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Vaccines | Review

Genetic engineering of *Salmonella* spp. for novel vaccine strategies and therapeutics

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ABSTRACT *Salmonella enterica* is a diverse species that infects both humans and animals. *S. enterica* subspecies *enterica* consists of more than 1,500 serovars. Unlike typhoidal *Salmonella* serovars which are human host-restricted, non-typhoidal *Salmonella* (NTS) serovars are associated with foodborne illnesses worldwide and are transmitted via the food chain. Additionally, NTS serovars can cause disease in livestock animals causing significant economic losses. *Salmonella* is a well-studied model organism that is easy to manipulate and evaluate in animal models of infection. Advances in genetic engineering approaches in recent years have led to the development of *Salmonella* vaccines for both humans and animals. In this review, we focus on current progress of recombinant live-attenuated *Salmonella* vaccines, their use as a source of antigens for parenteral vaccines, their use as live-vector vaccines to deliver foreign antigens, and their use as therapeutic cancer vaccines in humans. We also describe development of live-attenuated *Salmonella* vaccines and live-vector vaccines for use in animals.

KEYWORDS *Salmonella*, vaccines, anticancer therapy, *Salmonella*-delivered vaccines, recombinant-DNA technology

Salmonella spp. are Gram-negative, flagellated, intracellular, facultatively anaerobic bacilli classified as members of the family Enterobacteriaceae (1). The genus *Salmonella* is comprised of two species, *Salmonella enterica* and *Salmonella bongori*, of which species *S. enterica* is associated with global morbidity and mortality in humans (2). The species *S. enterica* is further classified into six subspecies including *S. enterica* subspecies *enterica* which includes more than 1,500 serovars based on typing of somatic (O) and flagellar (H) antigens (3). *S. enterica* serovars Typhi, Paratyphi A, and Paratyphi B are highly adapted to human hosts and cause typhoid and paratyphoid enteric fevers (4). The Global Burden of Disease Study estimated that there were 14.3 million cases and 135,900 deaths due to typhoid and paratyphoid fevers in 2017 (5). In contrast, non-typhoidal *Salmonella* (NTS) have broader host range and cause gastroenteritis in both animals and humans (4, 6). NTS serovars are mainly transmitted by animal-based foods such as contaminated raw eggs, beef, pork, and poultry (7–9). While NTS normally causes self-limiting gastroenteritis, certain serovars such as *Salmonella* Typhimurium and *Salmonella* Enteritidis are associated with invasive NTS (iNTS) disease in infants in sub-Saharan Africa (10). The global burden of iNTS disease is estimated to be 535,000 cases leading to 77,500 deaths in 2017 with most infections affecting infants and immunocompromised adults (5). Despite the significant burden of disease, there are no licensed vaccines against any NTS serovars for humans. The only vaccines licensed in the United States to protect against salmonellosis are Ty21a (Vivotif), a live oral vaccine, and Vi polysaccharide, which both prevent typhoid fever caused by *Salmonella* Typhi (11). Elsewhere, Vi conjugate vaccines are also available to prevent typhoid fever (11).

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S.M.T. and J.E.G. declare multiple patents to genetically engineer *Salmonella* for treatment and prevention of salmonellosis as well as other diseases.

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Salmonella can cause a variety of diseases in animals, including diarrhea, enteritis, septicemia, and even death. Among the numerous pathogenic *Salmonella* serovars, some are host-restricted, affecting a specific animal type, while others, known as generalist serovars, have a broad host range, and others are host-adapted and tend to be isolated only from certain hosts. For example, *Salmonella* Pullorum and *Salmonella* Gallinarum are host-restricted and specifically cause systemic infections in chickens; *Salmonella* Typhimurium is a generalist serovar and infects a variety of animals; *Salmonella* Choleraesuis and *Salmonella* Dublin commonly affect pigs and cattle, respectively, but can infect other animals (12, 13). Figure 1 shows the most common human-restricted and animal host-restricted serovars, generalist serovars, and serovars with a broad host range.

For host-restricted serovars that cause systemic infections in food animals, vaccines are used to prevent invasive disease, especially when these infections lead to significant production losses and death. Vaccines are particularly effective in mitigating *Salmonella* colonization and shedding in food animals, helping to limit the spread of NTS on farms and reducing the bacterial load from the animal products coming out of them. This reduction is vital since even stringent measures at slaughterhouses might not wholly prevent contaminated food from reaching the consumer. By reducing *Salmonella* levels in animals, vaccines can decrease the risk to public health, such as reducing contamination of poultry products and environmental sources (14). In addition, they may indirectly reduce the risk of antimicrobial resistance (AMR) development and spread by diminishing the need for antibiotics to treat salmonellosis. From a "One Health" perspective, vaccines against multiple serotypes of NTS with protective efficacy in multiple susceptible host species are desirable. However, the vaccines currently licensed for use in food animals generally only protect against a single serovar which limits their efficacy in environments where many serovars are circulating.

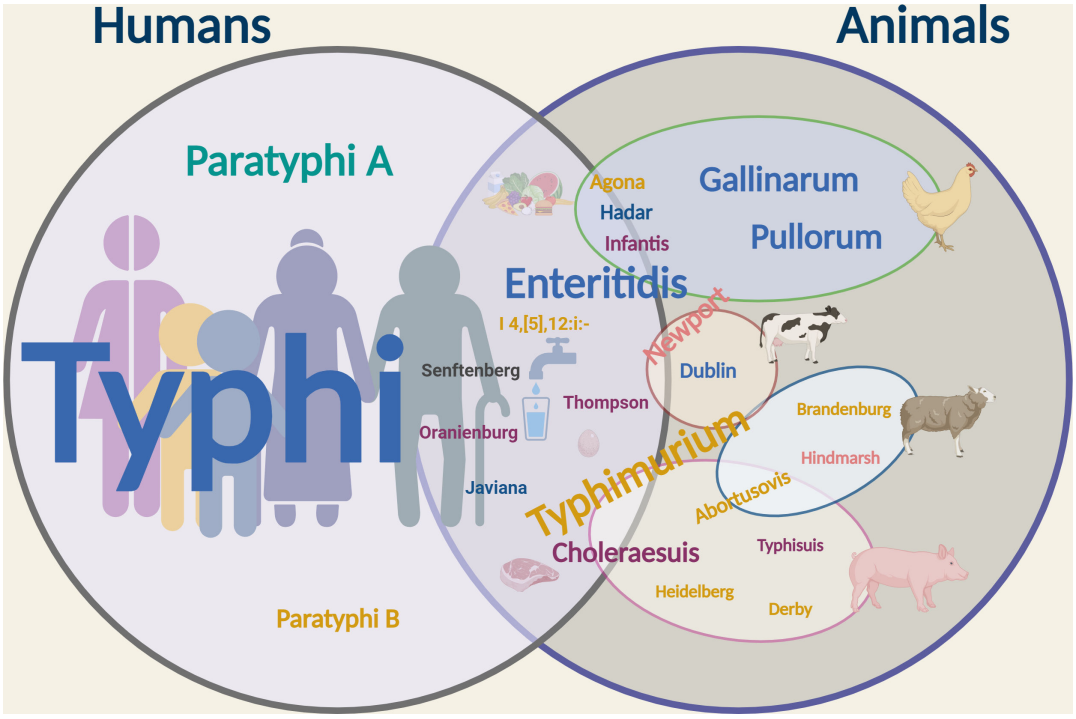

FIG 1 Human- and animal-associated *Salmonella* serovars. *Salmonella* Typhi, *Salmonella* Paratyphi A, and *Salmonella* Paratyphi B are human host-restricted (indicated in the left circle). NTS infect a wide variety of hosts (indicated in the right circle) including humans (middle). The text color indicates the serogroup: cyan, serogroup O:2; yellow, serogroup O:4; purple, serogroup O:7; light pink, serogroup O:8; blue, serogroup O:9; and dark grey, serogroup O:1,3,19. The font size of the serovar indicates its prevalence relative to other serovars. Common NTS serovars are depicted with the agriculturally important animal they are most closely associated with respect to disease burden. Serovars that cause infection in more than one animal span multiple spheres. Created by Biorender.com.

Salmonella is a well-studied model organism, which is easy to manipulate, and its pathogenic mechanisms are well understood. This has enabled the rational design of live-attenuated *Salmonella* vaccines (15, 16). Additionally, *Salmonella* can be genetically engineered to express single or multiple heterologous antigens from other bacteria, viruses, parasites, or eukaryotes, or they can be manipulated to serve as a source of antigen for *Salmonella* vaccines (17–19). In this review, we will describe genetically engineered *Salmonella* for use as live vaccines, live-vector vaccines, or reagent strains for purification of components used in parenteral vaccines for use in humans, their use in vaccination against cancer in humans, and their use as vaccines and live-vector vaccines in animals.

LIVE-ATTENUATED *SALMONELLA* VACCINES FOR HUMAN USE

Live-attenuated *Salmonella* vaccines are strains which are weakened by stable mutations, allowing them to colonize the target host transiently while eliciting a protective immune response. Live-attenuated vaccines have several advantages over other vaccine formats: (i) they mimic natural infection and therefore induce cell-mediated immunity in addition to antibody responses, (ii) they have the potential to induce mucosal immunity, (iii) they can be orally administered to large communities with no risk of hazardous waste, and (iv) they have relatively lower cost and easy storage (20, 21). Attenuated *Salmonella* strains can be constructed by well-defined deletions, insertions, or disruptions in virulence- and metabolism-related genes of pathogenic strains using site-directed mutagenesis. However, finding the perfect balance between vaccine immunogenicity and reactogenicity is a challenge (22). Below, we discuss the most widely and extensively characterized attenuation strategies. Candidate *Salmonella* vaccines related to each strategy are summarized in Table 1.

Auxotrophic mutants

Live-attenuated *Salmonella* vaccine strains with deletions in genes involved in biosynthesis of aromatic amino acids (*aroA*, *aroC*, and *aroD*), purines (*pur*), or guanine (*guaBA*) metabolic pathways are auxotrophic as they are unable to synthesize metabolites required for growth and are thus avirulent (56). Several pre-clinical studies have shown that *Salmonella* vaccine strains with mutations in *aro* genes have good potential as live-attenuated vaccines (56–58). For example, CVD 908 (*Salmonella* Typhi Δ*aroC* and Δ*aroD*) was well tolerated and highly immunogenic in clinical trials although subjects developed silent vaccinemia after vaccination (23, 24, 59).

Deletion of *guaBA*, which results in the inability of the bacterium to synthesize the nucleotide guanine, has been used as an attenuating mutation in multiple serovars, including Typhimurium, Enteritidis, Paratyphi A, Paratyphi B, and Newport (32, 34, 37, 38, 53). CVD 1931 (*Salmonella* Typhimurium D65 Δ*guaBA* Δ*clpX*) and CVD 1944 (*Salmonella* Enteritidis R11 Δ*guaBA* Δ*clpX*) are live-attenuated iNTS vaccines with deletion mutations in the *guaBA* and *clpX* genes. Both vaccines were proven to be immunogenic and protective against lethal challenge with the homologous serovar in mice (37). The live-attenuated *Salmonella* Paratyphi A vaccine (CVD 1902), (Δ*guaBA* Δ*clpX*), was evaluated in a Phase 1 clinical trial (NCT01129453; ClinicalTrials.gov), and results suggested that CVD 1902 was well tolerated and elicited $CD8^+$ and $CD4^+$ T-cell responses following a single dose [10^9 or 10^{10} colony-forming units (CFUs)] in human volunteers (32, 33). The *Salmonella* Paratyphi B vaccine, CVD 2005, was shown to induce antibody responses (serum IgG) against *Salmonella* Paratyphi B lipopolysaccharide (LPS) and conferred significant protection from both homologous challenge with *Salmonella* Paratyphi B *sensu stricto* (90%) and heterologous challenge (42%) with *Salmonella* Paratyphi B Java (34). Fuche et al. (38) constructed the *Salmonella* Newport (O:8) live-attenuated CVD 1966 vaccine (Δ*guaBA* Δ*htrA*) which showed protection against *Salmonella* Newport infection in a mouse model.

Similarly, deletion of *purB* (required for *de novo* synthesis of purine nucleotides) has been used as an attenuating mutation in *Salmonella* Typhimurium. The *Salmonella*

TABLE 1 Recombinant *Salmonella* strains for human vaccines

Vaccine strategy	Target serovar(s)	Vaccine or strain name	Genetic modification	Phase	Reference
Live-attenuated *Salmonella* vaccines	*Salmonella* Typhi	CVD 908	*Salmonella* Typhi Ty2; Δ*aroC*, Δ*aroD*	Phase 1	(23)
		CVD 908-*htrA*	*Salmonella* Typhi Ty2; Δ*aroC*, Δ*aroD* Δ*htrA*	Phase 2	(24–26)
		CVD 909	*Salmonella* Typhi Ty2; Δ*aroC*, Δ*aroD*, Δ*rpoS* P_{tac}-*tviA*, Δ*htrA*	Phase 2	(27)
		χ4073	*Salmonella* Typhi Ty2; Δ*cya* Δ*crp* Δ*cdt*	Phase 1	(28)
		Ty800	*Salmonella* Typhi Ty2; Δ*phoP* Δ*phoQ*	Phase 1	(29)
		M01ZH09	*Salmonella* Typhi Ty2; Δ*aroC* Δ*ssaV*	Phase1/2	(30, 31)
	Salmonella Paratyphi A	CVD 1902	ATCC 9150; Δ*guaBA* Δ*clpX*	Phase 1	(32, 33)
	Salmonella Paratyphi B	CVD 2005	CMF 6999; Δ*guaBA* Δ*clpX*	Pre-clinical	(34)
	Salmonella Typhimurium	WT05	TML; Δ*aroC* Δ*ssaV*	Phase 1	(35)
		LH1160	ATCC 14028; Δ*purB* Δ*phoP/Q*	Phase 1	(36)
		CVD 1931	D65; Δ*guaBA* Δ*clpX*	Pre-clinical	(37)
	Salmonella Enteritidis	CVD 1944	*Salmonella* Enteritidis R11; Δ*guaBA* Δ*clpX*	Pre-clinical	(37)
	Salmonella Newport	CVD 1966	*Salmonella* Newport; Δ*guaBA* Δ*htrA*	Pre-clinical	(38)
Live-vector vaccines—expression from plasmids	*Helicobacter pylori*	Ty21a (pDB1)	*Salmonella* Typhi Ty21a; Δ*thyA;* expresses *H. pylori* Urease A and B antigens from *thyA*-stabilized expression plasmids	Phase 1	(39, 40)
	Streptococcus pneumoniae	χ9640 (pYA4088)	*Salmonella* Typhi Ty2 $RpoS^+$; ΔP_{crp527}::TT *araC* P_{BAD} crp, ΔP_{fur81}::TT *araC* P_{BAD} fur, Δ*pmi-2426*, Δ(*gmd-fcl*)–26, Δ*relA198*::*araC* P_{BAD} *lacI* TT, Δ*sopB1925*, Δ*agfBAC811*, Δt*viABCDE10*, Δ*araE25*, and Δ*asdA33;* expresses *S. pneumoniae* surface protein antigen PspA from Asd^+ expression plasmid	Phase 1	(41)
Live-vector vaccines—expression from the chromosome	*Salmonella* Typhi and *S. sonnei*	Ty21a-Ss (MD77)	*Salmonella* Typhi Ty21a expressing *S. sonnei* O-antigen genes integrated into *tviD-vexA* locus on the chromosome	Pre-clinical	(42)
	Salmonella Typhi and *S. dysenteriae*	Ty21a-Sd (MD149)	*Salmonella* Typhi Ty21a expressing *S. dysenteriae* serotype 1 O-antigen genes integrated into *tviD-vexA* locus on the chromosome	Pre-clinical	(43)
	Salmonella Typhi and *S. flexneri*	Ty21a-2a (MD194)	*Salmonella* Typhi Ty21a expressing *S. flexneri* 2a O-antigen genes integrated into *tviD-vexA* locus on the chromosome	Pre-clinical	(44)
		Ty21a-3a (MD196)D	*Salmonella* Typhi Ty21a expressing *S. flexneri* 3a O-antigen genes integrated into *tviD-vexA* locus on the chromosome	Pre-clinical	(44)
	Salmonella Typhimurium and *Salmonella* Enteritidis	S1075	*Salmonella* Typhimurium S100; (Δ*abe:prt-tyv*$_{D1}$ Δ*crp* Δ*cya*) (O9); chromosomal deletion–insertion mutations resulting in O-serotype conversion	Pre-clinical	(45)
	Salmonella Typhimurium and *Salmonella* Choleraesuis	S1157	*Salmonella* Typhimurium S100; [Δ(*rmlB-wbaP*):(*wzy*$_{C1}$-*wzx*$_{C1}$) Δ*crp* Δ*cya*] (O7); chromosomal deletion–insertion mutations resulting in O-serotype conversion	Pre-clinical	(45)
	Salmonella Typhi, *Salmonella* Typhimurium, and *Salmonella* Enteritidis	S1163	*Salmonella* Typhimurium S100; (O9, Vi^+, ΔP_{tviA}::P_{ssaG} Δ*cya* Δ*crp*); chromosomal deletion–insertion mutations resulting in O-serotype conversion	Pre-clinical	(46)

(*Continued on next page*)

TABLE 1 Recombinant *Salmonella* strains for human vaccines (*Continued*)

Vaccine strategy	Target serovar(s)	Vaccine or strain name	Genetic modification	Phase	Reference
Live-vector vaccines mediating directed antigen delivery	*S. aureus*	MvP728 (p3635)	*Salmonella* Typhimurium NCTC 12023 Δ*htrA* Δ*purD* (carrier strain MvP728); pWSK29 P_{sseA} *sseJ*::lisA$_{51\text{-}363}$::HA (p3635); T3SS effector protein SseJ fused with *Listeria monocytogenes* LisA	Pre-clinical	(47)
		N19	*Salmonella* Typhimurium (Δ*aroA* Δ*pryF*) carrying PagC-*invB*-*sipA*-SaEsxA (ColE1 ori)	Pre-clinical	(48)
		N20	*Salmonella* Typhimurium (Δ*aroA* Δ*pryF*) carrying PagC-*invB*-*sipA*-SaEsxB (ColE1 ori)	Pre-clinical	(48)
	P. aeruginosa	SV9699	*Salmonella Typhimurium 14028 aroA551*::Tn*10* Δ*aroB*::Km^R; pWSK29-PsseA-SseJ-PcrV-FLAG (pIZ2267); T3SS effector protein SseJ fused with *P. aeruginosa* antigen PcrV	Pre-clinical	(49)
Designed to cause regulated delayed lysis	*Streptococcus pneumoniae*	χ8937 (pYA3685)	*Salmonella* Typhimurium UK - 1 *ΔasdA19::araC* P_{BAD} *c2* ΔP *murA7 ::araC* P_{BAD} *murA* Δ*(gmd - fcl) - 26;* Expression plasmid pYA3685 releases pneumococcal surface protein A (PspA) within the lymphoid tissues.	Pre-clinical	(50)
	M. tuberculosis	RASV χ11021	*Salmonella* Typhimurium ΔP$_{murA25}$::TT *araC* P$_{BAD}$*murA* Δ*asdA27*::TT *araC* P$_{BAD}$ *c2* Δ*araBAD23* Δ*(gmd-fcl)–26* Δ*pmi-2426* Δ *relA198*::TT *araC* P_{BAD} *lacI;* Asd$^+$/MurA$^+$ lysis vector expresses *M. tuberculosis* proteins Ag85A$_{294}$.	Pre-clinical	(51)
	Extraintestinal pathogenic *E. coli*	χ9558 (pYA4428)	*Salmonella* Typhimurium strain UK-1 (χ3761);Δ*pmi-2426* Δ*(gmd-fcl)–26* ΔP$_{fur81}$:TT *araC* P$_{BAD}$ *fur*ΔP_{crp527}:TT *araC* P_{BAD} *crp* Δ*asdA27*:TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198:araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811;* expresses *E. coli* EcpA and EcpD recombinant antigens	Pre-clinical	(52)
Reagent strains for parenteral vaccines	*Salmonella* Typhimurium and *Salmonella* Enteritidis	CVD 1925 (pSEC10 - *wzzB*) and CVD 1943	*Salmonella* Typhimurium I77 Δ*guaBA* Δ*clpP* Δ*fliD* Δ*fljB* (pSEC10-*wzzB*); *Salmonella* Enteritidis R11 Δ*guaBA* Δ*clpP* Δ*fliD;* OPS and flagellin for conjugate vaccines	Phase 2	(53, 54)
		STm$_G$ and SEn$_G$	*Salmonella* Typhimurium 1418 *tolR::aph; Salmonella* Enteritidis 618 *tolR::aph* for production of GMMA	Phase1/2	(55)

Typhimurium vaccine LH1160 (Δ*purB* Δ*phoPQ*) elicited robust vaccine-specific humoral and mucosal immune responses against *Salmonella* Typhimurium LPS and flagellin in adult volunteers after a single oral dose of 5–8 × 10^7 CFU (36).

Deletion and/or upregulation of virulence genes

The pathogenic mechanisms of *Salmonella* have been extensively studied, and multiple virulence genes have been targeted in live-attenuated vaccines. As described above, the *Salmonella* Typhi vaccine, CVD 908, elicited vaccinemia in volunteers and was attenuated further by deleting the *htrA* gene, which is a part of the stress response system and encodes a periplasmic serine protease required for survival in macrophages (24, 60–63). The resulting vaccine strain CVD 908-*htrA* (Ty2 Δ*aroC* Δ*aroD* Δ*htrA*) was well tolerated and showed robust immunogenicity in human volunteers in a Phase 2 study (25).

Another virulence gene which has been targeted is *ssaV*, which encodes a structural protein that is part of the *Salmonella* pathogenicity island 2 (SPI2) type III secretion system (T3SS), which is associated with virulence mechanisms of *Salmonella* infection (64). SsaV is an essential component of the T3SS machinery required for secretion of SPI2 effector proteins that mediate adhesion, invasion, and intracellular replication in host cells (65–67). The *ssaV* gene was deleted along with *aroC* in *Salmonella* Typhi and *Salmonella* Typhimurium to create vaccine strains, M01ZH09 and WT05, respectively. Both strains were well tolerated and highly immunogenic in a Phase 1 clinical study (30, 31, 35, 68). However, prolonged excretion of WT05 (up to 23 days) was observed (35).

Live oral *Salmonella* Typhimurium vaccines that are shed in stool for extended periods pose a significant safety concern as the live vaccine could be transmitted to household contacts. Live *Salmonella* Typhimurium vaccines with mutations in *shdA* and *misL*, which contribute to colonization of the intestine, have reduced shedding in mice but are still immunogenic and effective (69).

Virulence genes have also been upregulated in live-attenuated vaccines to enhance immune responses. For example, the Vi capsular polysaccharide is a key virulence determinant of *Salmonella* Typhi (70). To improve efficacy of the live *Salmonella* Typhi vaccine CVD 908-*htrA*, the strain was genetically engineered by replacement of the native P*tviA* promoter with the strong constitutive promoter P*tac* to constitutively express Vi (71). The resulting vaccine strain, designated as CVD 909, was found to be safe and immunogenic in a Phase 1 clinical trial and elicited serum IgG Vi antibody in addition to anti - LPS and antiflagellin responses (27).

Deletion of regulatory genes

The adenylate cyclase (*cya*) and the cyclic AMP receptor protein (*crp*) genes regulate the expression of many virulence genes in *Salmonella*. *Salmonella* Typhi vaccine strains carrying mutations in *cya* and *crp* have been shown to be safe and immunogenic in Phase 1 clinical trials (28). The *phoP–phoQ* two-component regulatory system has also been targeted in a live oral *Salmonella* Typhi vaccine (Ty800) which was shown to be well tolerated and highly immunogenic in a Phase 1 clinical trial (29). Similarly, Roland et al. (72) constructed the *Salmonella* Paratyphi A MGN10028 vaccine candidate carrying a deletion mutation in *phoPQ* and showed that this vaccine was attenuated and immunogenic in an oral rabbit model (72).

RECOMBINANT *SALMONELLA* AS LIVE-VECTOR VACCINES FOR HUMAN USE

Attenuated *Salmonella* strains have received attention over the last three decades as prospective carriers to deliver recombinant antigens to the immune system (22, 73). *Salmonella* can express heterologous antigens from other pathogens, stimulating innate immunity and activating the adaptive immune system (74, 75). Attenuated *Salmonella* Typhi and *Salmonella* Typhimurium vaccines have been engineered to express and deliver heterologous antigens from several pathogens including enteric bacterial pathogens, viral pathogens, and pathogenic protozoan parasites (76, 77). However, success with such hybrid vaccines depends on many factors including carrier strain

genetics, antigen expression strategies, immunization protocols, and human host factors (22, 75, 78, 79). Therefore, various strategies have been developed for the rational design of *Salmonella* live-vector candidates expressing heterologous antigens at sufficient levels to elicit an immune response (19, 22); candidate *Salmonella* vaccines related to these strategies are again summarized in Table 1.

Expression of foreign antigens from plasmids

The use of high-copy number plasmids for antigen expression by a carrier vaccine has been reported to improve foreign antigen-specific immunogenicity of the vaccine, but such plasmid-based expression is often associated with metabolic burden in the carrier vaccine, leading to over-attenuation and plasmid instability (80, 81). To overcome plasmid loss and reduced immunogenicity of the carrier vaccine, various "balanced lethal" plasmid stabilization systems based on *thyA* (39, 40), *asd* (41, 82), and *purB* (83) were developed and evaluated in clinical trials. Here, genes encoding essential factors required for growth are deleted from the bacterial chromosome and complemented with a wild-type copy of the corresponding gene on a plasmid, thereby constituting a balanced lethal combination in all surviving cells (84–86). For example, the enzyme aspartate β-semialdehyde dehydrogenase (Asd) is essential for the synthesis of several amino acids as well as the diaminopimelic acid (DAP) constituent of peptidoglycan in the cell wall of Gram-negative bacteria (85). Consequently, *Salmonella asd* mutant strains harboring multicopy *asd*-encoding plasmids constitute a balanced lethal combination in that all surviving cells would have to possess the recombinant asd^+ plasmid (77, 87). Using an *asd* balanced lethal system, highly immunogenic live-attenuated *Salmonella* Typhimurium strains were constructed for the heterologous expression of a viral peptide from hepatitis B virus (88) and F1-Ag and V-Ag antigens derived from *Yersinia pestis* (89).

Expression of foreign antigens from the chromosome

Chromosomal integration of expression cassettes has been successfully used to obtain stable expression of the foreign antigen in the absence of selective pressure (90–92). For example, Dharmasena et al. (42) developed a bivalent candidate vaccine against typhoid and shigellosis. Chromosomal integration of the *Shigella sonnei* O-antigen biosynthetic gene cluster (12 kb) into the genome of *Salmonella* Typhi Ty21a led to a recombinant Ty21a-Ss (Ty21a expressing *S. sonnei* O-antigen) strain, which was observed to be 100% genetically stable and elicited robust serum anti-*S. sonnei* LPS and anti-*Salmonella* Typhi LPS IgG antibody responses. Mice immunized with the Ty21a-Ss construct showed significant protection against a virulent *S. sonnei* challenge (42). This group further developed immunogenic candidate Ty21a vaccine vector strains expressing heterologous *Shigella dysenteriae* 1 O-antigens (Ty21a-Sd) or *Shigella flexneri* 2a (Ty21a-2a) or 3a O-antigens (Ty21a-3a) (43, 44).

In a study by Li et al. (45), the immunodominant O4 serotype of *Salmonella* Typhimurium was converted into O9, O7, and O8 serotypes by introducing chromosomal deletion–insertion mutations with an overall goal to develop live-attenuated *Salmonella* Typhimurium vaccines providing broad-spectrum protection against the major serovars responsible for NTS infections. Live-attenuated *Salmonella* Typhimurium vaccine strains S1075 (Δ*abe:prt-tyv*$_{D1}$ Δ*crp* Δ*cya*) (encoding serotype O9), S1157 [Δ(*rmlB-wbaP*):(*wzy*$_{C1}$-*wzx*$_{C1}$) Δ*crp* Δ*cya*] (encoding serotype O7), and S1116 [Δ(*wzx*$_{B1}$-*wabN*):(wzx$_{C2}$-wba*Z*) Δ*crp* Δ*cya*] (encoding serotype O8) were constructed using this novel O-serotype conversion strategy. All vaccinated mice survived challenge with 100 times the LD_{50} dose of the homologous wild-type *Salmonella* Typhimurium. Mice vaccinated with *Salmonella* Typhimurium S1075 (O9) also showed 100% protection against a heterologous lethal challenge with *Salmonella* Enteritidis. Similarly, complete protection was observed against *Salmonella* Choleraesuis in mice vaccinated with *Salmonella* Typhimurium S1157 (O7). Co-vaccination with *Salmonella* Typhimurium S1075 (O9) and *Salmonella* Typhimurium S1157 (O7) vaccine strains provided broad coverage and protective efficacy against *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Choleraesuis

(45). In a related study, live-attenuated *Salmonella* Typhimurium vaccine S1163 (O9, Vi^+, ΔP_{tviA}::P_{ssaG} Δ*cya* Δ*crp*) that is capable of producing both Vi capsular and O9 O-antigen polysaccharide antigens was shown to be immunogenic and conferred a high level of protection in mice against challenge with wild-type strains of *Salmonella* Typhimurium and *Salmonella* Enteritidis (46).

Directed presentation of antigens

Another important aspect for the successful delivery of heterologous antigen from a live carrier vaccine is the presentation of antigen to the appropriate immune inductive site(s), which will influence the type and strength of the immune response induced in the vaccinated host (93). Toward this end, *Salmonella* T3SS can be used to deliver foreign antigens (94). *S. enterica* encodes several virulence factors through SPI genes and, in particular, SPI1 and SPI2 that play important roles in different phases of pathogenesis. The SPI1-encoded T3SS delivers at least 13 effector proteins across the host cell membrane and plays an important role in contact and invasion of host intestinal epithelial cells (95). The SPI2 T3SS on the other hand delivers about 30 effector proteins into the host cytoplasm and causes systemic infections and proliferation within host cells (96). Therefore, SPI1 and SPI2 genes encode distinct T3SS that can be used for the efficient delivery of heterologous antigen to the cytosol of antigen-presenting cells leading to desired immune responses (97). T3SS-mediated heterologous antigen delivery by recombinant *Salmonella* has been shown to produce protective immunity against other bacterial and viral pathogens. However, selection of the appropriate SPI2 T3SS effector protein is a critical parameter for heterologous antigen fusions and construction of efficient recombinant vaccines (97). For example, Hegazy et al. (47) demonstrated that out of five SPI2 T3SS effector proteins tested for their efficiency of translocation of the model antigens ovalbumin and listeriolysin O (Llo) and elicitation of immune responses, including SifA, SteC, SseL, SseJ, and SseF, only an attenuated *Salmonella* Typhimurium strain construct MvP728 (p3635) in which SseJ was fused to listeriolysin ($Llo_{51–363}$) elicited potent T-cell responses *in vitro* as well as *in vivo* when tested in mice (47). In another study, an *Salmonella* Typhimurium-attenuated strain SV9699, expressing the SPI2 T3SS effector protein SseJ fused to the *Pseudomonas aeruginosa* antigen PcrV and placed under the transcriptional control of an *in vivo* inducible promoter P*sseA*, produced elevated levels of specific anti-PcrV IgG and protected mice against a lethal challenge with fully virulent *P. aeruginosa* (49). Finally, mice orally vaccinated with attenuated *Salmonella* Typhimurium vaccine strains N19 and N20, delivering Staphylococcal antigens SaEsxA and SaEsxB via the SPI-1 T3SS effector protein SipA, elicited both humoral and cell-mediated immune responses protecting mice against challenge with *Staphylococcus aureus* strains (48).

Programmed delayed cell lysis approach

For the rational design of recombinant attenuated *Salmonella* vaccine (RASV) strains with improved safety, Curtiss III et al. (50) developed a system of "programmed delayed cell lysis." Under this system, the *Salmonella* Typhimurium host vector strain χ8937(pYA3685) was constructed by introducing arabinose-regulated expression of the *asdA* and *murA* genes. The Asd enzyme is required for the synthesis of DAP, and the MurA enzyme controls the expression of muramic acid. Both DAP and muramic acid are components of the peptidoglycan layer of the bacterial cell wall. In addition, another deletion mutation Δ(*gmd-fel*) was introduced to repress the synthesis of colonic acid which is a polysaccharide made in response to stress associated with cell wall damage. Mice orally inoculated with RASV *Salmonella* strains did not show any lethality even when delivered at high doses; once in the host tissue, these strains undergo several rounds of replication, then programmed lysis occurs due to the absence of arabinose, and the strains are subsequently cleared from the lymphoid tissue (50).

RASV strains have also been used to deliver recombinant antigens to the immune system. Juárez-Rodríguez et al. (51) constructed the RASV strain χ11021 expressing

Mycobacterium tuberculosis antigens ESAT-6 and culture filtrate protein 10 (CFP-10) which induced both humoral and cellular immune responses in mice. The RASV χ11021 strain showed regulated delayed lysis and regulated delayed antigen synthesis *in vivo* and conferred significant protection against mycobacterial infection (51). In another study, a RASV strain was designed to provide protection against extraintestinal pathogenic *Escherichia coli* infections. Mice orally immunized with RASV χ9558 vaccine strain expressing *E. coli* major pilin (EcpA) and tip pilus adhesin (EcpD) antigens produced both anti-*E. coli* EcpA and EcpD IgG and IgA antibodies as well as anti-*Salmonella* LPS antibodies (52).

Genetically controlled induction of regulated attenuation, delayed antigen expression, and delayed lysis in these RASV strains is regulated at the level of transcription by sugar-inducible promoters that can easily be activated by the addition of one or more sugars to the growth medium during preparation of the live-vector vaccine. Following administration of the vaccine, the availability of these supplemented sugars vanishes *in vivo*. The vaccine strain gradually becomes attenuated as the strain replicates and intracellular levels of the inducing sugars drop to non-inducing levels; as intracellular sugar levels decrease, foreign antigen expression is derepressed, and high levels of antigen are efficiently released to immune inductive sites through delayed lysis of the vaccine strain.

A particularly remarkable example of this approach is found in a recent report by Wang et al. (98) in which attenuation, antigen synthesis, and delayed lysis of an *Salmonella* Typhimurium live-vector vaccine strain are simultaneously regulated by supplementation with three exogenous sugars. Delayed attenuation is accomplished at two levels. Chromosomal deletion of the *pmi* gene encoding phosphomannose isomerase (required for proper synthesis of LPS O-antigen side chains) attenuates the vaccine strain; propagation of the vaccine strain therefore requires supplementation with *mannose* to properly synthesize full-length LPS. A second level of attenuation is achieved by integrating a *rhamnose*-regulated promoter into the chromosome of the *Salmonella* Typhimurium live-vector strain to control expression of WaaL. Since Waal is an O-antigen ligase that is necessary to ligate polysaccharide to the lipid A-LPS core moiety, removal of rhamnose results in attenuation of the strain due to the complete loss of O-antigen. Coordinated regulation of delayed lysis and foreign antigen synthesis is accomplished by *arabinose*-regulated promoters controlling synthesis of Asd as well as MurA, both involved in biosynthesis of the bacterial cell wall, as well as arabinose-controlled expression of foreign antigen(s) from multicopy plasmids. Therefore, growth of the vaccine strain in medium supplemented with mannose, rhamnose, and arabinose results in metabolically fit live-vector strains displaying full-length LPS and no foreign antigen production *in vitro*; after immunization, vaccine strains become attenuated upon replication *in vivo*, with a rise in foreign antigen synthesis and efficient delivery of immunogens to immune inductive sites upon subsequent lysis of the vaccine. This novel vaccine design was applied to the development of a *Salmonella* Typhimurium-based poultry live-vector vaccine against necrotic enteritis caused by the Gram-positive pathogen *Clostridium perfringens* Type G (98). This approach could similarly be applied to *Salmonella* live-vector vaccines for use in humans.

RECOMBINANT *SALMONELLA* AS REAGENT STRAINS FOR HUMAN VACCINES

The development of various parenteral vaccines may be facilitated using attenuated *Salmonella* vaccines as a source of purified antigens. These vaccines include conjugate as well as outer membrane vesicle (OMV)-based vaccines. We have previously engineered *Salmonella* Typhimurium CVD 1925 (pSEC10 - *wzzB*) and *Salmonella* Enteritidis CVD 1943 reagent strains which could efficiently produce high concentrations of flagellin monomers and core and O-polysaccharide (COPS) for use in conjugate vaccines (53, 54, 99). *Salmonella* Typhimurium and *Salmonella* Enteritidis COPS:FliC glycoconjugate vaccines were constructed using components purified from these reagent strains and were shown to be immunogenic and protective against lethal challenge (99–103). These

vaccines have been combined with Bharat Biotech's Typbar TCV as a trivalent *Salmonella* conjugate vaccine which is currently being evaluated in a Phase 2 clinical trial (ClinicalTrials.gov ID NCT05784701).

OMVs are spherical exosomes of about ~25–250 nm in size, which are naturally released by Gram-negative bacteria (104). Their composition includes antigens found in the bacterial outer membrane, such as LPS, endogenous proteins, lipoproteins, and peptidoglycan; these vesicles are being investigated as vaccines (105). GSK Vaccines Institute for Global Health has developed OMV-based *Salmonella* vaccines which they call Generalized Modules for Membrane Antigens (GMMA) (106, 107). They have genetically engineered *Salmonella* Typhimurium and *Salmonella* Enteritidis to produce large amounts of GMMA by deletion of the *tolR* gene. In an elegant study reported by Micoli et al. (55), GMMAs from *Salmonella* Typhimurium (STm_G) or *Salmonella* Enteritidis (SEn_G) were constructed and directly compared to the homologous conjugate vaccine comprised of purified O-antigen conjugated to CRM197. Immunization with purified GMMAs induced higher anti-O-antigen IgG than glycoconjugate administered without Alhydrogel adjuvant; when the glycoconjugate vaccines were administered with Alhydrogel, antibody levels were similar. O-antigen-specific antibody responses were also accompanied by a reduction in bacterial colonization (55). As a result, such OMV-based vaccines against iNTS have now progressed to clinical studies (108).

RECOMBINANT *SALMONELLA* AS A THERAPEUTIC CANCER VACCINE IN HUMANS

The remarkable versatility of *Salmonella* used as a prophylactic vaccine against infectious diseases has also been investigated in attempts to develop therapeutic interventions against non-infectious diseases such as cancer (109–113). As opposed to prophylactic vaccines designed to *prevent* disease, immunotherapeutic *Salmonella* vaccines are intended to disrupt *established* disease caused by tumors and to block metastasis of cancer cells to new anatomical sites. Despite recent success with immune checkpoint inhibitors that break tolerance against tumors and reactivate the immune system to target non-solid tumors, attempts to apply this approach to solid tumor tissue have been disappointing in multiple clinical trials. Solid tumors present a daunting challenge to immunotherapeutic approaches due to the robust immunosuppressive microenvironment surrounding established tumors, which blocks productive innate and adaptive tumor-specific immunity. However, it has been shown in experimental murine models that systemic infection with *Salmonella* can result in specific colonization of tumor tissue and subsequent reactivation of innate immunity, leading to a robust cytotoxic T-cell response targeting the clearance of growing tumors.

Salmonella Typhimurium

Most pre-clinical studies conducted thus far have focused on intravenous (IV) administration of attenuated *Salmonella* Typhimurium to mice subcutaneously implanted with various tumor cell lines. The IV route has been intensively investigated based on work repeatedly demonstrating that systemic administration of attenuated strains of *Salmonella* Typhimurium can preferentially colonize tumor tissue at ratios of 1,000:1 versus normal tissues (114, 115), resulting in robust anti-tumor immunity with minimal off-target side effects. Although the mechanisms behind this observation have not been conclusively defined, several factors have been implicated including (i) the hypoxic necrotic environment of tumors preferentially supporting the growth of facultative anaerobic *Salmonella* Typhimurium strains, (ii) chemotaxis toward necrotic tissues releasing nutrients, and (iii) active invasion of tumor cells to escape immune surveillance (109, 112, 116).

To date, the most extensively studied attenuated strain of *Salmonella* Typhimurium examined in both pre-clinical mouse studies and subsequent clinical trials is VNP20009, carrying attenuating mutations in *purM* (conferring a purine auxotrophy) (117, 118), *msbB* (reducing the acylation of lipid A) (114, 118), and a cryptic 128-kb chromosomal

deletion that phenotypically suppresses the salt sensitivity associated with deletion of *msbB* (119). The *msbB* deletion is intended to reduce potential endotoxic inflammatory responses associated with IV delivery of therapeutic bacteria. This strategy of using attenuated strains of *Salmonella* Typhimurium to reduce potentially toxic systemic inflammatory responses associated with IV delivery, while maintaining tumor-specific inflammatory responses triggered by specific colonization of tumor tissues, is central to all systemic *Salmonella*-based immunotherapeutic approaches. For example, several excellent studies by Kong et al. (120, 121) have demonstrated that detoxification of lipid A through both dephosphorylation and reduced acylation strategies can be achieved while still maintaining the immunogenicity and efficacy of the modified vaccine strain. The most reactogenic form of lipid A is a hexa-acylated species that triggers TLR4-mediated proinflammatory responses through the TLR4-MD2-CD14 pathway (122). Significant reductions in proinflammatory cytokines can be achieved through genetic manipulation of the vaccine strain to synthesize only penta-acylated lipid A. Kong et al. (121) demonstrated that deletion of both *msbB* (addition of a C14 myristate acyl chain) and *pagP* (addition of a C16 palmitate acyl chain) reduced acylation of the wild-type *Salmonella* Typhimurium hepta-acylated lipid A, resulting in synthesis of only a penta-acylated but fully phosphorylated species of lipid A. Significant reduction in proinflammatory responses was demonstrated *in vitro* using cultured human Mono Mac 6 cells, while impressive antigen-specific immunity and efficacy against challenge were still observed in a mouse model.

Pre-clinical therapeutic efficacy studies in mice experimentally challenged with various tumor cell lines (e.g., B16F10 melanoma cells) and subsequently treated with VNP20009 strains strongly suggested that therapeutic success in clinical trials might be realized (114, 123). Despite such promising results in mice, when VNP20009 was tested intravenously in Phase 1 clinical trials, similar levels of efficacy were not observed. Toso et al. (124) reported that IV infusion of 3×10^8 CFU/m^2 of VNP20009 as a single dose into six patients with metastatic cancer during a 30-minute period proved to be remarkably safe, with no serious side effects. However, no clinical benefit from the treatment was reported; modest tumor accumulation was inconsistently observed, and no patient experienced objective tumor regression. It was also noted in this study that VNP20009 was cleared from the blood within 60 minutes of infusion. A second study was therefore initiated in which four additional metastatic cancer patients received infusions of 3×10^8 CFU/m^2 of VNP20009, this time administered IV over an extended 4-hour period in an attempt to maintain a high circulating level of bacteria intended to potentially improve tumor-specific accumulation. Again, the treatment was well tolerated, but bacteria were cleared from the bloodstream within 2 hours post-infusion, again with negligible tumor colonization and no objective clinical responses to treatment (125). Subsequent attempts to improve the therapeutic efficacy of engineered VNP20009 involving co-administration with chemotherapeutic agents again proved very promising in mouse models but failed to elicit objective tumor responses in patients (126, 127).

To improve both tumor targeting of therapeutic strains, intratumoral replication, and increased oncolysis, new auxotrophic strains of VNP20009 have been engineered for methionine dependency (128), taking advantage of the methionine metabolic dependence (Hoffman effect) common to cancer cells (129). In mouse models using both syngeneic and xenograft tumor cells, colonizing auxotroph effectively outcompeted tumor cells for methionine, resulting in tumor regression and suppression of metastasis (128). Still other strategies have explored co-administration of therapeutic *Salmonella* strains along with immune checkpoint PD-L1 inhibitors (130) or using bacteria delivering oncolytic prokaryotic (131) or eukaryotic payloads (132) to targeted tumor tissues. Excellent reviews discussing in greater detail these and other novel engineering strategies have been previously published (111–113, 127).

Salmonella Typhi

One parameter often overlooked in pre-clinical studies is the host specificity of the *Salmonella* serovar being investigated. Although wild-type *Salmonella* Typhimurium is pathogenic for humans, it is not human host-adapted and therefore cannot mount a sustained systemic infection in human hosts as it can when infecting mice and other warm-blooded animals. Failure in clinical trials of non-invasive attenuated strains of *Salmonella* Typhimurium administered intravenously may therefore be partially explained by an inherent inability of even the wild-type strain to persist in deep human tissues. However, the wild-type serovar *Salmonella* Typhi is exquisitely human host-restricted and can efficiently invade into deep tissues, leading to systemic typhoid disease and a potentially protracted carrier state in which pathogenic organisms are continually shed. This insight has spawned new efforts to explore attenuated strains of *Salmonella* Typhi, including the licensed typhoid vaccine Ty21a (Vivotif), as therapeutic agents against cancer.

Unmodified Ty21a has been recently tested against non-muscle-invasive bladder cancer (NMIBC) using intravesical introduction of the vaccine strain directly into the bladder. This approach is based on standard-of-care treatment of these cancers based on intravesical administration of the Bacillus–Calmette–Guerin (BCG) tuberculosis vaccine to prevent recurrence and/or progression (133, 134). Pre-clinical testing was conducted in an orthotopic MB49-bladder cancer model that closely reproduces NMIBC in mice (135, 136). Results indicated that Ty21a was more effective than BCG at inducing regression of established bladder tumors after intravesical administration. When tested in Phase 1 clinical trials by intravesical administration of Ty21a into patients with NMBIC, the treatment was found to be safe, with no serious side effects and a lower risk of undesirable bacterial persistence than in patients treated with BCG (137).

Phase 1 clinical trials have also been reported in which Ty21a was used as a DNA vaccine delivery platform (138–140). The therapeutic vaccine strain VXM01 was derived from Ty21a into which a DNA vaccine encoding human vascular endothelial growth factor receptor 2 (VEGFR2) was introduced. VXM01 was designed to compete with rapidly proliferating tumor tissues as an antiangiogenic therapy leading to the breakdown of nascent vasculature and tumor necrosis (141). Of note, no pre-clinical efficacy studies in mice were reported for this candidate therapeutic vaccine. In Phase 1 clinical trials, patients with advanced pancreatic cancer received four oral priming vaccinations of VXM01 at doses up to 10^{10} CFUs per dose in addition to gemcitabine as standard of care. No dose-limiting toxicities were reported, and modest reductions of tumor perfusion were observed in some patients, potentially linked to an observed reactivation of pre-existing antiangiogenic memory T cells triggered by the vaccine (139). A follow-up study was conducted to potentially improve T-cell responses by again orally priming patients with four doses of vaccine between 10^6 and 10^7 CFUs spaced every 2 days, followed by monthly booster doses for a further 6 months; patients also received gemcitabine across the duration of the study. Although some improvement in VEGFR2-specific T cells was again observed, no objective clinical improvements were noted (140).

LIVE-ATTENUATED *SALMONELLA* VACCINES FOR USE IN ANIMALS

As noted above, NTS cause a wide range of diseases in animals, particularly those of agricultural and economic importance. The range of host adaptation within NTS also makes agriculturally important animals a reservoir for human NTS infections. Vaccine development strategies, similar to those developed for *Salmonella* vaccines intended for human use, have been employed to limit the burden of NTS disease particularly in chickens, pigs, and cattle. Reduction in the colonization of *Salmonella* in various organs like the cecum, liver, and spleen and reduced shedding in feces are among the more critical readouts in assessing vaccine efficacy. Live-attenuated *Salmonella* animal vaccines, developed using various strategies, aim to provoke adaptive immunity while ensuring they cannot revert to a pathogenic form or pose environmental risks (142).

TABLE 2 Candidate and currently available *Salmonella* vaccines in agriculturally important animals

Target species	Vaccine serovar(s)	Vaccine name/strain	Target serovar(s)	Parental strains and genetic modification	Target gene class	Effect	Reference
Chickens	Enteritidis	Gallivac SE/Salmovac SE	Enteritidis and Typhimurium	*Salmonella* Enteritidis strain 441/014 adenine-histidine auxotroph	Metabolic pathways	Reduced colonization of liver and cecum following challenge with wild-type nalidixic acid resistant strains of *Salmonella* Enteritidis and *Salmonella* Typhimurium	(145)
		Z11 Δ*rfbG*	Enteritidis	*Salmonella* Enteritidis Z11; Δ*rfbG*	Virulence factor	Reduced colonization in liver, spleen, and cecum following challenge with wild-type *Salmonella* Enteritidis	(146)
		SE 147N Δ*phoP* Δ*fliC*	Enteritidis	*Salmonella* Enteritidis 147N; Δ*phoP* Δ*fliC*	Global regulators	Reduced cecal colonization and dissemination of *Salmonella* Enteritidis following challenge	(147)
		JOL919	Enteritidis	*Salmonella* Enteritidis JOL860; Δ*lon* Δ*cpxR*	Global regulators	Reduced colonization and protected from lethal challenge with *Salmonella* Enteritidis	(148)
	Typhimurium	Poulvac ST	Typhimurium	Lyophilized form of *Salmonella* Typhimurium strain AWC 591; Δ*aroA* Δ*serC*	Metabolic pathways	Protected from lethal challenge with *Salmonella* Heidelberg after multiple doses and partial protection after one dose. It also reduced shedding of wild-type *Salmonella* Typhimurium	(149, 150)
		MT2313	Typhimurium, Enteritidis, O:6,14,24:e,h:monophasic	*Salmonella* Typhimurium UK-1; *dam102*::Mud-Cm	Global regulators	Protected against colonization of homologous and heterologous challenge serovars	(151)
		χ3985	Typhimurium, Heidelberg, Agona, Bredeney	*Salmonella* Typhimurium strain X3761; Δ*cya* Δ*crp*	Metabolic pathways	Protected against colonization of homologous and heterologous challenge serovars	(152)
	Gallinarum	SG-VAC	Gallinarum	*Salmonella enterica*, subsp. *enterica*, serovar Gallinarum, Live strain SGP695AV	Information not publicly available	Information not publicly available	(153)
		Nobilis SG 9R	Gallinarum	*Salmonella* Gallinarum *rfaJ* point mutation	Virulence factor	Protected against fowl typhoid (*Salmonella* Gallinarum infection)	(154)
		χ11387	Gallinarum and Enteritidis	*Salmonella* Gallinarum 287/91 ΔP_{crp527} ::TT *araC* P_{BAD} *crp*	Metabolic pathways (amino acid synthesis, transporters, and metal uptake)	Reduced colonization of challenge strain in liver and spleen	(155)
		JOL2841	Gallinarum	*Salmonella* Gallinarum JOL 2624 (Δ*lon* Δ*cpxR* Δ*rfaL*); Δ*arnT*	Global regulators	Reduced colonization in liver and spleen following challenge with	(156)

(*Continued on next page*)

TABLE 2 Candidate and currently available *Salmonella* vaccines in agriculturally important animals (*Continued*)

Target species	Vaccine serovar(s)	Vaccine name/strain	Target serovar(s)	Parental strains and genetic modification	Target gene class	Effect	Reference
						Salmonella Gallinarum; reduced liver pathology compared to SG 9R vaccine and protected from lethal challenge	
		χ11797	Gallinarum	*Salmonella* Gallinarum 287/91; Δ*fur-712*	Metabolic pathways	Protected from lethal challenge against *Salmonella* Gallinarum	(157)
		1009 Δ*spiC* Δ*crp*	Gallinarum and Pullorum	*Salmonella* Gallinarum 1009; Δ*spiC* Δ*crp*	Virulence factor	Vaccine strain persisted in liver and spleen; reduced clinical symptoms and mortality after lethal challenge with wild-type *Salmonella* Gallinarum	(158)
		HG212	Gallinarum	*Salmonella* Gallinarum strain 9; Δ*aroA*	Metabolic pathways	Protected from lethal challenge after multiple doses, partially after one dose. Protective by intramuscular delivery but not oral delivery	(159)
	Pullorum	S06004ΔSPI2	Gallinarum and Pullorum	*Salmonella* Pullorum S06004; ΔSPI2	Virulence factor	Vaccine strain persisted in liver and spleen; reduced clinical symptoms and mortality after lethal challenge with the parental *Salmonella* Pullorum strain and wild-type *Salmonella* Gallinarum	(160)
		S06004 Δ*spiC* Δ*rfaH* (DIVA strain)	Pullorum	*Salmonella* Pullorum S06004; Δ*spiC* Δ*rfaH*	Virulence factor	Reduced colonization and protected from lethal challenge against wild-type parental *Salmonella* Pullorum	(161)
	Enteritidis, Infantis, Typhimurium	*Salmonella* Enteritidis 147 SPI1-*lon-fliC*, *Salmonella* Typhimurium 16E5 SPI1-*lon-fliC-fljB*, and *Salmonella* Infantis 18G6 SPI1-*lon-fliC-fljB*	Enteritidis, Infantis, Typhimurium, Agona, Dublin, and Hadar	*Salmonella* Enteritidis 147; ΔSPI1 Δ*lon* Δ*fliC* *Salmonella* Typhimurium 16E5 and *Salmonella* Infantis 18G6; ΔSPI1 Δ*lon* Δ*fliC* Δ*fljB*	Virulence factor	Reduced cecal colonization following homologous challenge; some protection observed against heterologous strains (*Salmonella* Hadar and *Salmonella* Dublin)	(162)
Turkey	Typhimurium	Elanco AviPro Megan Egg	Hadar and Infantis	*Salmonella* Typhimurium; live culture	Information not publicly available	Reduced intestinal colonization (cecum, cecal tonsils, and cloaca) and systemic dissemination to spleen following challenge with wild-type *Salmonella* Infantis and *Salmonella* Hadar	(163)
Swine	Cholerasuis	Enterisol Salmonella T/C	Choleraesuis and Typhimurium	*Salmonella* Choleraesuis variety Kurzendorf SC-54 strain and	Loss of *spv* on virulence plasmid by serial passage in neutrophils	Significantly reduced the seroprevalence and *Salmonella* isolation in pigs at slaughter	(164)

(*Continued on next page*)

TABLE 2 Candidate and currently available *Salmonella* vaccines in agriculturally important animals (*Continued*)

Target species	Vaccine serovar(s)	Vaccine name/strain	Target serovar(s)	Parental strains and genetic modification	Target gene class	Effect	Reference
				Salmonella Typhimurium strain 421/125			
		NOBL SC-54	Choleraesuis	*Salmonella* Choleraesuis variety Kurzendorf SC-54 strain	Loss of *spv* on virulence plasmid by serial passage in neutrophils	Significantly reduced clinical symptoms and bacterial burden in organs post-challenge with *Salmonella* Choleraesuis	(165)
		Argus SC/ST	Cholerasuis and Typhimurium	Δ*cya*, Δ*crp*	Metabolic pathways	Significantly reduced *Salmonella* infections in swine farm	(166)
		C500	Choleraesuis	*Salmonella* Choleraesuis strain C78-1; attenuated by chemical methods	Unknown	Significantly reduced *Salmonella* infections in swine farm	(167)
	Typhimurium	Δ*znuABC*	Typhimurium	*Salmonella* Typhimurium ATCC14028; Δ*znuABC*	Metabolic pathways	Induced humoral and cellular immune responses; reduced viability of vaccine strain in feces and the environment	(168)
		BBS 866	Typhimurium and Choleraesuis	*Salmonella* Typhimurium strain BSX 8 (χ4232); Δ*rfaH*; deletion mutation in multiple sRNA genes (Δ*omrA*, Δ*omrB*, Δ*rybB*, Δ*micA*, and Δ*invR*)	Virulence factor	Reduced colonization of wild-type strain after challenge, fecal shedding, and reduced clinical symptoms of infection post-challenge	(169–171)
		JOL911	Typhimurium	*Salmonella* Typhimurium strain JOL401;Δ*cpxR* Δ*lon*	Global regulators	Immunogenic in pregnant sows; protected piglets from clinical symptoms of wild-type *Salmonella* Typhimurium infection	(172)
		Salmoporc	Typhimurium	*Salmonella* Typhimurium strain 421/125; Δ*his* Δ*ade*	Metabolic pathways	Reduced tissue colonization of *Salmonella* Typhimurium challenge strain	(173)
Cattle	Dublin	EnterVene-d	Dublin	*Salmonella* Dublin *aro* mutant	Metabolic pathways	Reduced mortality risk in immunized calves	(174)
	Dublin	Sdu189 Δ*spiC* Δ*aroA*	Dublin	*Salmonella* Dublin Sdu189; Δ*spiC* Δ*aroA*	Virulence factor, global regulators	Protected mice from lethal challenge with wild-type parental strain	(175)
	Typhimurium	MT2313	Typhimurium and Newport	*Salmonella* Typhimurium UK-1; *dam102*::Mud-Cm	Global regulators	Reduced tissue colonization of *Salmonella* Newport challenge strain in calf organs; protected calves from clinical symptoms of *Salmonella* Newport infection	(176)

(*Continued on next page*)

TABLE 2 Candidate and currently available *Salmonella* vaccines in agriculturally important animals (*Continued*)

Target species	Vaccine serovar(s)	Vaccine name/strain	Target serovar(s)	Parental strains and genetic modification	Target gene class	Effect	Reference
Sheep	Abortusovis	SSM189	Abortusovis	*Salmonella* Abortusovis strain SS44; Δ*cya* Δ*crp* Δ*cdt*	Metabolic pathways	Improved lambing performance in immunized sheep after challenge with *Salmonella* Abortusovis	(177)
		SU304	Abortusovis	*Salmonella* Abortusovis strain SS44; Δ*aroA*	Global regulators	Improved lambing performance in immunized sheep after challenge with *Salmonella* Abortusovis	(177)
	Typhimurium	MT2313	Typhimurium	*Salmonella* Typhimurium UK-1; *dam102*::Mud-Cm	Global regulators	Reduced tissue colonization of wild-type *Salmonella* Typhimurium and protected sheep from lethal challenge	(178)

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While many live *Salmonella* vaccines with different mutations have undergone efficacy testing in different animals as discussed here and elsewhere (143, 144), only a handful are currently available commercially. Table 2 lists by host species some of the more promising vaccine candidates either commercially available or in the research pipeline. As with the human vaccines, these live-attenuated vaccines can also serve as carriers to boost immunity against various pathogens (142), and future multitarget vaccines could offer wider protection with a single dose (142). Below, we discuss development of NTS vaccines for the most agriculturally important animal species.

Poultry

In response to rising demand for poultry products and stricter consumer and regulatory demands, vaccination has become a cornerstone in integrated control programs for poultry including chickens, turkeys, especially for breeders, and layers (179). Vaccination reduces the risk of human foodborne infections by decreasing *Salmonella* colonization, organ invasion, and environmental contamination (180–182). Additionally, vaccination against host-restricted *Salmonella* serovars protects the health of chickens and prevents economic losses due to disease or mortality. Diseases caused by *Salmonella* Pullorum and *Salmonella* Gallinarum in chickens are effectively managed in high-income countries like the United States through robust eradication and surveillance initiatives implemented within commercial chicken production systems. Consequently, countries adopting this approach do not employ vaccines targeting these host-restricted strains. The poultry industry in resource-poor nations across Africa and Asia encounters persistent challenges posed by these strains. The primary focus of vaccine development efforts against *Salmonella* Pullorum and *Salmonella* Gallinarum is therefore directed toward these developing regions, where the impact of these bacterial strains remains a significant concern. Generalist serovars have also been targeted in efforts to reduce disease burden in animals but also limit transmission of strains to humans.

There are several commercially available live-attenuated vaccines for use in poultry (Table 2). Nobilis SG 9R has a long history of use and is a rough mutant of a *Salmonella* Gallinarum strain (154). However, there have been reports showing high similarity between field strains isolated during outbreaks and the vaccine strain indicating possible reversion to the virulent wild-type (183, 184). Another *Salmonella* Gallinarum vaccine (SG-VAC) is only available in Poland (153). *Salmonella* Typhimurium vaccines Poulvac ST (150) and AviPro Megan Egg (163) and the *Salmonella* Enteritidis vaccine Gallivac SE/Salmovac SE are more widely available commercially for use in poultry (145). Their availability varies in different countries based on the epidemiological context and local regulations.

A variety of vaccines have been engineered by targeting genes linked to bacterial antigens, synthesis pathways, regulatory functions, and virulence factors (Table 2). Generalist serovars like *Salmonella* Enteritidis are a major concern in poultry flocks not only because of the morbidity and mortality of flocks but also due to the associated food safety risks and the potential for transmission to human hosts. Vaccine candidates targeting this serotype have been engineered to be deficient in global regulators of protein expression and/or deficient in virulence factors. For example, *Salmonella* Enteritidis strain Z11 Δ*rfbG* does not make LPS, a key virulence factor for invasion, and was developed as a Differentiating Infected from Vaccinated Animals (DIVA) vaccine strain (146). The deletion of the *rfbG* gene produces a truncated rough LPS that does not react with O antibodies and can be distinguished from wild-type strains during routine sampling and surveillance; it can also be distinguished from wild-type strains based on morphology. Chickens immunized intramuscularly with Z11 Δ*rfbG* produced *Salmonella* Enteritidis-specific IgG, with reduced bacterial colonization of the liver, spleen, and cecum after challenge with wild-type *Salmonella* Enteritidis Z11. *Salmonella* Enteritidis strain JOL919 is deficient in both Lon protease and CpxR, a global regulator and response regulator in a two-component signal transduction pathway, respectively (148). Chickens immunized with JOL919 and challenged with wild-type *Salmonella* Enteritidis had

reduced gross lesion scores on the liver and reduced colonization of the wild-type strain in the liver, spleen, and cecum. Live vaccines deficient in genes involved in global gene expression have also been paired with competitive exclusion (CE) cultures which limit the capacity of wild-type infectious strains to colonize the intestinal tract. Oral administration of candidate vaccine SE 147N Δ*phoP* Δ*fliC* alone or with subsequent administration of a CE culture induced cytokine responses. After challenge with wild-type *Salmonella* Enteritidis, chicks receiving SE 147N Δ*phoP* Δ*fliC* alone or with CE had reduced cecal colonization of the wild-type strain, and the effect was additive when chicks received both the vaccine and CE (147). Another example of generalist serovars being engineered as vaccine strains for use in animals is the *Salmonella* Typhimurium strain MT2313. Chicks immunized at 10 hours post-hatching and subsequently challenged with wild-type *Salmonella* Typhimurium had significantly reduced colonization; moderate protection was also observed following challenge with heterologous *Salmonella* Enteritidis and *Salmonella* O:6,14, 24:e,h-monophasic strains (151).

Live-attenuated vaccines targeting host-restricted serovars in chickens like *Salmonella* Gallinarum and *Salmonella* Pullorum encompass a range of genetic alterations across multiple strains. For instance, the *Salmonella* Gallinarum JOL2841 vaccine strain harbors deletions in the *cpxR* and *arnT* genes, which has multiple impacts on strain viability and pathogenicity; the absence of *cpxR* affects stress, intracellular survival, and biofilm formation, while deletion of *arnT* alters the charge and stoichiometry of lipid A, decreasing toxicity and virulence (156). Chickens immunized with JOL2841 had significant protection from death upon challenge (156).

The T3SS has also been a target for vaccines administered to animals. Vaccine strains targeting poultry-adapted serovars have been engineered to be deficient in T3SS effector proteins. *Salmonella* Gallinarum 1009 Δ*spiC* Δ*crp*, *Salmonella* Pullorum S06004 ΔSPI2, and *Salmonella* Pullorum S06004 Δ*spiC* Δ*rfaH* (Table 2) lack the SpiC effector protein; intracellular trafficking of host proteins is disrupted and prevents the fusion of *Salmonella*-containing vacuoles with lysosomes, consequently leading to *Salmonella* with reduced virulence (160, 161). Chickens immunized with *Salmonella* Gallinarum 1009 Δ*spiC* Δ*crp* had increased antibody and cellular responses to whole *Salmonella* Gallinarum as an antigen. This vaccine also demonstrated 100% efficacy against lethal challenge with the wild-type strain *Salmonella* Gallinarum SG9 (158). *Salmonella* Pullorum S06004 ΔSPI2 also elicited protective cellular and humoral responses and protected chickens from challenge with wild-type *Salmonella* Pullorum and *Salmonella* Gallinarum strains (160). The protective efficacy of *Salmonella* Pullorum S06004 ΔSPI2 was found to be 90% when the birds were challenged with wild-type *Salmonella* Pullorum. In comparison, vaccine efficacy was 70% in birds challenged with strain *Salmonella* Gallinarum SG9 (160). *Salmonella* Pullorum S06004 Δ*spiC* Δ*rfaH* refines this strategy by deleting a single gene in the SPI2 region (*spiC*) and deleting a gene involved in LPS synthesis to generate a DIVA vaccine strain making it distinguishable from wild-type strains during routine sampling and surveillance. Chickens immunized with the *Salmonella* Pullorum strain S06004 Δ*spiC* Δ*rfaH* produced *Salmonella* Pullorum-specific IgG (assessed against whole bacteria as antigen) and showed increased proliferation of peripheral blood mononuclear cells (PBMCs) and increased cytokine expression compared to non-immunized controls. Upon challenge with wild-type *Salmonella* Pullorum strain S06004, chickens immunized with *Salmonella* Pullorum S06004 Δ*spiC* Δ*rfaH* had an 86% (13/15) survival rate compared to only 20% survival in non-immunized birds (161).

Auxotrophic and global regulator mutants appear to be highly immunogenic live vaccines against multiple *Salmonella* serovars including *Salmonella* Gallinarum (185), *Salmonella* Abortusequi (186), *Salmonella* Dublin (187), *Salmonella* Typhimurium (188), *Salmonella* Enteritidis (189), and *Salmonella* Choleraesuis (190) and in different animal species. Early research and development of *aroA* mutants in host-restricted strains led to promising candidates like HG212, a mutant of *Salmonella* Gallinarum strain 9; however, this vaccine strain only showed protection from lethal challenge with wild-type *Salmonella* Gallinarum strain 9 when the vaccine was delivered intramuscularly and

not orally (159). *Salmonella* Typhimurium-based vaccine candidates like χ3985, a Δ*cya* Δ*crp* mutant, have been able to reduce tissue colonization of challenge strains from homologous and heterologous serovars (152). However, the challenge strains assessed were not chicken-adapted. Candidate vaccine χ11387, a modified *Salmonella* Gallinarum strain, required arabinose for expression of the *crp* gene which encodes CRP, a global regulator of virulence genes. This vaccine conferred homologous (vs wild-type *Salmonella* Gallinarum) and heterologous (vs wild-type *Salmonella* Enteritidis) protection with reduced bacterial load of the liver and spleen as a readout (155). Another *Salmonella* Gallinarum candidate vaccine strain developed by this group, χ11797, had the ferric uptake regulator (*fur*) gene deleted. After challenge, it reduced the enlargement of livers and spleens in vaccinated chickens compared to non-vaccinated controls, but this effect was only observed when the vaccine was delivered intramuscularly and not orally (157).

Notably, genetically modified live-attenuated *Salmonella* vaccines can offer a degree of cross-protection against multiple serovars due to shared somatic and flagellar antigens (152, 191–193). The commercially available Poulvac ST vaccine is comprised of the *Salmonella* Typhimurium strain AWC 591 with deletions of *aroA* and *serC*; it is marketed as a vaccine that can induce homologous protection against *Salmonella* Typhimurium and heterologous protection against serovars Enteritidis and Heidelberg (145, 149, 150). Cross-protective efficacy of Poulvac ST has been assessed and verified in studies using locally circulating field strains. In one example, chickens immunized with Poulvac ST and subsequently challenged with a Brazilian field strain of *Salmonella* Heidelberg had reduced colonization of the liver and spleen 3 days after challenge and reduced cecum colonization 21 days post-challenge (150). However, making a broadly protective vaccine is a challenge. A trivalent vaccine comprised of *lon* and *fliC/fljB* mutants of *Salmonella* Enteritidis 147, *Salmonella* Typhimurium 16E5, and *Salmonella* Infantis 18G6 conferred protection in chickens challenged with wild-type versions of the vaccine strains. However, heterologous challenges with serovars Agona, Hadar, and Dublin showed mixed results (162). While the number of animals assessed in this study was small, it indicates that more broadly protective vaccines that target host-restricted and generalist strains could be better developed.

The potential for shedding of live vaccines into the environment remains a concern (194). Live oral *Salmonella* vaccines activate immunity and can defend poultry against *Salmonella* colonization, but they need to be cleared or reduced to undetectable levels in poultry before they reach processing for safety and regulatory purposes (142, 182). Oral vaccination post-hatch can offer swift protection via a colonization–inhibition mechanism (195, 196). Methner et al. (196) demonstrated protection against superinfection by *Salmonella* strains within hours following the administration of live-attenuated vaccines to day-old chicks, showing a colonization inhibition mechanism that provides immediate partial protection before developing an adaptive immune response. The effectiveness varies across vaccine strains, underscoring the importance of carefully choosing strains that hinder colonization while avoiding over-attenuating modifications (142). For instance, pre-treatment of chicks with mutants of *Salmonella* Typhimurium lacking *ompC* decreased the ability of the challenge strain to colonize the liver and ceca compared to control strains. While *rpoS* and *phoP* deletions (either alone or in combination) in *Salmonella* Typhimurium and *Salmonella* Enteritidis also reduced the challenge strain's ability to colonize these tissues, the resulting vaccine strains were significantly attenuated, limiting the capacity of the vaccine strain to colonize the cecum and more fully inhibit colonization and growth of the challenge strain (196). Some studies suggest the potential benefits of combining live vaccines with probiotics for more comprehensive protection with CE (196, 197).

Swine

The urgency to find effective interventions against *Salmonella* in swine has also become paramount with the global expansion of food animal production systems and growing restrictions on antimicrobial usage. Among the intervention strategies, vaccination

stands out due to its potential to mitigate the spread of *Salmonella*. This not only reduces dissemination within herds but also diminishes the need for antibiotics against salmonellosis, further reducing the risk of AMR spread (198). In swine, live vaccines can be administered to the sows to enhance passive immunity for piglets or may be administered to the piglets directly (198).

In China, *Salmonella* Choleraesuis strain C500 has been developed by chemical mutagenesis as a live oral vaccine for swine. This strain has demonstrated both efficacy and safety, improving the prevention and control of piglet paratyphoid in China for over four decades (199). Despite the complete genome characterization of C500 (200), the precise molecular mechanism responsible for its virulence attenuation remains unclear (167). Furthermore, C500 exhibits residual toxicity, which limits its practical utility as a live oral vaccine vector. Therefore, many researchers have explored modifying it to increase its potential as a live oral vaccine vector for delivering heterologous antigen (167, 201–203).

In the European Union, the Salmoporc vaccine was the first licensed live-attenuated vaccine against *Salmonella* Typhimurium for administration to sows and piglets. It consists of the live *Salmonella* Typhimurium strain 421/125 with histidine–adenine auxotrophic mutations for attenuation. Several studies have demonstrated its effectiveness in reducing *Salmonella* concentrations in pigs (204–206). In the United States, the Center for Veterinary Biologics at the United States Department of Agriculture-Animal and Plant Health Inspections Service (USDA-APHIS) has licensed several live *Salmonella* vaccines for use in swine, though availability varies for administration (198, 207). Some of the prominent vaccines, like Enterisol *Salmonella* T/C and Argus SC/ST, which consist of attenuated strains of *Salmonella* Choleraesuis, have yielded encouraging results in reducing the prevalence of *Salmonella* Choleraesuis and *Salmonella* Typhimurium in vaccinated pigs (164). Enterisol *Salmonella* T/C and NOBL SC-54 employ live strains of *Salmonella* Choleraesuis that lack the *spv* genes found on the virulence plasmid (164, 165, 208). The *spv* genes encode proteins that play crucial roles in various intracellular processes and contribute to increased virulence of *Salmonella* within the host (209). Argus SC/ST utilizes a live-attenuated strain of *Salmonella* Choleraesuis with deletion mutations in the *cya* and *crp* genes, impacting its virulence and growth (210). This vaccine is indicated for preventing pneumonia, diarrhea, septicemia, and mortality caused by *Salmonella* Choleraesuis and helps in controlling disease and shedding caused by *Salmonella* Typhimurium.

Similarly, vaccine candidates against the generalist serovar *Salmonella* Typhimurium have also been developed for use in pigs but are not available commercially. Examples of these are *Salmonella* Typhimurium JOL911 (172), *Salmonella* Typhimurium *ΔznuABC* (168), and the DIVA strains BBS 202 (169) and BBS 866 (170). Strain JOL911 is a *cpxR* and *lon* deletion mutant; *cpxR* is required for invasion of host cells and *lon* regulates SPI-1 invasion genes. Two orally administered doses of *Salmonella* Typhimurium JOL911 induced significant LPS-specific IgG and IgA responses in serum and colostrum. Piglets born to sows primed and boosted with *Salmonella* Typhimurium JOL911 also had increased LPS-specific antibody titers compared to control piglets and more importantly exhibited no clinical signs of infection until 21 days after challenge with a wild-type *Salmonella* Typhimurium strain (172). *Salmonella* Typhimurium *ΔznuABC* lacks the entire *znuABC* operon, leading to an inability to retrieve metals from the host, which causes a loss of virulence. It generated vaccine-specific antibodies and protected immunized pigs from clinical symptoms of infection (168). Additionally, the environmental presence of this strain remained below detectable levels by day 14 post-inoculation. *Salmonella* Typhimurium BBS 202 has a mutation in the Δ*rfaH* gene, and its derivative, BBS 866, has additional mutations in five small regulatory RNA (sRNA) genes —*omrA, omrB, rybB, micA,* and *invR*—that negatively regulate conserved outer membrane proteins (169, 170). Vaccination with *Salmonella* Typhimurium BBS 202 reduced clinical symptoms such as fever and diarrhea, decreased *Salmonella* fecal shedding, and minimized intestinal colonization upon exposure to virulent *Salmonella* Typhimurium

(169). Moreover, immunization with *Salmonella* Typhimurium BBS 866 provided broader protection, showing efficacy against challenge with wild-type *Salmonella* Typhimurium and *Salmonella* Choleraesuis (170).

Cattle

Like other animals, cattle can be infected by both host-restricted and generalist *Salmonella* serovars. The host-restricted *Salmonella* Dublin strain causes various clinical indications in cattle. Calves commonly experience respiratory symptoms, whereas older cattle experience fever; depression; foul-smelling diarrhea containing mucus, blood, and fragments of intestinal lining; and even abortions. In the United States, EnterVene-d, an auxotroph for aromatic amino acids, is a commercially available live-attenuated vaccine specifically targeting *Salmonella* Dublin and is administered to cattle 2 weeks and older (174). A randomized field study with calves receiving two doses of EnterVene-d orally, intranasally, or no vaccine (control group) determined that while there was no significant difference in the incidence of pneumonia in the calves, vaccinated calves had a significantly reduced risk of mortality (174). A promising live-attenuated vaccine candidate in development is *Salmonella* Dublin Sdu189 Δ*spiC* Δ*aroA*. This strain induced serum IgG antibodies against whole *Salmonella* Dublin and also stimulated increased cytokine expression in BALB/c mice intramuscularly immunized with the vaccine (175). Mice were also completely protected upon lethal challenge with wild-type *Salmonella* Dublin Sdu189 (175). *Salmonella* Dublin is also associated with invasive disease in humans making control of this serovar in cattle important for human health.

Among the generalist *Salmonella* serovars, *Salmonella* Typhimurium and *Salmonella* Newport are frequently associated with cattle and their products. Currently, there are no live-attenuated vaccines available on the market for these strains for use in cattle. However, strain MT2313, which is a *Salmonella* Typhimurium vaccine deficient in DNA methylation, has been tested against serovars Typhimurium, Dublin, and Newport in a challenge study conducted in Australia. *Salmonella* Typhimurium MT2313 conferred considerable reduction in clinical symptoms, fecal shedding of the challenge strain, and tissue colonization in calves (151, 176, 211).

Sheep

In sheep flocks, *Salmonella* outbreaks are commonly linked to serovars Abortusovis, Dublin, and Typhimurium. *Salmonella* Abortusovis stands as the predominant cause of ovine salmonellosis in southern Europe, often resulting in abortions, as its name implies (212). Currently, there are no commercially available live-attenuated vaccines against salmonellosis for use in sheep in the United States. However, strains such as SSM189 (Δ*crp* Δ*cdt* Δ*cya*) and SU304 (Δ*aroA*), both derivatives of wild-type *Salmonella* Abortusovis strain SS44, have been evaluated in sheep for homologous protection from disease (177). Mutations in *aroA* alone or *crp*, *cdt*, and *cya* result in avirulence of *Salmonella* Abortusovis and complete elimination of its ability to induce abortions. Immunization with either strain resulted in reduced abortions and fewer infected ewes at parturition. However, the reduction in pregnancy failures, while promising, was not statistically significant.

Salmonella Typhimurium vaccine strain MT2313 was also evaluated in sheep (178). Animals were fasted prior to being offered water containing the vaccine strain for 24 hours. Sheep were challenged 24 hours, 7 days, or 28 days after in-water immunization. Colonization of the mesenteric lymph nodes, ileum, liver, spleen, and lung was significantly reduced in sheep immunized 28 days before challenge; fewer bacteria were also recovered from the organs of sheep immunized 7 days before challenge. *Salmonella* Typhimurium MT2313 completely protected sheep immunized 7 and 28 days prior to lethal challenge with a wild-type *Salmonella* Typhimurium strain (178).

LIVE-VECTOR *SALMONELLA* VACCINES FOR ANIMAL USE

Many live-attenuated *Salmonella* vaccines, previously developed and tested against homologous challenge in experimental animal models, are now being further engineered as live vectors to vaccinate against multiple animal pathogens including other bacteria, parasites, and viruses. For example, chickens vaccinated orally with *Salmonella* Typhimurium live-vector RASV strains c4550 and c3987, expressing the *Campylobacter jejuni cjaA* gene, encoding an N-glycosylated lipoprotein of the inner membrane, elicited serum IgG and mucosal IgA antibody responses against *C. jejuni* membrane proteins; this immune response led to reduced colonization of heterologous wild-type *C. jejuni* in the cecum of immunized chickens (213).

The RASV system has also been used to immunize against Gram-positive infections. An attenuated *Salmonella* Typhimurium vaccine strain has been employed as an oral live-vector vaccine to protect chickens from necrotic enteritis by expressing the antigens fructose-1,6-bisphosphate aldolase, α-toxoid, and NetB toxoid from *C. perfringens* (214). More recently, AVERT NE became the only mass-administered commercial vaccine based on RASV technology, licensed for use in chickens in the United States, for controlling necrotic enteritis caused by *C. perfringens* Type A. It expresses *C. perfringens* genes coding for a nontoxic C-terminal adhesive part of α-toxin and a fusion of glutathione S-transferase (GST) with NetB toxin (GST-NetB) (98).

Salmonella Choleraesuis has been investigated as a potential live-vector vaccine for delivery of heterologous antigens in swine because of its host restriction and pathogenic mechanisms. Two promising vaccine candidates (SCL 00031 and SCL 00032) with mutations in Δ*rpoS* and Δ*phoP* (215) were developed for use in pigs against *Salmonella* Choleraesuis. However, they were highly attenuated and were unable to colonize deep tissue organs when competing against the wild-type parent strain; these candidates have not advanced in development.

Similar strategies have been applied to combat infections by parasitic and viral pathogens. Another RASV *Salmonella* Typhimurium vaccine strain was engineered to deliver the *Eimeria acervulina* merozoite antigen EAMZ250 expressed from plasmid pYA3658 as a peptide fused to the translocation domain of the T3SS effector protein SptP (216). Chickens immunized with the vaccine strain expressing EAMZ250 and challenged with *E. acervulina* oocysts showed significant weight gain compared to vector control groups. The strain was also able to colonize the bursa, intestines, and spleens of the chickens indicating that long-lasting immunity from *Eimeria* infection may be possible with an easily administered oral vaccine (216). This strategy has also been used to target *Eimeria tenella* by expressing its SO7 antigen from an RASV strain. Chickens immunized with this SO7-expressing strain showed a reduction in oocyst shedding and both humoral and cellular immune responses to immunization (217).

New technologies have also broadened the development landscape. A *Salmonella* Typhimurium strain, modified with deletions in the *lon* and *sifA* genes, was employed to deliver a CRISPR/Cas9 plasmid encoding the virulence gene *pp38* from Marek's disease virus (MDV), a herpes virus that causes tumors and paralysis in chickens; *pp38* is crucial for the infection and maintenance of B cells by MDV (218). *Salmonella*-mediated delivery of the CRISPR::*pp38* plasmid was confirmed by isolation of PBMCs from vaccinated chickens for analysis by PCR. *In vivo* plasmid delivery was also confirmed by fluorescence-activated cell sorting analysis of spleen and liver cells from chickens that received a green fluorescent protein-expressing version of the plasmid. This genetic interference strategy was used against highly pathogenic MDV, resulting in significant resistance to MDV infection in *Salmonella*-vaccinated chickens (219).

CONCLUSION

Comprehensive knowledge of *Salmonella* pathogenesis, gene regulation, and virulence along with the advent of modern recombinant DNA technology permits the possibility of genetic engineering of *Salmonella* with great efficiency and efficacy. Many new

strategies to develop live-attenuated vaccine strains of *Salmonella* spp. can be harnessed to reduce the burden of *Salmonella* infections in both humans and animals. The development of recombinant *Salmonella* strains as vaccine vectors against heterologous pathogens constitutes a promising approach toward the development of safe, effective, and affordable vaccines against a number of infectious diseases. Recently, genetically engineered *Salmonella* have also been used to generate components for other vaccine modalities such as conjugate vaccines and OMV-based vaccines. In addition, *Salmonella*-mediated antitumor therapy is an attractive strategy for the control of cancer. As research progresses, modern technologies that offer safer, effective, and more broadly protective vaccines have the potential to decrease morbidity and mortality due to *Salmonella* and other pathogens in humans as well as animals.

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