouse models for *Salmonella*. One uses mice that are intrinsically susceptible to *Salmonella* infections, and the other uses mice that are resistant. Susceptibility is bestowed by mutations in genes that are important for innate or acquired immunity to intracellular pathogens. These mutations include those in the divalent cation transporter Nramp, Toll-like receptor 4, and others. Among these susceptible strains, Nramp1-deficient strains ($Nramp1^S$; e.g., BALB/c, C57BL/6) have been used extensively to elucidate *Salmonella* pathogenesis. The Nramp1 transporter is an intracellular protein that is recruited to the endosome, where it functions as a transporter for divalent cations, including Fe^{2+} and Mn^{2+} (39). There are several ways this transporter is thought to impact *Salmonella* survival, including denying the bacterium access to iron and activating inflammatory responses such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (40–42). For susceptible ($Nramp1^S$) mice, infection via the orogastric route leads to a systemic infection and is ultimately lethal in several days or up to 2 weeks (depending on the dose). These mice succumb following parenteral infection with very low doses (<10 CFU) of highly virulent strains. Similar results can be produced with resistant ($Nramp1^R$) mice by using intraperitoneal or intravenous infection with moderate bacterial doses (e.g., 10^5 CFU), or via oral infection with very high bacterial doses (e.g., 10^8 CFU). In either case, infection leads to a systemic spread of the bacteria that is similar in clinical presentation to typhoid in humans (35). There are pros and cons to both the susceptible and resistant mouse models, as reviewed by Simon et al. (36). When the route of infection is important, susceptible mice may be preferred, as oral infection is the natural route for *Salmonella* infection. However, some resistant mouse strains, such as CD-1, have the advantage of being outbred stocks, which is useful for evaluating candidate vaccines, as the bias of major histocompatibility complex (MHC) type is diminished relative to that in inbred strains.

While typhoid pathogenesis and immunity can be investigated in the mouse by using *Salmonella* Typhimurium, for vaccine safety and efficacy studies experiments need to be performed with the ultimate vaccine strain. *Salmonella* Typhi and *Salmonella* Paratyphi A are human host restricted and are normally asymptomatic in mice. However, a lethal infection can be produced in mice by suspending the bacteria in 5 to 10% (wt/vol) hog gastric mucin and then injecting the suspension intraperitoneally (43–46). In immunization studies, dosing mice with live attenuated *Salmonella* Typhi vaccines was ineffective by the orogastric route but highly immunogenic when vaccines were given intranasally (47). By utilizing this combination of intranasal immunization and hog gastric mucin challenge, researchers have assessed the safety and efficacy of several live attenuated vaccines for *Salmonella* Typhi and *Salmonella* Paratyphi A. This includes *Salmonella* Typhi CVD 908, *Salmonella* Typhi CVD 908-*htrA*, and *Salmonella* Paratyphi A CVD 1902, which have been shown to be well tolerated and immunogenic in clinical trials (48–50) (K. Kotloff, personal communication) (clinical trial registration number NCT01129453 [https://clinicaltrials.gov]). However, while a *Salmonella* Typhi Δ*phoP* mutant was shown to be virulent in the hog gastric mucin model (51), this same mutation (*Salmonella* Typhi Ty800) was found to be attenuating and safe in a human clinical trial (52).

Although the systemic mouse model does not accurately recapitulate disease caused by either typhoidal or nontyphoidal *Salmonella* serovars, it has a number of advantages. First, the model is very robust, and the high lethality of the infection provides a strong endpoint from which vaccine efficacy can be calculated. The ease of working with mice also allows experiments to be performed quickly and with large sample sizes, increasing the statistical power of the studies. This allows vaccine researchers to quickly identify lead vaccine candidates and assess dosage, immunization schedule, and the requirement for adjuvants. Finally, these early experiments in mice may allow researchers to characterize correlates of protection, allowing them to establish whether the vaccine could be effective in humans. Many *Salmonella* vaccine candidates have been tested in this model, including whole killed, live attenuated, subunit, conjugate, and *Salmonella* live vector vaccines (28, 36, 53–55).

(ii) Immunodeficient mouse models. Immunocompromised mice have been extensively used in *Salmonella* pathogenesis studies to investigate the relative contributions of different elements of the immune system to bacterial clearance (56–61). In vaccine research, these models have been used both to test the safety of live

attenuated vaccines and to determine the ability of vaccines to elicit a response in immunodeficient hosts (56, 62–65).

Immunodeficiency is of particular concern for the development of live attenuated vaccines, as *Salmonella* vaccines are expected to be used in populations where coinfections or underlying pathologies affect immune status, and immunocompromised individuals may be susceptible to even significantly weakened vaccine strains. To address this, *Salmonella* mutants that were attenuated both in wild-type and ROS-deficient (*gp91*$^{-/-}$ *phox*) mice were identified (66). One attenuating mutation, Δ*cysE*, caused a delayed presentation of symptoms in immunodeficient mice compared to Δ*aroC* mutants yet was also protective (86 to 97% vaccine efficacy) in vaccination-challenge studies with wild-type mice. By incorporating mutations identified in this manner, researchers can be more confident in the safety of live attenuated vaccines in diverse populations.

One additional model that must be mentioned is the TLR11$^{-/-}$ mouse model. Mathur et al. reported in 2012 that TLR11-deficient mice (*tlr11*$^{-/-}$) are highly susceptible to infection with *Salmonella* Typhi (67). Knockout mice infected perorally with 5×10^8 CFU of *Salmonella* Typhi Ty2 developed a systemic illness similar to human typhoid. Those authors also showed that *tlr11*$^{-/-}$ mice could be protected from lethal challenge by intraperitoneal immunization of a heat-killed *Salmonella* Typhi vaccine. While this model would be a great step forward for *Salmonella* Typhi vaccine testing, issues have arisen surrounding its reproducibility. It has since been reported that five separate groups at four institutions have been unable to replicate these results (68). While standing by their results, the original authors have also noted high variability in the susceptibility of the *tlr11*$^{-/-}$ mice over time (69). Thus, whether this represents a useful model for *Salmonella* Typhi vaccine testing is yet to be established.

(iii) Humanized mouse models. Looking to the future, humanized mouse strains may become a useful tool for better predicting immune responses to *Salmonella* vaccines in humans, making the determination of vaccine efficacy and immune correlates simpler and more relevant. Humanized mouse models have recently been developed for *Salmonella* Typhi but may also be applicable to other *Salmonella* serovars. In these mice, human hematopoietic stem cells are introduced into an immunodeficient mouse background (70–72). This is sufficient to make the mice susceptible to *Salmonella* Typhi, permitting dissemination of bacteria throughout the spleen, liver, and gallbladder. Analysis of the immune response to infection identified upregulation of a number of human cytokines, in addition to the intrinsic mouse immune response, indicating that the introduced cells are able to respond to infection. This could be advantageous to vaccinologists who are looking to predict the human immune response to vaccination.

(iv) Other small animal models. In addition to the mouse model, there are also guinea pig, rat, and rabbit models of systemic *Salmonella* infection. The guinea pig model is similar to the mouse model in that animals succumb to a systemic illness within approximately 3 days. However, this model also induces intestinal inflammation and pathology, including blunting of the villi and disruption of the epithelial cell brush border (73, 74). Guinea pigs can be made susceptible to oral infection with *Salmonella* Typhimurium via the administration of opium following infection. While this model was important for initial studies of *Salmonella* pathogenesis, it is no longer widely used.

The rat model, on the other hand, is still in limited use. This model shares many parallels with the mouse model, including the systemic spread of bacteria, although death is rare unless overwhelming challenge doses are given (75, 76). As with mice, the rat strain that is used affects the outcome of an infectious challenge, with Hooded-Lister rats found to be particularly susceptible to systemic spread (75). Little work has been performed to characterize the contribution of bacterial virulence determinants to pathogenesis in either the guinea pig or rat models, although it is known that flagellum and fimbria genes are not implicitly required for virulence of *Salmonella* Enteritidis in rats (75). The rat model has been used to determine immune responses to *Salmonella* vector vaccines (77–79) and has in one case been used to establish immunogenicity and protection of a *Salmonella* Enteritidis ghost particle vaccine (80).

Two rabbit models have been described for *Salmonella*: one using peroral and the other intraperitoneal infection. The peroral model has been used to evaluate *Salmonella* Paratyphi A vaccine candidates. Four vaccine strains carrying mutations in *phoPQ* were assessed for reactogenicity and immunogenicity in orally immunized New Zealand White rabbits (81). One strain, MGN10028, was particularly well tolerated, even at very high doses (2×10^{10} CFU). Despite being less immunogenic than the other three (more reactogenic) strains, rabbits given two doses of this vaccine strain were protected from the most severe symptoms of challenge, including lethargy, anorexia, and decreased water intake.

More recently, an intraperitoneal rabbit model was established for nontyphoidal *Salmonella* (82). Animals were infected intraperitoneally with high doses (10^{12} to 10^{13} CFU) of *Salmonella* Enteritidis strain CVD J73 and monitored daily for signs of illness, including weight loss, fever, lethargy, and dehydration. There was a 100% attack rate in infected animals, with all showing some clinical signs of disease. Bacteria were also found in distant organs, such as the liver, spleen, heart, lungs, and kidneys. This model may be used in the future to model invasive NTS infections in vaccine studies.

GASTROENTERITIS MODELS

Unlike the systemic models described above, the following models are designed to mimic a natural human infection with NTS. The focus for these models is therefore on gastrointestinal symptoms, including diarrhea, dehydration, and intestinal inflammation and pathology.

(i) Streptomycin mouse model. To model gastroenteritis in mice, animals are pretreated with streptomycin to deplete the normal flora prior to orogastric infection (83). Several different serovars have been investigated using this model, including serovars Typhimurium, Enteritidis, Dublin, Gallinarum, and Pullorum (84). The depletion of the normal flora allows *Salmonella* to colonize the cecum and colon, where it grows rapidly to high density. *Salmonella* then invades the intestinal mucosa, causing localized inflammation, before spreading systemically. In addition to gut pathology, there is some evidence of increased water content in the stool. The response of the intestinal mucosa to infection in this model is similar to the bovine ileal loop and primate orogastric infection models (85). For these reasons, this model has been used to investigate the contribution of different mutations to disease in both the bacterium and the host. While this model is simple and easily implementable, it has some drawbacks, specifically in the

relatively limited level of fluid accumulation induced in mice. This makes it difficult to investigate fluid secretion phenotypes in this model. Thus, this model is an excellent tool for investigation of colonization and mucosal inflammation but may not be suitable for evaluation of fluid secretion and other gastroenteritis phenotypes.

The streptomycin mouse model has been used to test safety and efficacy of several live attenuated vaccines (86–88). In one example, researchers examined the efficacy of a *Salmonella* Typhimurium strain carrying a deletion of the zinc transport operon, *znuABC*. This vaccine strain had previously been shown to be safe and efficacious in the systemic mouse model (89). Mice immunized orally with 2×10^7 CFU of attenuated strain SA186 were shown to be protected from homologous challenge with virulent *Salmonella* Typhimurium post-streptomycin treatment (87). In addition to decreased mortality, immunized animals also showed decreased bacterial burden in the spleen and decreased bacterial burden and inflammation in the cecum.

An additional advantage of this model is that it can be performed using immunodeficient mice. In two separate studies, safety of *Salmonella* Typhimurium vaccine candidates was examined in streptomycin-pretreated mice carrying mutations affecting interferon gamma signaling (*ifng1*$^{-/-}$), complement activity (C3$^{-/-}$), tumor necrosis factor receptor signaling (*tnfr*$^{-/-}$), caspase-1 signaling (*casp1*$^{-/-}$), B and T cells (*rag1*$^{-/-}$), interleukin-10 signaling (*IL-10*$^{-/-}$), T cell costimulation (*CD40L*$^{-/-}$), and nitric oxide synthase (*cybb*$^{-/-}$ *nos2*$^{-/-}$) (86, 88). While the two vaccine candidates were sufficiently attenuated in some immunodeficient mouse strains (*cybb*$^{-/-}$ *nos2*$^{-/-}$, *tnfr*$^{-/-}$), they were still virulent in highly immunodeficient backgrounds, such as *rag1*$^{-/-}$ and *ifng*$^{-/-}$.

(ii) Suckling mouse model. Complementary to the streptomycin pretreatment approach, the infant mouse model has been used to specifically address fluid accumulation phenotypes for *Salmonella* mutant strains (90). In this model, suckling mice are infected intragastrically with *Salmonella* at a dose of up to 5×10^7 CFU. At 2.5 hours postinfection, the alimentary canals are removed and weighed to determine fluid accumulation. Results are presented as the ratio of alimentary weight to total body weight. Compared to mock-infected mice, mice receiving wild-type *Salmonella* had significantly higher alimentary weight ratios. In contrast, *Salmonella pef* (fimbrial) mutants were attenuated in their ability to cause fluid accumulation. These results were consistent with those from a mouse ileal loop experiment, suggesting that this model serves as an effective proxy for intestinal fluid accumulation caused by *Salmonella*. Due to the young age of the mice, this model is not appropriate for vaccine efficacy testing, but it may be useful for establishing vaccine safety.

(iii) Oral calf infection model. One of the most robust models for *Salmonella* gastroenteritis is the calf infection model. Cows are natural hosts for *Salmonella* Typhimurium as well as other serovars, such as *Salmonella* Dublin and *Salmonella* Newport (91). Infection of calves with *Salmonella* Typhimurium causes a limiting gastroenteritis characterized by diarrhea, dehydration, and intestinal pathology. Likewise, *Salmonella* Dublin infection produces extensive intestinal pathology; however, it also disseminates to sterile sites, such as the spleen and liver, leading to systemic illness (92). *Salmonella* Dublin is found in both young and adult cows, with subclinical infections in heifers being a cause of abortion (93). In contrast, *Salmonella* Typhimurium is virulent predominantly in calves under 2 months of age (94, 95). This matches the age-dependent susceptibility observed in humans, where children constitute the majority of invasive cases (4). The incubation period for both serovars is around 48 h in calves, similar to humans and nonhuman primates (96). In addition, the intestinal pathology observed upon infection of calves with *Salmonella* Typhimurium is similar to that seen in rhesus macaques and humans (97, 98). These factors combined make calves a relevant model for investigation of *Salmonella* pathogenesis. Many virulence factors for *Salmonella* have been identified or confirmed in this model (99). Interestingly, there are a number of discrepancies between the genes required for virulence in calves versus those identified in mice. The most striking examples of these are the *spv* operon, which is absolutely required for systemic spread in mice (100) but dispensable in calves (101), and SPI-2, mutants of which are highly attenuated in mice (102) but only mildly attenuated in calves (101). Both of these examples refer to genes that are important in the systemic phase of infection. In contrast, mutations in genes involved in the intestinal phases of infection, such as *pipA*, have been shown to be attenuating in calf ileal loop models (15) but not in a systemic mouse model (E. Higginson and S. M. Tennant, unpublished data).

Salmonella Typhimurium live attenuated vaccines carrying mutations in the *aro* gene locus have been shown to be safe and effective in multiple studies using the calf infection model (103–105). Specifically, Δ*aroA* and Δ*aroA* Δ*aroD* mutants were shown to be well tolerated and only transiently excreted in orally vaccinated calves (103, 105). In these two studies, vaccination was shown to be highly effective at protecting animals from diarrhea and ultimately death. Interestingly, while animals immunized with a heat-inactivated vaccine showed greater antibody responses to lipopolysaccharide (LPS) and outer membrane porins, those given the live attenuated *aro* mutant vaccines were significantly better protected (104). This model is useful in determining not only protection against gastroenteritis but also key vaccine characteristics, such as shedding and immune correlates.

(iv) Ileal loop model. The ileal loop model has been optimized for many different species, including cows, rabbits, pigs, and primates (15, 106–108). With this model, animals undergo a surgical procedure in which the ileum is tied into multiple equal loops. These loops are then injected with bacteria, and the animal is left to recover for up to 18 h. Within this closed system, fluid accumulates over the course of the incubation period. The level of fluid that accumulates can be used as a proxy for the induction of diarrhea. This system for measuring fluid secretion has been used for other bacterial pathogens (109–111), as well as toxins that act on the mucosa, such as cholera toxin (112) and toxin A from *Clostridium difficile* (113).

Besides fluid accumulation, other aspects of the early infection process can be assessed using this model, including inflammation of the mucosa, presence of immune cell infiltrate, and bacterial adherence and invasion (106). The major advantage of this model is the potential to assess multiple experimental conditions in the same animal, thus increasing the number of experimental replicates without increasing the number of animals required. However, the obvious limitation is that it can only model the early stages of the infection process.

This model has been used to assess safety of live attenuated vaccine candidates. Specifically, researchers identified one gene, *invA*, which when deleted led to significantly decreased fluid se-

cretion, tissue damage, and inflammation in ileal loops, compared to an *aroA* mutant and the wild-type parental *Salmonella* Typhimurium strain SL1344 (114).

(v) Nonhuman primate models. Due to the close genetic relationship with humans, nonhuman primates provide the most relevant model for determination of vaccine efficacy. *Salmonella* Typhimurium has been evaluated in rhesus macaques (98, 115, 116), while *Salmonella* Typhi has been tested in chimpanzees (117–119). In both models, animals are infected via the oral route and show symptoms similar to those in humans, such as diarrhea, weight loss, and fever. In a study published in 1970, researchers showed a decrease in clinical symptoms (fever, dehydration, weight loss) upon challenge of chimpanzees immunized with a live attenuated *Salmonella* Typhi vaccine (119). Unfortunately, due to the small sample size and high biological variation in response to challenge, there was difficulty in determining the relevant endpoint criteria for calculating vaccine efficacy. This remains a significant concern for those looking to use nonhuman primate models, for which group sizes are often restricted due to the high cost of such studies.

More recently, we used the rhesus macaque model to evaluate the live-attenuated *Salmonella* Typhimurium vaccine candidate CVD 1921. This novel strain was shown to be significantly attenuated, with decreased systemic spread, shedding, and clinical disease manifestations (116). Preliminary analysis of the humoral immune response to vaccination showed seroconversion for both anti-LPS and anti-FliC (flagellin) serum IgG in three (out of three) immunized rhesus macaques. We also showed that the vaccine was safe in simian immunodeficiency (SIV)-infected rhesus macaques. These promising preclinical data provide a strong rationale for advancement of this vaccine candidate. This ability to accurately model human disease and the potential interactions with other infections thus makes the primate model very persuasive in the submission of investigational new drug applications to regulatory authorities, a key step in advancement of promising candidates into clinical investigations.

INSECT, NEMATODE, AND FISH MODELS

Although experiments conducted in higher vertebrates are more medically relevant, there are advantages to lower-order animal models. First, they are favorable from an animal use ethics standpoint. Second, the model organisms can be easily propagated in the laboratory, thereby decreasing costs and increasing the number of replicates. Finally, they can be used in situations where larger models are not practical, such as in space (e.g., on space shuttles or the International Space Station) (120) or when high-throughput analysis is required. It is in this capacity for use in a high-throughput screening assay that these models may be most useful in vaccine research.

Caenorhabditis elegans is the most highly used invertebrate model for bacterial pathogenesis. This free-living nematode has been used for virulence assessment of other pathogens, including *Pseudomonas aeruginosa* and enteropathogenic *Escherichia coli* (121–123). Worms fed a diet of *Salmonella* Typhimurium have significantly shorter time to death than worms fed laboratory *E. coli* strain OP50 (124, 125). This time-to-death model has allowed researchers to identify several genes that are required for virulence in *C. elegans*, including genes located in SPI-1, SPI-3, SPI-4, and SPI-5 (126).

The *Galleria mellonella* wax moth model has recently been used to evaluate the pathogenesis of bacterial species such as *P. aeruginosa*, *Acinetobacter baumannii*, and *Listeria monocytogenes*, among others (127–129). The wax moth larvae are infected by injecting bacterial suspensions into the hindmost proleg and assessing viability over time. When infected with *Salmonella* Typhimurium at various doses, larvae showed a clear dose-dependent response, with a 50% lethal dose of 3.6×10^3 CFU (130). This ability to control the bacterial dosage is one advantage over the *C. elegans* model, which relies on worms grazing on a bacterial lawn.

A second insect model is that of the fruit fly, *Drosophila melanogaster*. Flies infected by injection of 10^4 CFU of *Salmonella* Typhimurium succumbed to infection within 7 to 9 days (131). When infected with *Salmonella* Typhimurium, strains carrying mutations in SPI-1 (*orgA*::Tn*10*) or SPI-2 (*ssrA*::miniTn*5*), the time to death for flies was significantly longer; however, the bacterial load in the flies was almost 4-fold higher than for wild-type-infected flies. The authors postulated that the expression of secreted effectors in the wild-type infection activated the fly immune response, limiting bacterial burden but also causing damage and eventually death.

Zebrafish may be even more relevant to vaccine research, as they have both innate and adaptive immune systems. To assess virulence in real time, fluorescently tagged *Salmonella* Typhimurium cells are injected into the axial vein of zebrafish embryos (132). As with *Salmonella* infections in humans, the bacterium resides in macrophage-like cells, which are not able to clear the bacterium. Embryos given a comparatively high dose of bacteria (50 CFU) succumbed to infection within 2 days, while a percentage of those given lower doses survived, suggesting that the zebrafish are able to resist infection. When infected with *Salmonella* LPS mutant strains, the zebrafish embryos were able to quickly kill bacteria, thus showing an active immune defense.

COMORBIDITY MODELS

As a vaccine candidates moves closer to clinical testing, it becomes important to assess how the vaccine will fare in the broader population, where underlying comorbidities, such as HIV infection, may be present. In recent epidemiological studies in Malawi, the prevalence of invasive NTS (iNTS) infections was found to be intrinsically entwined with rates of HIV, malaria, and malnutrition (133). This supports earlier reports from the Gambia and Kenya, where decreases in iNTS infections were noted upon implementation of effective malaria interventions (134, 135). As such, determining the safety and efficacy of vaccines in populations with these underlying conditions represents a new challenge.

It is well known that underlying malnutrition significantly impairs a person's ability to respond effectively to vaccination. Although malnutrition models for other pathogens, such as *Cryptosporidium* and *Leishmania*, have been developed (136–138), as of yet there is no such model for *Salmonella*. These models use combined protein, iron, and zinc depletion to induce a state of malnutrition in mice. Malnourished mice are then compared to healthy controls in their ability to respond to immunization and mount an effective immune response to challenge. This is important because several *Salmonella* vaccines in development are live attenuated and thus rely on a functional gut immune system. These vaccines will likely be used in areas where malnutrition and other enteropathies are rife. Experience has taught us that oral vaccines often show reduced efficacy when introduced in developing countries (139). Thus, the development of a model to pre-

dict vaccine performance in malnourished children (and/or those with environmental enteropathy) is an area of *Salmonella* research that merits further investigation.

Several groups have examined the effect of malaria coinfection on *Salmonella* in mice. In one model, researchers used simultaneous intraperitoneal coinfection with *Salmonella* Typhimurium and *Plasmodium berghei* to establish bacterial and parasite loads, as well as investigate the impact on the immune response (140). Both the parasite and bacterial loads were found to be significantly increased in coinfection, compared to those from single infections. This increased burden was also associated with higher levels of tissue damage and mortality. The authors postulated that this outcome was driven largely by uncontrolled oxidative stress brought on by an unchecked immune response. In a second study, the authors recapitulated the likely infection process in areas where the pathogens are endemic by first inducing parisitemia and anemia in mice before orogastrically challenging animals with *Salmonella* Typhimurium (141). In this staggered secondary infection model, researchers similarly observed high systemic levels of bacteria and parasites, with bacterial replication in the liver significantly increased. This unchecked liver replication was linked to parasite-induced IL-10 production, which blunted the immune response to *Salmonella*.

Finally, HIV-*Salmonella* coinfection has been modeled using SIV-infected rhesus macaques. Animals primarily infected with SIV and subsequently infected with *Salmonella* Typhimurium had much greater systemic spread of bacteria than did immunocompetent animals (142). This was linked to the depletion of Th17 cells in SIV-infected animals. The contribution of Th17 cells to the intestinal barrier function was confirmed by those authors by using IL-17 knockout mice (*Il17ra*$^{-/-}$), which also showed increased systemic dissemination of *Salmonella* compared to immunocompetent mice. As described above, the SIV coinfection model has also been used to evaluate safety of a live attenuated *Salmonella* Typhimurium vaccine candidate, CVD 1921 (116). This vaccine was well tolerated in SIV-infected macaques, with the vaccine strain being cleared within 4 days. One out of three rhesus macaques seroconverted for LPS and flagella, proving that induction of an immune response is possible. This number could potentially be increased by vaccinating with additional doses, as is required for the *Salmonella* Typhi live attenuated vaccine Ty21a, which is administered in three or four doses.

CONCLUSIONS

The animal models for human salmonellosis are many and varied. Thus, there is a plethora of options for researchers looking to evaluate new vaccine candidates, but care must be taken to select the most appropriate model to address the research question at hand. While mouse models have been, and likely will continue to be, the most highly utilized models, integrating mouse data with results from other models may provide a more complete assessment of vaccine candidates. In addition, implementation of larger animal models and comorbidity models should be prioritized for vaccine candidates that have already been evaluated in smaller animal models, in order to advance these candidates toward clinical trials and to predict their efficacy in target populations.

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