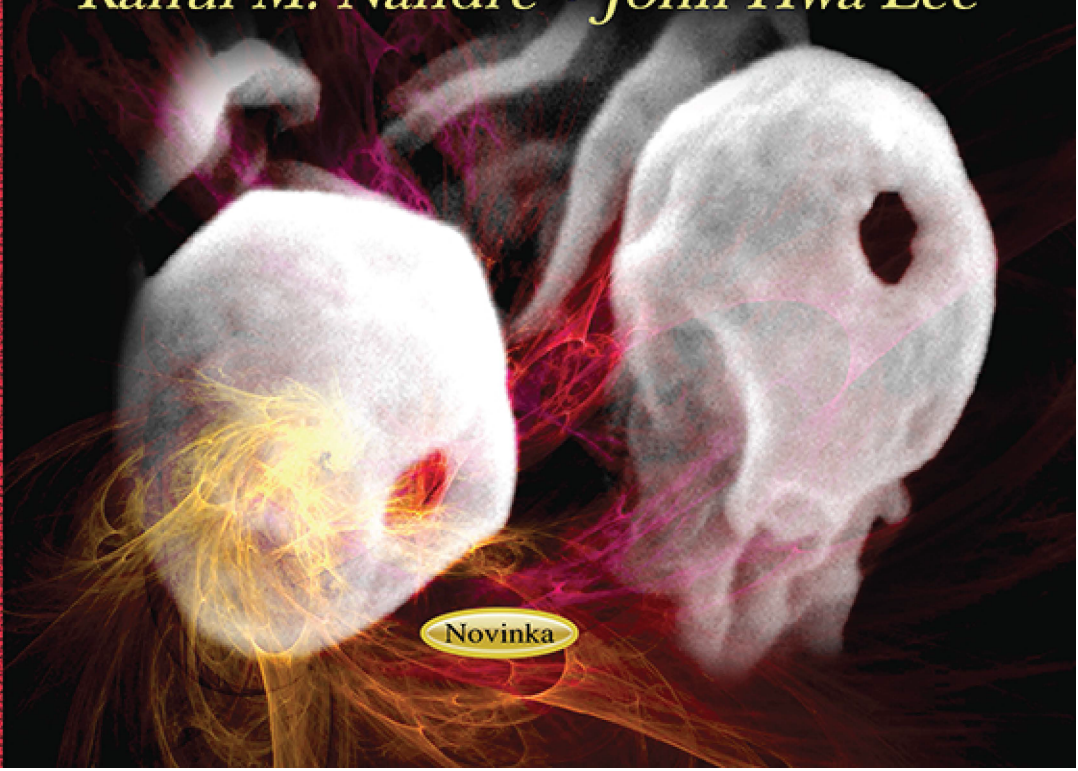
Veterinary Sciences and Medicine

Salmonella Pathogenesis and Progression in the Development of Human and Veterinary Non-Typhoidal *Salmonella* Vaccines against Human Salmonellosis

Rahul M. Nandre • John Hwa Lee



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INTRODUCTION

Salmonella is a rod-shaped, gram-negative facultative anaerobe in the family of enterobacteriaceae. Intracellular *Salmonella* pathogens are important zoonotic agents in cold-blooded and warm-blooded animals [1]. More than 95% of *Salmonella* infections are foodborne in humans [2]. Acquisition of *Salmonella* from pets, direct personal contact, nosocomial transmission and waterborne transmission are less common modes of transmission [3]. Presently, more than 2500 different serovars have been characterized, with most (1531) classified as part of the *Salmonella subsp. Enterica* is the main causative agent for more than 99% of disease cases in humans [4, 5]. Among the *Salmonella subsp. Enterica*, the most common broad host range serovars are *Salmonella* Enteritidis and *Salmonella* Typhimurium, which mainly cause disease in a variety of animals [6, 7]. These diseases are associated with gastrointestinal inflammation and diarrhea and are usually self-limiting. However, systemic infections by non-typhoidal *Salmonella* (NTS) can occur. *Salmonella* Virchow often causes invasive disease [8]. The bovine-adapted *Salmonella* Dublin and the porcine-adapted *Salmonella* Choleraesuis are occasionally seen in humans, mostly causing bacteremia [9]. *Salmonella* Newport causes septicemic illness in animals and humans [10]. *Salmonella* Heidelberg causes gastroenteritis in humans [11]. *Salmonella* London leads to meningitis and gastroenteritis in humans [12, 13]. In addition, *Salmonella* Isangi causes enteric encephalopathy with paralytic ileus in humans [14]. The NTS disease syndrome and its causative agents are mentioned in Figure 1. NTS pathogens lead to gastroenteritis, bacteremia, and subsequent focal infection in humans. Most NTS serotypes cause gastroenteritis, in which the infection remains localized to the terminal ileum, colon, and mesenteric lymph node of immunocompetent individuals. NTS gastroenteritis has a short incubation period, averaging less than one day [15], followed by the development of diarrhea, fever,

and intestinal inflammatory infiltrates that are dominated by neutrophils [16]. NTS serotypes produce a more severe infection in infants, the elderly, and individuals with debilitating illnesses, and are associated with considerable mortality rates. As a result, a recent estimate puts the global burden of NTS gastroenteritis at 93.8 million cases, resulting in 155 000 deaths annually [17]. Notable recent outbreaks of NTS infection have been associated with eggs, cheese, dry cereal, ice cream premix, a variety of fresh sprouts, juices, cantaloupes, and other fresh vegetables [3]. Undercooked eggs have been linked to sporadic transmission, because eggs can be contaminated with *Salmonella* by direct contamination from infected reproductive organs of hens or by penetration through the egg shell from contaminated feces [18, 19]. The cumulative global death toll from NTS gastroenteritis and bacteremia is considerable, thus highlighting the need for efficient vaccines to protect against NTS infections. Human NTS vaccines are needed to protect against NTS infections. In addition, the heavy damage to national and regional economies caused by some highly contagious animal diseases has forced the implementation of vaccination programs against zoonotic diseases in animals to protect the human population [20]. In order to combat *Salmonella* gastroenteritis, the vaccination of livestock animals appears to be more suitable than vaccination of humans [21]. Veterinary NTS vaccines in animals can prevent these organisms from entering the human food chain via contaminated meat, eggs or dairy products.

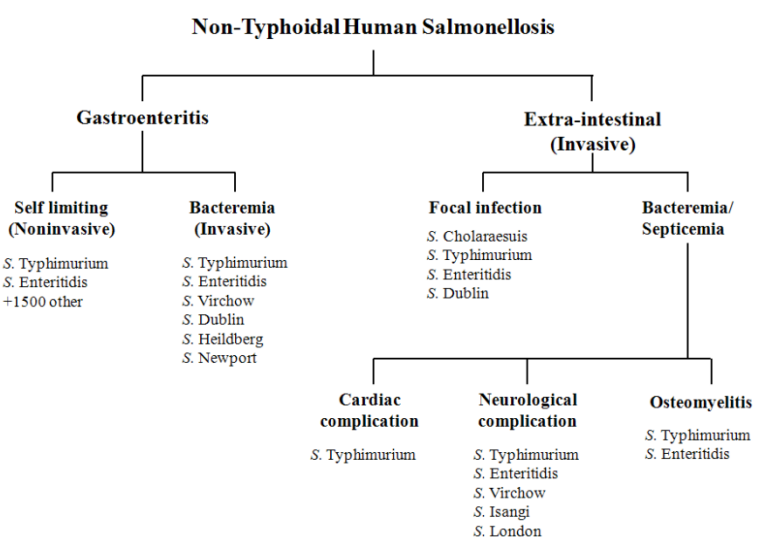

Figure 1. NTS disease syndrome and its causative NTS subspecies.

In food manufacturing, prevention of *Salmonella* infection by implementation of biosecurity or hygiene measures is expensive and inadequate and is especially undermined by the expansion of free-range production. Antibiotic therapy is not routinely recommended for the control of mild to moderate presumed or proven *Salmonella* gastroenteritis in healthy individuals. Antimicrobial resistance against clinically important 'first-line' drugs is developing among *Salmonella* worldwide [3]. The emergence of multidrug-resistant NTS serotypes with resistance to ciprofloxacin and expanded-spectrum cephalosporins threaten to limit antimicrobial treatment options [22]. Vaccination remains the ideal strategy to counteract *Salmonella* enteric infections. However, currently licensed vaccines for humans and domestic animals are far from optimal. These vaccines are mostly based on very old technology and are excessively reactogenic. At present there are no ideal vaccines or delivery systems against NTS gastroenteritis or septicaemia [23].

There is a need to generate new, advanced vaccine candidates and vaccination strategies and the understanding of within-host population dynamics of NTS infections is important for allowing delivery of targeted interventions. An understanding of within-host dynamics of *Salmonella enterica* interactions with eukaryotic cells could shape the development of vaccines. Specifically, it is important to understand: 1) entry of NTS bacteria into the body; 2) localization of the NTS bacteria at the various stages of the infection; 3) NTS bacterial spread from cell to cell in the whole body; 4) NTS disease progression; and 5) NTS bacterial death. Understanding these aspects of the infection is a prerequisite for the development of novel vaccine strategies to: 1) target and act at the right sites of NTS infection; 2) use existing vaccines sensibly against NTS infection; and 3) design new advanced vaccines in a rational way for the prevention of NTS infection.

This review focuses on non-typhoidal *Salmonella* pathogenesis in the application of an immunization strategy to study the precise determinants of bacterial growth, spread and distribution at the single-cell level, and the understanding of infection dynamics for vaccine development. In addition, recent advances with future prospectus in *Salmonella* vaccines are reviewed to assess the current status in safety, immunogenicity and protection efficacy of vaccines against NTS gastroenteritis.

Chapter 1

CLINICAL FEATURES OF NON-TYPHOIDAL *SALMONELLA* (NTS)

Areas of Southeast Asia and India have the highest prevalence of disease caused by *Salmonella enterica* serovars, followed by sub-Saharan Africa and South America. Acute gastroenteritis is the most common clinical manifestation of foodborne NTS pathogen [24]. However, invasion beyond the gastrointestinal tract occurs in approximately 5% of the patients with NTS gastroenteritis, resulting in bacteremia [3]. Patients with invasive disease frequently present with apparent focal infection, which is commonly attributed to co-infection with other pathogens such as *Mycobacterium* Tuberculosis [25] and *Streptococcus* Pneumonia [26]. A predisposition to NTS bacteremia and a trend towards higher mortality have been observed in immunosuppressed patients [24]. In addition to bacteremia, invasive NTS can also cause meningitis, especially in children [27]. Information about the prevalent serotypes of NTS with high invasive potential is of epidemiological and public health importance. One of the foremost obstacles is antibiotic-resistant *Salmonella* strains, which create a significant threat to the development of reliable therapies against *Salmonella* infection [28]. As the clinical prognosis for patients infected with resistant *Salmonella* strains is poor [29], new advanced therapies must be developed. To develop new therapies, the pathogenesis of NTS needs to be studied in great detail.

Chapter 2

SALMONELLA PATHOGENICITY

VIRULENCE FACTORS AND FACULTATIVE INTRACELLULAR LIFESTYLE OF *SALMONELLA*

Although animal models have successfully been used to understand the pathogenicity of intestinal *Salmonella* [30, 31], this approach has inherent limitations. In mice, *Salmonellae* appear to preferentially adhere to and enter the M cells of the intestinal epithelium [32]. In addition, *Salmonella* Typhimurium disseminates systemically in both genetically resistant and genetically susceptible mouse lineages, thus making it difficult to study mucosal barrier functions in the mouse model [33]. In bovine epithelium, however, *Salmonellae* do not appear to interact preferentially with M cells, and the relative roles of M cell and enterocyte invasion in different animal hosts and *Salmonella* disease syndromes require additional exploration [34]. Limitations of the calf model include the scarcity of reagents available and limited availability of animal facilities to perform the research [33]. The variation in the repertoire, sequence or expression of effector proteins for SopE/E2 and SipC may explain differences in the ability of serovars to induce enteritis in animal models [35, 6]. Similarly, SlrP mutants exhibit a colonization defect in mice but are not impaired of their ability to colonize the intestines of calves [37], or induce enteritis in bovine ileal loops [38]. Each animal model has shortcomings that limit its usefulness for studying *Salmonella* disease manifestations. Current limitations of animal models have contributed to the relative paucity of knowledge about NTS bacteremia [33].

Many NTS serovars harbor isolates that are capable of infecting different animals, which can spread zoonotic diseases to humans [39]. During evolution, *Salmonella* has acquired many virulence factors that enable it to gain access to

new niches in a host during the complex pathogenesis. Over 100 of 4500 *Salmonella*-harbored genes have been implicated as virulence-associated genes [6, 40]. Principal virulence-associated genes with their functions are described in Table 1. Many of the virulence factors are encoded on large genetic elements, termed *Salmonella* pathogenicity islands (SPIs), which often show a distinct base composition of DNA and are absent in non-pathogenic relatives of *Salmonella* [65]. To date, 17 SPIs have been identified in *Salmonella enterica* [66, 67]. Although SPI-1 and SPI-2 are central to *Salmonella* pathogenesis, many other classes of genes, which are involved in metabolism or biosynthesis, are also required [68]. Both SPI-1 and SPI-2 encode type III secretion systems (T3SSs) for the translocation of effector proteins to the host cell membrane and the cytosol [69]. The SPI1-encoded T3SS (SPI1-T3SS) is involved in the invasion of nonphagocytic cells by *Salmonella* [70] and the elicitation of intestinal inflammation [71]. In addition, the SPI-2-encoded T3SSs is required for intracellular survival in macrophages [72]. It is important to note that T3SSs, including SPI-1 and SPI-2, can encode needle-like complexes that can 'inject' bacterial proteins directly into host cells [73]. These injected proteins, often referred to as effector proteins, can hijack host cell functions. Hence, *Salmonella* can remodel their targeted host cells [31].

In addition to these two major SPIs, SPI-3, SPI-4 and SPI-5 also has importance for the virulence and survival of the bacterium [74]. SPI-3 has ten open reading frames (ORFs) in six transcriptional units, and encodes proteins including the most important Mg^{2+} transporter, a putative ToxR regulatory protein and a putative AIDA-I adhesion [75]. SPI-4 contributes to intestinal inflammation in the mouse model [76]. SPI-4 encodes a type I secretion system, T1SS, and a substrate protein of the T1SS, SiiE [77]. SPI-5 was first located in *Salmonella* Dublin and mainly contains effector proteins [54]. Another important island is SPI-7, which encodes a variety of putative virulence-associated gene clusters, including a type IV pilus. The type IV pilus is involved in aiding attachment to eukaryotic cells [78-81].

Salmonella is well-adapted to an intracellular lifestyle, in which it invades and persists in mammalian cells such as macrophages and other immune cells [39, 82, 83]. This characteristic has been genetically linked to virulence factors based on mutants that are unable to survive in such cells, and have a reduced or lack of ability to cause infection [84]. Most of these mutants are required at both early (1 week post-infection) and late (2–4 weeks post-infection) stages of infection, which is expected for intracellular survival. However, two SPI2-dependent effectors, SseK2 and SseI (SrfH), were only required for the late stages of

Table 1. Important NTS Virulence-Associated Genes and their Functions

NTS Strain	Prinicipal virulence-associated genes	Functions	Reference
SE	Type 1 Fimbrae subunit (*FimA*)	Mediate adhesion or virulence in the hosts?	[41]
ST	Long Polar Fimbrae (*lpf*)	Mediate adhesion to the cells of Payers' patches of the small intestine	[42]
ST	Plasmid Encoded Fimbrae *(pef)*	Necessary for the attachment of the small intestine	[43]
SE	Thin Aggregative Fimbrae (*agf*)	Enhances survival of *Salmonellae* facing hostile barriers such as stomach acid, and mediate binding of *Salmonella* to the fibronectin (Eucaryotic extracellular matrix)	[44]
ST	Apparatus Genes *(invH)*	Attachment and invasion factor	[45]
ST	Apparatus Genes *(invG)*	Required for the secretion of proteins by the SPI1 type III apparatus	[46]
ST	Apparatus Genes *(invA)*	Involved in the biosynthesis of the flagella.	[47]
ST	Apparatus Genes *(invE)*	Indirectly involved in the $[Ca^{2+}]_i$ levels of host cells, which is known to be an important regulator of various cellular functions, including phagocytosis	[48, 49]
ST	Apparatus Genes *(invC)*	May interact with other components of the type III secretion apparatus to facilitate the translocation of proteins out of the cell	[50]
ST	Oxygen Reulated Gene *(orgA)*	Required for invasion into epithelial cells and for cytotxicity to macrophages	[51]
ST	*Salmonella* Invasion Protein or *Salmonella* Secreted Protein (*sip/ssp*)	Required for invasion, translocation of effector proteins	[52,53]
SD	*Salmonella* Outer Protein (*sopB*)	Required for the inflammation and fluid accumulation in bovine ileal loops and is believed to stimulate the recruitment of PMNs to the site of a *Salmonella* infection	[41,54]
ST and SD	*Salmonella* Outer Protein (*sopE*)	Required for efficient entry of *Salmonella* into host cells	[55,56]
ST	Hyperinvasive Locus *(hilA)*	An SPI1-encoded protein required for the expression of the type III secretion apparatus and Invasion into epithelial cells and induction of apoptosis of macrophages	[57]
SC, SD and ST	*Salmonella* Plasmid Virulence (*spv*)	Enhance the ability to proliferate at extraintestinal sites, most likely within tissue macrophages.	[58]
ST	Acid Tolerance Response (*atr*)	Log-phase acid tolerance response in stomach	[59]
ST	PhoP Activated Genes *(pags)*	Resistance to cationic peptides and killing by macrophages and PMNs	[60]
ST	Flagella Master Regulator (f*lhD*)	Fluid secretion and neutrophil recruitment	[61]
SE	The Nucleases (*yafD* and *xthA*)	Required for survival of *S.* Enteritidis in egg albumen	[62,63]
ST	A Cellular Kinase (*akt1*)	Controls intracellular replication of *Salmonella.*	[64]

ST - *Salmonella enterica* serovar Typhimurium; SE - *Salmonella enterica* serovar Enteritidis; SD - *Salmonella enterica* serovar Dublin; SC - *Salmonella enterica* serovar Choleraesuis

persistent infection [85]. The function of SseK2 (SPI2-dependent factor) during persistent *Salmonella* infection remains unknown and is the subject of future studies. The SPI2-dependent factor, SseI, translocates into phagocytes and inhibits the ability of these cells to adhere [86] and migrate efficiently, thereby disrupting their ability to effectively communicate with other cells of the host's immune system [87]. SseI suppresses migration in part by associating with the host protein IQGAP1, an important regulator of the cytoskeleton and cell migration [87-89].

During infection, the bacterium may be starved of nutrients, including essential amino acids, which are in short supply in host tissues. Furthermore, *Salmonella* that are auxotrophic for these limited nutrients may be attenuated in terms of their ability to cause infection [90-92]. Such attenuated strains are frequently exploited to extend the period of bacterial persistence in susceptible animals [93].

SALMONELLA INGESTION AND INFECTION THROUGH NATURAL ROUTES

Although *Salmonella enterica* serovars are some of the best studied bacterial pathogens, the field still needs a deeper study, especially when one considers that (i) they cause significant morbidity and mortality worldwide; (ii) they have broad host ranges; (iii) they can establish persistent infections, which serve as reservoirs for transmission/shedding; and (iv) they are developing resistance against many antibiotics [94].

In natural infection, *Salmonella* are typically acquired from the environment by oral ingestion of contaminated food/water or by contact with a carrier. Following ingestion in sufficient numbers, a proportion of the inoculum survives the low pH environment of the stomach to enter the small intestine where infection can be established. Conditions that increase the pH of the stomach can decrease the infective dose. However, *Salmonella* do have an adaptive acid tolerance response, which may aid survival in this environment [95]. The bacterial adaptive responses may sense phagosomal milieu, low pH and low magnesium ion contents, and consequently *Salmonella* tightly regulate expression of virulence determinants to survive in this endosomal compartment [39]. Stomach acid and competition with resident microbial flora constitute early bottlenecks to the infection process of *Salmonella* in the host [96]. After ingestion, the organisms replicate in the small intestine over a period of 1-3 weeks, breech the intestinal

wall, and spread to other organs. In addition, *Salmonella* interacts with nonphagocytic cells and phagocytic cells [74].

INVASION AND COLONIZATION IN THE GASTROINTESTINAL TRACT

Many commensal bacteria persist in the intestinal lumen without causing any significant interaction with the epithelia or deeper tissues. In contrast, *Salmonella* are predominantly regarded as 'invasive' bacteria as they encode multiple systems (including SPI-1) for interaction and penetration into mucosal epithelia. Indeed, *Salmonella* encode specific proteins such as ShdA, which enhance the persistence of colonization [97]. Once *Salmonella* reach the submucosa, some bacteria are captured and killed by phagocytes. For successful invasion of the epithelium, they must avoid the neutralizing effects of the immune system, including anti-microbial peptides and immunoglobulin A [98], as well as chemical barriers such as bile salts [39]. A proportion of the bacteria evade phagocyte killing by the induction of caspase-1-mediated cell death of resident macrophages mediated through the SPI-1-encoded T3SS [84]. The adhesions such as fimbriae of *Salmonella* mediate adherence to the apical membrane surface of epithelial cells (enterocytes), which is a prerequisite for effective invasion [99].

Gastrointestinal infection induces enteritis through a combination of the actions of secreted effectors of the SPI-1 T3SSs and recognition of flagella and lipopolysaccharide (LPS) via Toll-like receptors (TLRs) [100-102]. This initiates a pro-inflammatory cytokine and chemokine response leading to the recruitment of neutrophils [103]. The *Salmonella enterica* SPI-1 *sipA* and *sopABDE2* genes also contribute to the inflammation process by enhancing the production of GROα, GROγ, IL8 and GCP-2 chemokines [16]. *Salmonella* invasion and gut inflammation have a negative effect on the resident microflora of the host, and *Salmonella enterica* triggers and exploits inflammation to compete with the intestinal microbiota [104]. Bile affects the expression of *Salmonella* Typhimurium genes, which have been proposed to enhance colonization and persistence within the gallbladder [105]. *Salmonella* Typhimurium forms biofilms on the surfaces of human gallstones that may contribute to the development of the carrier state of bacteria [106].

After passage of the mucosal barrier, *Salmonella* spreads to deeper tissue [107]. There is evidence that some *Salmonella* prefer to exploit the microfold (M) cells, which are specialized epithelial cells [108-110]. In the distal ileum and

caecum, *Salmonella enterica* invades epithelial cells, and M cells in the Peyer's patches (PPs), using a T3SS encoded by genes within SPI-1 [31]. *Salmonella* can be taken up by cells including those that express CD11c and other dendritic cell markers within the gut-associated lymphoid tissues (GALT) [111-113]. Some bacteria can bypass the PPs and transport to the spleen and liver directly from the intestinal lumen through the blood within CD18+ cells [114].

SYSTEMIC SPREAD IN RETICULO-ENDOTHELIAL SYSTEM (RES)

After invasion and colonization into the PPs, *Salmonella* migrate within phagocytic cells to mesenteric lymph nodes, followed by primary bacteremia and dissemination to the intracellular location within phagocytes of the RES organs, such as the spleen, liver, bone marrow, etc. [31, 115,116]. In the RES system, *Salmonella* replicate in cellular niches, the macrophages [83]. In early infection, red pulp macrophages and marginal zone macrophages of the spleen appear to contain most of the bacteria [107]. In the liver, *Salmonella enterica* localizes preferentially in the resident Kupffer cells. A proportion of bacteria are also observed within PMNs [83]. In some cases, bacteria are found in dendritic cells (DCs) or B cells [117, 118].

To infect systemically, *Salmonella* undergoes either passive or active macropinocytosis to gain entry into targeted cells, such as macrophage and dendritic cells. In these targeted cells, *Salmonella* does not only escape NADPH oxidase- and inducible nitric oxide synthase (iNOS)-dependent killing [119, 120], but it can modify the normal maturation of the phagosome to form a *Salmonella*-containing vacuole (SCV) permissive for survival, persistence and eventually replication [107, 111]. The functional Nramp1 protein expression by macrophages restrains the division of *Salmonella* Typhimurium *in vivo* [121]. The survival within macrophages is essential for efficient systemic infection [39]. Continuous spread of the bacteria from infected cells to new infection foci is one of the key characteristics of systemic *Salmonella* infections. A high intracellular bacterial density within the phagosomal compartment renders the bacteria either nutritionally or spatially constricted. This situation affects bacterial spread in tissues. *Salmonella* leads the progressive local activation of the inflammatory response from established infection foci to new immunologically unprimed sites [122-125].

BACTERIAL SURVIVAL AND GROWTH IN THE PHAGOSOMES

The interaction of bacterial species such as *Salmonellae* with macrophages is a complex process involving the coordinated orchestration of signals and responses involving thousands of bacterial gene products and tens of thousands of mammalian gene products [126]. After the spread of *Salmonella* to the spleen and liver, the initial foci of infection consist of spatially separated phagocytes with a single bacterium carriage in each [122]. SPI-2 is optimally expressed after bacterial phagocytosis and phagosome trafficking has been controlled. SPI-2 remodels the vacuole into an intracellular replication niche by altering endocytic trafficking, the vacuolar membrane, and modifying the vacuolar-associated actin polymerization and formation of tubular lysosomes, extending from the vacuole. SPI-2 translocates at least 20 effector proteins through the phagosomal membrane into the eukaryotic cell cytoplasm to enhance intracellular replication and systemic spread [127-129]. These effector proteins have diverse subcellular localizations after translocation including the SCV, nucleus, and actin cytoskeleton. Intracellular *Salmonella* induces a variety of regulatory systems in macrophages, which promote bacterial surface remodeling of the protein, carbohydrate, and membrane components of the bacterial envelope. These are bacterial receptors that induce bacterial defense responses to protect the bacteria from macrophage-killing mechanisms. These responses are important to prevent killing by cyclic AMPs and nitrogen and oxygen radicals. Regulators important to this response are numerous and are part of the two component systems: OmpR and PhoPQ [92, 130, 131]. OmpR is essential for the expression of genes that encode a second TTSS located within SPI2 that is required for intracellular survival [72, 131, 132]. The PhoPQ system is made up of a membrane-bound sensor kinase PhoQ and the cytosolic response regulator PhoP [133]. The PhoPQ system regulates genes to increase resistance to macrophage anti-microbial defenses and decreased sensing of *Salmonella* through alteration of bacterial molecules recognized by macrophage innate immune receptors [134]. PhoP-repressed genes composed of the SPI-1 TTSS and flagellar genes [135,136] may decrease the host immunostimulatory activity [137]. PhoP-activated genes modulate resistance to antimicrobial peptides and reactive oxygen species [138]. After activation of PhoQ within the macrophage phagosome, resistance to anti-microbial peptides is acquired by modifications of the bacterial cell surface including modifications of LPS and membrane protein composition [139-141]. PhoQ-activated genes are also important in altering host processing and presentation of *Salmonella* antigens [142]. In addition, differential resistance/susceptibility of chicken lines to systemic salmonellosis has encouraged

the identification of host loci associated with disease outcome. The *SAL1* locus has been mapped to chicken chromosome 5 and is thought to be involved in survival of *Salmonellae* within macrophages [143,144]. Additional candidate loci involved in the resistance to systemic salmonellosis in the chickens include regions containing the *Nramp1* and TLR4 [145, 146].

The bacterial net growth of *Salmonella enterica* is controlled mainly by bacterial division [147]. *Salmonella enterica* bacterial division rate is dependent on the bacterial virulence, and is controlled by both ROS [119, 125, 148] and the *Slc11a1* gene [149]. The *Slc11a1* gene encodes a phosphoglycoprotein of 90–100 kDa, which is preferentially localized in membranes of phagosomes containing bacteria and functions as a divalent metal (Fe2+, Zn2+, Mn2+) ion pump at the membrane of the late endosome [150, 151]. In addition, the *Salmonella* metal ion transporters, MntH, SitABCD, and FeoB are shown to be required for intracellular replication [152, 153]. Furthermore, manganese, iron, magnesium and zinc are also critical for intracellular *Salmonella* growth [154]. During persistent *Salmonella* infection, certain metabolic pathways increase in importance. Isocitrate lyase (AceA) catalyzes the first step in the glyoxylate shunt pathway, enabling the bacteria to utilize fatty acids as a carbon source [155].

FORMATION OF PATHOLOGICAL LESIONS WITH RECRUITMENT OF INFLAMMATORY CELLS

The number of multicellular pathological lesions increases in parallel with the microbial burden, resulting in a small increase in the size of the lesion [122]. The bacteria escape from the discrete infection foci of the organ to disseminate throughout the organ, and establish new foci of infection at distant sites. The formation of pathological lesions in *Salmonella* infection is a dynamic process, which requires an influx of inflammatory cells [96] and the presence of adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) [156]. Polymorphonuclear cells (PMNs) are the first inflammatory cells to influx at the infection foci to form pathological lesions. These PMNs have a function in host resistance at the early stages of infection [83, 157]. Subsequently, mononuclear cells migrate into the organs to gradually replace PMNs in the pathological lesions [96]. TNFα is also an important mediator for cell recruitment into the lesions [158].

Salmonella enterica infections of humans, pigs and cows manifest as enteritis with persistent association with the gastrointestinal epithelium. In the chicken, as

in mammals, infection in the GI tract with *Salmonella* Typhimurium results in an influx of heterophils and inflammation [159]. The recruitment of CD3 cells leads to a dramatic elevation in caecal inflammation and neutrophil infiltration in the caecal mucosa [160]. An obvious augmentation of $CD4^+$ T cells, $CD8^+$ T cells and an increased number of $TCR2^+$ T cells in the caecum were observed after *Salmonella* Enteritidis infection [161, 162]. The highest gene expression values for IFN-γ and IL-12 were also found in the caecum of *Salmonella* Enteritidis-infected birds [163]. IFN-γ produced by NK cells and T cells activates the recruited cells to enhance antimicrobial functions [119]. IFN-γ enhances the ability of macrophages to kill intracellular *Salmonella* [163]. In addition, bacteria stimulate the production of the chemokine IL-8 in tissue, which directly recruits granulocytes in mammals [164, 165] and heterophils in chickens [166]. IL-8 exposed granulocytes are activated to generate oxygen radicals and nitric oxide, and thus contribute to inflammation and tissue destruction [167, 168]. The failure of innate immunity to control *Salmonella* replication in the spleen and liver leads to pronounced hepatosplenomegaly, with necrotic lesions in these organs [103, 162]. Initial *Salmonella* Typhimurium infection induces the expression of pro-inflammatory cytokine, especially high levels of IL1-β, and results in a transient hepatosplenomegaly [169-171].

SALMONELLA-INDUCED APOPTOSIS

In vivo, *Salmonella* Typhimurium induces cell death in mouse liver neutrophils and macrophages [83] and is cytotoxic to cultured macrophages [172]. Activated macrophages are more cytotoxic than unactivated cells after *Salmonella* infection, which indicates that both the host and pathogen contribute to the cell death process [51, 173]. This cytotoxicity is independent of intracellular bacterial replication, but is dependent on SPI-1 T3SSs [51, 174]. Bacteria inside macrophages delay the onset of apoptosis to allow sufficient time to replicate, escape and invade new macrophages in the systemic phase [51]. However, apoptosis induction in macrophages is a very rapid process as compared to the epithelial cells [175]. A small proportion of *Salmonella* were associated with epithelial cells of the intestinal microvilli, where apoptosis is suppressed to maintain an intracellular niche [173]. Apoptosis induction was dependent upon intracellular invasion and the synthesis of bacterial proteins. In addition, TNF-α and nitric oxide also mediate epithelial cell apoptosis [176].

Salmonella can invade and survive within dendritic cells. The interaction between *Salmonella* and dendritic cells has also been explored aside from the interaction with macrophages and epithelial cells during infection. Dendritic cells are antigen-presenting cells, which stimulate B and T lymphocytes to initiate immune responses against bacteria. In addition, dendritic cells can also produce cytokines and ingest apoptotic and necrotic bodies [177-180]. In contrast, surface receptors and ligands required for apoptotic cell death are expressed on dendritic cells [181]. By inducing the premature death of these antigen-presenting cells, *Salmonella* could severely interfere with a host immune response development to increase their chance for survival in the host. Subsequently, *Salmonella* can effectively escape from the host cell and re-infect new cells [182]. The cell death is associated with the mitochondrial release of cytochrome *c*, and the activation of caspase-2 and -3, followed by the activation of caspase-6 and -8. However, caspase-3-mediated apoptosis is a rare event and correlates with intracellular bacterial density, whereas the more common necrotic lysis and extracellular release of individual bacteria are independent of intracellular bacterial numbers [125].

PATHOGENICITY AND ITS RELATIONSHIP WITH ANTIMICROBIAL RESISTANCE

Antimicrobials are used in mammals and birds to treat or prevent disease. The link between antimicrobial drug usage in livestock including birds and the emergence of antimicrobial drug resistance in human pathogenic bacteria has been well described [183-186]. Many antibiotic regimens fail to completely clear an infection. In addition, bacteria can also evade antibiotic therapy [96]. In the l990s, the clinical isolates of non-typhoidal *Salmonella* species showed increasing resistance to ampicillin, chloramphenicol, and TMP-SMZ [3]. Unlike other *Salmonella* Typhimurium phage types, *Salmonella* Typhimurium DT104 is widely distributed in food animals such as cattle, pigs and chickens [187]. The zoonotic *Salmonella* Typhimurium DT104 has acquired the ACSSuT-resistance phenotype, which includes four of the five most common drug classes used in veterinary medicine (tetracycline, β-lactams, aminoglycosides, and sulfonamides) [188]. The multi-drug resistant (MDR) region in *Salmonella* Typhimurium DT104 is located in the *Salmonella* genomic island 1 (SGI1) region [187]. The 43-kilobase (kb) genomic island SGI1 has 44 ORFs, many of which are known genes and some have unknown functions. The MDR region with antibiotic resistance

genes is localized in a 13-kb segment of the SGI1 [189, 190]. The resistant *Salmonella* bacteria have been implicated in increased morbidity and mortality compared to pan-susceptible *Salmonella*. This was supported by studies showing that outbreaks caused by antimicrobial-resistant *Salmonella* were associated with an increased rate of hospitalization and bacteremia compared with outbreaks caused by pan-susceptible *Salmonella* strains [191, 192]. SGI1 could be conjugally transferred from *Salmonella enterica* donor strains to non-SGI1 *Salmonella enterica* recipient strains where it is integrated into the recipient chromosome in a site-specific manner [193]. It is noteworthy that *Salmonella* Typhimurium DT104 infection results in a higher egg contamination rate than *Salmonella* Enteritidis PT4 [194]. In addition, a secreted cytotoxin in MDR *Salmonella* Typhimurium DT104 is capable of inflicting damage on tissue culture cells and murine enterocytes [195, 196]. SGI1-harboring MDR *Salmonella enterica* leads to the enhancement of pathogenicity and invasion after exposure to rumen protozoa [197]. The enhancement of invasion was also associated with hyper-virulence in a bovine infection model. The protozoan-mediated hyper-virulence phenotype was found in *Salmonella* Typhimurium DT104, *Salmonella* Agona and *Salmonella* Infantis, which all possess SGI1 [187].

In a previous study, antimicrobial resistance and its genetic basis were studied in strains of *Salmonella* Enteritidis recently isolated from chicken meat, feces and eggs, which contribute to foodborne salmonellosis in humans. Researchers also investigated the presence of virulence-associated genes in these isolates by examining the SPI-1-associated genes (*invA, hilA, sipA, sopA, sopB, sopD* and s*opE2*), the SPI-2-associated genes (*ssrA* and s*saR*), and the *spvC* gene from the *spv* operon [198]. In an earlier study, most *Salmonella* Enteritidis clinical isolates were drug-susceptible [199], with antimicrobial resistant and multi-drug resistant (MDR) *Salmonella* Enteritidis isolates being rarely reported [200, 201]. In contrast, all examined *Salmonella* Enteritidis isolates were resistant to at least one antibiotic. In addition, many (65.2%) of the isolates were resistant to three or more antimicrobial drugs. Notably, a high resistance rate was observed to sulfisoxazole, nalidixic acid, ampicillin, piperacillin and ticarcillin antimicrobials [198]. Several *Salmonella* Enteritidis isolates were also resistant to cefazolin and cephalothin. These resistances are of great concern as these expanded-spectrum cephalosporins (ESCs) and quinolones have been recommended as drugs of choice for treatment of human *Salmonella* infections [202, 203]. Furthermore, β-lactams are used to treat complicated cases of salmonellosis in children and the elderly [184-186, 202]. However, more than 50% of the isolates were resistant to β-lactams, including ampicillin, piperacillin and ticarcillin [198]. In general, β-lactamases in the enterobacteriaceae are primarily TEM and SHV enzymes,

which confer resistance to β-lactams antibiotics and older generation cephalosporins [204, 205]. The high rates of resistance and MDR *Salmonella* Enteritidis strains increase concerns about future public health problems associated with salmonellosis, which would complicate the treatment of human *Salmonella* Enteritidis infections [198].

Chapter 3

OBSTACLES IN THE CONTROL MEASURES AND VACCINATION TO PREVENT SALMONELLOSIS

The major obstacle in control measures for salmonellosis is the ubiquitous presence of *Salmonellae* in the animal farm industry. Once *Salmonellae* enter into the farm, they spread rapidly because infected animals serve as carriers. *Salmonella* Enteritidis is unlikely to be cleared from birds by relying solely on the test-and-slaughter method of disease control, because *Salmonella* Enteritidis can be reintroduced into flocks from rodent reservoirs. Infected animals constantly shed *Salmonellae* and contaminate the feeding and watering systems of the farms. Current prevention measures are inefficient, inadequate and expensive for controlling *Salmonella* diseases. Antibiotic usage has decreased because of complications resulting from the development of antibiotic-resistant *Salmonella* strains [206-208] and the risk of consuming meat and poultry products contaminated with antibiotic residue. Sterilization of finished meat and poultry products with irradiation is an emerging approach, but it is an expensive method of controlling food poisoning related to *Salmonella* [209]. These control measures will not guarantee *Salmonella*-free animals because of the prolific nature of *Salmonellae* in infected animals, so all control efforts are useless if *Salmonella* proliferation cannot be prevented. Therefore, a critical need exists to develop an effective vaccine against these NTS.

An ideal strategy for the control of *Salmonella* may be the establishment of immunity in animals through vaccination [210]. Killed and live vaccines have been used worldwide for controlling *Salmonella* infection. The killed vaccines are able to induce a systemic level of immune responses [211], but are ineffective in

increasing proliferation of immune system cells [170]. In addition, previous live vaccines have potential for virulence reversal, though horizontal gene transfer remains a concern for live vaccines [212]. Previously reported live *Salmonella* vaccines have shown bacterial persistence in internal organs [213, 214] and fecal shedding in environment [215], which may cause undesirable effects to host and public health**.** Furthermore, a previously reported attenuated live vaccine did not trigger an innate immune response and cytokine production [216]. In addition, some *Salmonella* vaccine candidates did not protect against colonization of the intestines after a challenge with a virulent *Salmonella* strain [217]. Many *Salmonella* mutant vaccines fail due to either over- or under-attenuation of the vaccine strain [218, 219]. Besides the attenuation of systemic virulence, the control of gastrointestinal symptoms caused by the vaccine is another complication. Many promising candidate strains failed in clinical trials due to subsequent intestinal inflammation and diarrheic disorders in the host after vaccine administration [21].

Vaccination should also prevent transmission of salmonellosis from carriers, which is one of the major challenges to develop an effective vaccine. Another obstacle in the development of vaccines is the variety of non-typhoidal *Salmonella* diseases caused by various serovars and strains. Vaccination only induces immunity against specific antigens of an individual serovar and strain [94].

Chapter 4

CURRENT PROGRESS IN NTS VACCINE DEVELOPMENT

CHARACTERISTICS OF AN IDEAL VACCINE

An ideal vaccine must be inexpensive, safe, have minimal side effects, induce strong immunity, and provide life-long protective efficacy. It should be a single-dose oral vaccine, but still be safe at inducing a durable mucosal and cellular immunity. However, it should not cause any disease in progeny of vaccinated animals either through vertical or horizontal transmission. A good vaccine candidate must easily be differentiated from wild-type bacteria by antigenic or genetic or phenotypic markers. An ideal vaccine should not loose potency over long storage if killed, and should be stable and non-reverting to pathogenic if live. It should not interfere with colonization of normal gastrointestinal flora necessary for pathogen exclusion mechanisms in healthy individuals. In addition, it should not result in tolerance on overuse, and must not interfere with other tandemly used vaccines [220].

An ideal vaccine against *Salmonella enterica* pathogens should satisfy the following key requirements: 1) terminates the overall increase in bacterial load in tissues; 2) prevents the spread of bacteria in the body; 3) is safe for host and prevents fecal shedding of bacteria to avoid undesirable effects; 4) rapidly clears the pathogen from the infection site; and 5) is a cost-effective application.

Vaccine-induced immune responses require a suitable anatomical location at the infection site. For example, antibodies can only target bacteria in the extracellular space, while cell-mediated immunity can also target bacteria in the intracellular compartment. Extracellular bacterial spread (following necrosis) and

intracellular bacterial spread (following apoptosis) provide a rationale for the requirement of both antibodies and T cells to control *Salmonella enterica* [96]. Development of a reliable vaccine is critical, as salmonellosis has global effects on human health. Vaccines against *Salmonella* in food animals are an important step in preventing the spread of infection to humans. However, as the efficiency of the vaccine strain decreases, virulence of *Salmonella* increases [94].

ATTENUATED LIVE VACCINES

In order to combat *Salmonella* gastroenteritis, the vaccination of livestock animals appears to be more convenient than vaccination of humans. However, the increasing rate of identification of antibiotic-resistant foodborne zoonotic *Salmonella*e is a major challenge for the development of improved *Salmonella* vaccines [220]. Attention has been paid to the construction of avirulent *Salmonella* vaccine strains because such *Salmonella* strains are more immunogenic than killed or subunit vaccines in animals [221]. Live attenuated *Salmonella* Typhimurium is one of the most promising candidates for the engineering of live recombinant mucosal vaccines [222]. Current commercially available live *Salmonella* Typhimurium and *Salmonella* Enteritidis vaccine strains are either developed on the principle of metabolic drift mutations [223-225] or auxotropic double-marker mutants obtained through chemical mutagenesis [226]. These are null mutations in essential enzymes and metabolic regulatory centers, as a consequence of which the resulting metabolic processes lead to prolonged generation times and decreased virulence [224]. The genome of NTS facilitates the construction of completely rational mutations in virulence genes [6, 227], which are useful for the generation of attenuated NTS mutants as potential vaccine candidates (Table 2). However, mutant vaccine strains should retain their capacity of invasiveness in order to stimulate sufficient immunity to be protective. At the same time, the vaccine strain needs to be eliminated before slaughter age in animals and before onset of lay in layer and breeder chickens [252]. Antibiotic resistance genes widely used to select new mutants should not be present in the genome of the final vaccine candidate. The final constructed strain should be safe and highly immunogenic for application in immuno-compromised individuals. However, finding the right balance between attenuation and immunogenicity is a major challenge. Nevertheless, the recent observations of the host–pathogen interactions and bacterial mechanisms can contribute to the rational design of

Table 2. Recent Veterinary NTS Killed/Ghost/Subunit Vaccines in Food Animals

NTS Strain	Type of Mutant/Attenuation	Route	Animal	Reference
ST and SE	*ΔaroA* or Δ*cya* Δ*crp*	Oral	Pig	[228]
ST	Δ*cya* Δ*crp*	Oral	Chicken	[169]
SH	Δ*cya*, Δ*crp*, Δ*phoP*	Oral	Chicken	[229]
ST	Δ*gyrA*, Δ*cpxA*, Δ*rpoB*	Oral	Piglet	[230]
SC	Δ*gifsy-1 or* Δ*ssaV*	Oral	Pig	[231]
SE	ΔaroC	Oral	Chicken	[232]
SE (TAD *Salmonella* Vac® E)	metabolic drift mutant	Oral	Chicken	[233]
ST (TAD *Salmonella* Vac® T)	metabolic drift mutant	Oral	Chicken	[233]
ST	Δ*dam*	Oral	Calves, Sheep	[234, 235]
SC	Δ*crp* Δ*asd*	Oral	Piglet	[236]
SC	Δ*crp*	Oral	Pig	[237]
ST	Δ*htrA* Δ*clpP*	Oral	Pig	[238]
ST	Δ*ompD*	Oral	Pig	[239]
SD	metabolic drift mutant	IM*	Calves	[240]
SE	Δ*hilA*	Oral	Chicken	[241]
SC	Δ*asd*	IP¶	Pig	[242]
SC	Δ*slyA*	Oral	Pig	[243]
SE	Δ*aroA* Δ*htrA*	Oral	Turkey	[244]
SE	Δ*phoP* Δ*fliC*	Oral	Chickens	[245]
SE	Δ*lon* Δ*cpxR*	Oral/IM	Chickens	[246, 247]
ST	Δ*lon* Δ*rfj*	IM	Pig	[248]
SE	SPI-1 and SPI-2	Oral	Chickens	[249]
SE	Δ*hilA* Δ*ssrA*Δ*fliG*	Oral	Chickens	[250]
SE	Δ*lon* Δ*cpxR*Δ*asd* expressing LTB protein	Oral	Chickens	[251]

ST - *Salmonella enterica* serovar Typhimurium; SE - *Salmonella enterica* serovar Enteritidis; SH - *Salmonella enterica* serovar Hadar; SC - *Salmonella enterica* serovar Choleraesuis; SD - *Salmonella enterica* serovar Dublin

* IM - Intramuscular route; IP - Intraperitoneal route.

immunogenic recombinant *Salmonella* strains [21]. In addition to developing classical adaptive immunity to infection, the oral live *Salmonella* vaccines to day-old chicks may also induce a microbiologically-based intestinal colonization-inhibition effect [253] and an innate immunity-based invasion-inhibition effect, which resists systemic dissemination to other *Salmonella* organisms [254].

After oral vaccination, *Salmonella* invade and replicate in the mucosa-associated lymphatic tissues (MALT) and gut-associated lymphatic tissues (GALT) such as Peyer's patches and then reach systemic sites through the mesenteric lymph nodes. This dissemination pattern permits *Salmonella* to induce cell-mediated, humoral and secretory antibody immune responses. Thus, major emphasis should be placed on developing live vaccine candidates, capable of invasion in GALT and MALT to stimulate a protective immune response [220]. The mechanism of action of live *Salmonella* vaccines via oral and parenteral routes is briefly shown in the figure 2. In addition, live *Salmonella* vaccines have also been proven effective and compatible with probiotics and prebiotics [255].

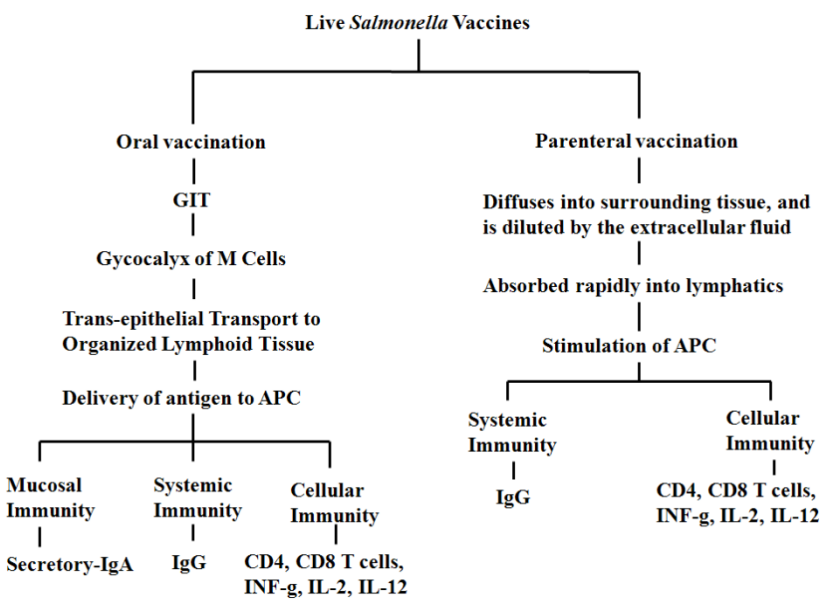

Figure 2. Mechanism of action of live *Salmonella* vaccines via oral and parenteral routes.

The live attenuated *Salmonella* strains are used to deliver recombinant antigens to the immune system, which is an attractive additional strategy for the creation of multivalent vaccines for animals. Sustained expression of the

heterologous antigen to induce a protective immune response is the main target during development of *Salmonella* recombinant vaccines. The number of vaccinations would decrease in the field through the use of multivalent vaccines [256]. Since SPI2 and probably also other virulence factors interrupt cytokine signaling pathways [131, 257, 258], two different recombinant *Salmonella* vaccines differing only by their distinct mutation within the SPI2 locus were used to modulate the immune response against a heterologous antigen [259].

In addition, recombinant DNA with a defined gene deletion has enabled the development of attenuated vaccines against a broad range of human and animal pathogens. An inactivation of the DNA adenine-methylase (Dam) results in attenuation of *Salmonella* Typhimurium, probably because the resulting strain has lost the ability to control the expression of virulence genes and stress response genes. The use of dam mutant strains of *Salmonella* Typhimurium as live vaccines was confirmed to protect against *Salmonella* Typhimurium and other *Salmonella enterica* serovar infections [260]. The vector potential of *Salmonella* vaccine strains has been manipulated for expression of a number of bacterial antigens [261, 262]. *Salmonella* vaccines are one of the most potent vectors for oral delivery of multivalent DNA and plasmid-vectored vaccines [263, 264]. This type of vaccine may induce immunity against the *Salmonella* carrier and heterologous antigen(s) from other *Salmonella* serotype [265]. In addition, some attention has been given to future modulation of the immune response by the co-expression of cytokines. A number of cytokines have been expressed in *Salmonella* vaccine strains, some of which have shown to have an immune-modulatory effect [266, 267].

The genetically engineered vaccines for control of *Salmonella* infection should have at least one suitable marker. The World Animal Health Organization (WAHO) has recommended that a vaccine must have both differentiating infected from vaccinated animals (DIVA) and marker(s) qualities before being released in the veterinary industry [220].

MUCOSAL VACCINES

The majority of infections originate from mucosal areas such as respiratory, gastrointestinal, and urogenital tracts [268]. Mucosal vaccines are less expensive, provide easy accessibility and needle-free administration, and the ability to perform mass immunizations during pandemics. Traditional injected vaccines are generally weak inducers of mucosal immunity and are therefore less efficient against mucosal site infections [269]. In contrast to injected vaccines, mucosal

vaccines have been reported to show secretory antibody-mediated protection against pathogens at the mucosal site of entry [270]. However, mucosa-administered antigens induce tolerance, since the host struggles to maintain mucosal homeostasis by responding to mucosal antigens with tolerance [271]. In this case, potent mucosal adjuvants, vectors or other special delivery systems are required for successful induction of mucosal immunity through vaccination [272]. The purpose of mucosal vaccines is to induce broad potent protective immunity by specific neutralizing antibodies at mucosal surfaces and by induction of cellular immunity. The ideal mucosal vaccine should protect vaccine antigens from enzymatic or chemical degradation, and enhance the preferential uptake of antigen by specialized lymphoid tissue M cells in order to target antigen presenting cell (APC), DCs or epithelial cells. The mucosal vaccine should also facilitate the co-uptake of both antigen and adjuvant to APCs in order to stimulate the production of neutralizing secretory IgA and/or helper and cytotoxic T lymphocytes. Secretory antibodies may block the colonization of pathogens in the mucosal epithelium. They can also prevent attachment of microbial toxins on epithelial cells. Subsequently, cytotoxic T cells eliminate infected cells and prevent microbial invasion [273].

Based on mucosally administered live attenuated strains, the NTS vaccines can diminish the NTS disease burden globally [274]. Mucosal IgA has an important role as the first line of defense at the mucosal epithelial surface through inhibition of *Salmonella* invasion into the Peyer's patch after oral infection [98, 275]. MALT is the main mucosal inductive site for initiating immune responses. The lamina propria is an important effector site for expansion and terminal differentiation of B cells [276]. Mucosal effector sites are formed by a surface epithelium with an aggregation of intraepithelial T lymphocytes (IEL) and secretory IgA antibodies. In addition, the sub-epithelial compartment is also an effector site, where NK-like cells, macrophages, B and T cells are present. APC including dendritic cells (DCs) are also present in the mucosal lymphoid tissue to detect foreign agents [273]. Recombinant or attenuated strains of *Salmonella* have also been employed as vectors to deliver antigens into the GALT [277]. Mucosally administered immunogens with an appropriate adjuvant can stimulate the most effective systemic immune response against not only mucosa-invading pathogens, but also those pathogens with predilection sites and invasion sites remote from the gut [220]. Few NTS mucosal vaccines are also available for humans (Table 2). *Salmonella* vaccines as a vector for DNA and plasmid-vectored vaccines have tremendous potential. Live oral *Salmonella* vaccines may be the future prototype vaccine vector for mucosal delivery of a battery of antigens [278-281].

KILLED VACCINES

The development of a *Salmonella* vaccine was initiated with an attenuated vaccine in the late nineteenth century [282]. However, the use of killed organisms was introduced as a safer vaccine to avoid the risk of live vaccines. Various NTS serovars were used to develop killed bacterins for veterinary use (Table 3). To inactivate *Salmonella* in killed vaccines, different agents such as heat, formaldehyde [300], ß-propiolactone (BPL) and glutaraldehyde [301] have been used to preserve the antigenicity and increase the efficacy of vaccines. The major drawbacks of these vaccines are that their immunogenicity usually has to be enhanced by co-administration with an adjuvant. In addition, multiple doses are necessary to obtain long-term protective immunity, and they may contain immunosuppressive antigens [302]. Different adjuvants such as, Chrome alum, alhydrogel, mineral oil [303,304], potash alum, Freund's incomplete [305] and Freund's complete adjuvant (FCA) [303] have been used in different killed *Salmonella* vaccines to increase immunogenicity. To improve antigenicity of the vaccine, the expression of better immunogenic antigens was enhanced in *Salmonella* during *in vitro* growth of the vaccine candidate. Iron-restriction is known to upregulate bacterial factors for virulence, which may stimulate important immunogens [306]. This method was used to produce Selanvac, a commercially available *Salmonella* Enteritidis PT4 bacterin.

Another attempt to enhance the efficacy of vaccines involved the usage of immunopotentiators such as thymulin, zinc [303], levamesol and vitamin E [307]. The criteria for an ideal killed vaccine should satisfy the following key points: 1) provides effective host protection through both humoral and cellular immune responses; 2) has high efficacy in reducing intestinal colonization, and thus reduced environmental contamination, and egg contamination; 3) is compatibility with other control measures; 4) is safe with no side effects after administration; and 5) is a cost-effective application. However, killed vaccines are unable to induce these key effects. Some inactivated whole-cell vaccines cause local inflammation, pain, systemic fever and malaise in some recipients. Thus, a whole-cell inactivated vaccine is not considered suitable for mammals [308]. It seems unlikely at the moment that more effective killed vaccine candidates will be developed in the future to identify the major protective immunogens and the nature of the immune response in animals.

Table 3. Recent Attenuated Live Mutant NTS Vaccines in Food Animals

NTS Strain	Characteristics	Route	Animal	Reference
Killed vaccines				
ST	Inactivated whole cell vaccines	SC[¶]	Pigeon	[283]
SE	Acetone Killed vaccine	SC	Chicken	[284]
SE	Formalin inactivated vaccine, water-in-oil emulsion	IM[#]	Chickens	[285]
SE (Poulvac® SE)*	Water-in-oil emulsion	IM	Chickens	[286]
SE	Formalin inactivated vaccine, water-in-oil emulsion	SC	Sheep	[287]
SE and ST (Oilvax SET)*	Bivalent Killed vaccine	SC	Hens	[212]
SE (Corymune® 4K and 7K)	Inactivated multivalent	IM	Hens	[288]
ST and SI (Salenvac®)*	Autologous killed Vaccine	IM	Hens	[289]
SE (Layermune SE or MBL SE4C)*	Water-in-oil emulsion	SC	Hens	[290]
SE	Formalin inactivated vaccine	SC	Chicks	[291]
SE and ST	Inactivated vaccine	IM	Chickens	[292]
ST	Inactivated vaccine	IM	Piglets	[293]
Ghost vaccines				
SE	mE-mediated lysis	SC	Chicks	[291]
SE	Gene *E* mediated lysis	IM/SC/Oral	Chickens	[294]
Subunit vaccines				
SE	OMP proteins	SC	Chicken	[295]
SE	Fimbrial subunit Mutant vaccine	Oral	Chicken	[296]
SN (Epitopix Inc.)*	Siderophore Receptor and Porins (SRP® Technology)	SC	Cattle	[297]
SE	Poplypetide part of antigen site of *Fli C*	SC	Chicken	[298]
SE	SPI-1 Protein	SC	Chicken	[299]

ST - *Salmonella enterica* serovar Typhimurium; SE - *Salmonella enterica* serovar Enteritidis; SI - *Salmonella enterica* serovar Infantis; SN - *Salmonella enterica* serovar Newport

*Commercial available Killed or Subunit vaccines

[¶]SC - Subcutaneous route; IM - Intramuscular route

Previously, a killed but metabolically active (KBMA) *Salmonella* Typhimurium strain was reported as a *Salmonella* vaccine [309]. However, the safety of KBMA is speculated to be similar to that of killed micro-organisms. *Salmonella* KBMA was developed from Δ*phoP/phoQ*Δ*aroA.* The *phoP/phoQ* deletion removes a major virulence regulatory locus in *Salmonellae,* and the *aroA* deletion renders the organism auxotrophic for aromatic amino acids, which are not available in mammalian tissues. Further, this mutant vaccine strain was devoid of the *uvrAB* gene, which is involved in DNA repair mechanisms. Photochemical treatment of Δ*uvrAB* mutant bacteria renders the organisms "killed, but metabolically active". This strain can not be able to replicate after UVA light treatment. The KBMA vaccine strain was markedly less reactive and stimulated a humoral immune response equivalent to its live counterpart [310].

Despite the many limitations, killed vaccines are often preferred. The importance of killed vaccines is apparent in the control of salmonellosis in animals. Although killed vaccines are not very effective, they are still the best vaccines for use where the disease is eradicated and they are the preferred vaccines for the eradication of an endemic strain of *Salmonella* from a herd. Under these conditions, herd-specific killed vaccines have been found to be more effective than the established live attenuated vaccines [311,312].

GHOST VACCINES

Inactivated bacterial vaccines are not able to stimulate the production of high titer antibodies due to destruction or deletion of some surface antigens during the preparation of vaccines. Genetically inactivating pathogenic Gram-negative bacteria by controlled expression of the cloned bacteriophage PhiX174 lysis gene *E* represents an innovative approach in non-live vaccine technology for developing safe and potent vaccines against bacterial infectious diseases including NTS [291,294,313]. A lysis plasmid carrying the bacteriophage PhiX174 lysis gene *E* and the lambda P_R-cI857 regulatory system were used to produce the *Salmonella* Enteritidis ghost vaccine [294]. Gene *E* codes for a 91-amino acid polypeptide that assembles and penetrates the inner and outer membranes of Gram-negative bacteria, leading to the formation of a transmembrane tunnel structure of 40–200 nm through the cell envelope (Figure 3). The high internal osmotic pressure in the cell expels the bacterial genome and cytoplasmic contents through the tunnel, leaving an empty cell envelope, known as a bacterial ghost (BG) [291,314, 315]. Ultrastructural studies indicate that the E protein-mediated transmembrane tunnel is preferentially formed at the septum or the polar regions

of the cell [316]. Since lysis tunnel formation is restricted to only a small part of the total cell surface, the resulting empty cell envelopes share the functional and antigenic determinants of the envelope with their living counterparts and are termed BG. The retained antigens on BG can be recognized and engulfed by dendritic cells and macrophages in immunized animals, thereby stimulating humoral and cell-mediated immune responses [317-319] and inducing Th1/Th2 T lymphocytes to secrete cytokines, such as IFN-γ and IL-4, which promote protective effects [291,318]. Systemic antibodies are essential to target *Salmonella* bacteria, which escape from infected cells to establish new infection foci at distant tissue sites [31]. The pathogen primarily targets the intestinal tract, where mucosal immunity acts as a first line of defense [320]. The secretory IgA in the intestinal mucus may restrict the mucosal colonization of *Salmonella* by controlling adherence and subsequent invasion of the bacteria [321]. In addition, cell-mediated immune responses are pivotal to resolving intracellular *Salmonella* infections [103, 212]. In some studies, BG vaccines induced not only a good immune response but also resistance to infection against highly virulent strains [322-324], including the protective effect of the BG vaccine against NTS disease [291,294] (Table 3). The BG vaccination did not show any adverse reactions, which suggests that the ghost vaccine could be safely administered without any detrimental effects to animals or the environment. An effective immunization of *Salmonella* Enteritidis ghost reduces the *Salmonella* Enteritidis load in the food chain and may lead to a decrease in cases of human salmonellosis caused by food poisoning. Therefore, the BG system represents a novel platform for genetically engineered vaccine technology against *Salmonella* diseases and contributes significantly to establish consumer confidence in safe food products. [291,294].

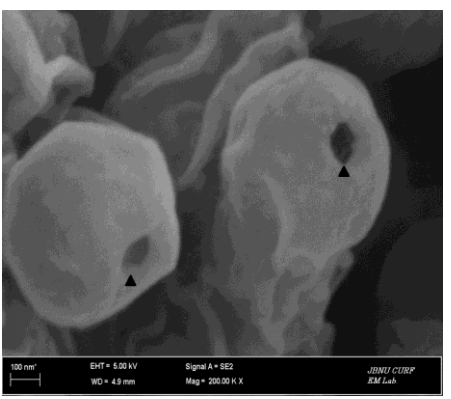

Figure 3. Electron microscopic analysis of *Salmonella* Enteritidis. Arrowhead showed the presence of transmembrane tunnels after lysis.

SUBUNIT VACCINES

In the 1980s, there were several attempts to use sub-cellular components of *Salmonella* to develop subunit vaccines to overcome the poor performance of killed vaccines. To develop subunit vaccines, common sub-cellular components of *Salmonella* are used, including outer membrane proteins (OMPs), porins, toxins and ribosomal fractions [220]. Characterization of the pathogen's antigens that are involved in protection is needed to avoid immunosuppressive proteins [302]. However, the production of subunit vaccines often requires purification of the immunogens from large quantities of the pathogenic organism, which is not safe and is significantly expensive [325]. Such vaccines have been administered in different animals with varying success [262,326-328]. Subunit vaccines have also been used in poultry and cattle (Table 3). The OMP vaccines with adjuvant have been administered to decrease bacterial shedding of *Salmonella* Enteritidis in poultry [329]. Immunization with the outer membrane protein-based vaccines decreases caecal colonization of virulent *Salmonella* Enteritidis strain [330].

In modern immunology and protein engineering, an isolation of the genes encoding epitope-carrying protein immunogens and their expression in heterologous hosts form the basis of recombinant-subunit-vaccine development. The main advantage of single proteins displaying immunodominant epitopes as vaccines is that they induce protective immunity without adverse effects and immune reactions caused by other parts of the pathogenic organism. Since subunit vaccines cannot replicate in the host, there is no risk of pathogenicity. However, these vaccines are poorly immunogenic and have short *in vivo* half-lives. Subunit vaccines often require multiple doses, adjuvant, and potent delivery systems to elicit a vigorous immune response [325, 331]. New generation adjuvants are essential to induce minimal side effects and concurrently stimulate humoral, cellular, and mucosal immune responses. These adjuvants should be biodegradable, economical, and simple to manufacture [302]. In addition, subunit vaccines often elicit only strain-specific protection. To induce full protection to a disease caused by several related strains, combinations of immunogens from the different strains might be needed [325].

Chapter 5

FUTURE PROSPECTUS FOR THE DEVELOPMENT OF NON-TYPHOIDAL VACCINES

The food animal vaccine industry is needed a remarkable innovation in the vaccination strategies considering the economic constraints of mass immunization. In this case, modified adjuvant technologies with several innate immune stimulators are needed to implement in the food animal vaccine industry. In some cases, vaccines are also being targeted for delivery via DNA, bacterial or viral vectors.

ADJUVANT TECHNOLOGIES AND INNATE IMMUNE STIMULATOR

Immunostimulatory adjuvants are either toxin-based or cytokine based. Cholera toxins (CT) and *E.coli* heat labile (LT) enterotoxins are effective musosal adjuvant, which promote mucosal and systemic immunity to co-administered protein antigens via oral route [332]. However, both enterotoxins cause sever diarrhoea and lead a potential threat to the central nervous system. To overcome these problems, nontoxic mutant derivatives of CT and LT have been reported [334, 334]. A genetically engineered *Salmonella* Typhimurium secreting *Escherichia coli* heat-labile enterotoxin B subunit protein has shown protection against salmonellosis in pigs [198]. Furthermore, cytokine based adjuvants, such as IL-1, IL-6, IL-12 and Type I IFNs have been shown to possess mucosal adjuvant activity. In addition, innate immune associated adjuvants such as

Lymphotactin/RANTES, CpG dinucleotide and saponin adjuvants may have important implications for the design of future oral vaccine formulations [335].

THE VACCINE DELIVERY VEHICLE

Naked plasmid DNA immunization resulted in the expression of the encoded protein, which can induce systemic immunity [336]. To enhance immunogenicity, the mucosal delivery via DNA vehicle for protein antigen has been successfully adapted [337-339]. This vaccine technology offers numerous advantages including preservation of antigen conformation, better antigen presentation via MHC class I and II molecules and incorporation of multiple vaccine antigens on the same construct. However, DNA vaccines are limited in their protective capacity to the encoded proteins on their vector and pose the risk of integrating into the host genome [340]. A novel approach for the development of an M cell-targeted DNA mucosal vaccine has been reported [339]. A new generation vaccine such as M cell targeted DNA vaccine can be used to combat *Salmonella* associated zoonotic diseases globally and to mitigate the threat of public health concerns. The use of DNA vaccines can be a promising technology with the potential for further research in the food animal vaccinology.

In addition, *Salmonella* Typhimurium-based vector in chickens expressed an immunogenic protein of *Clostridium* Perfringens. Vaccinated chickens showed protection against *Salmonella* Typhimurium and *Salmonella* Enteritidis colonization with lower levels of necrotizing enteritis [341]. These types of vector systems and its potential implications can promote the development of multivalent vaccines. Furthermore, a novel recombinant viral vector expressing protective *Salmonella* antigens may prove beneficial to the food animal industry, especially if it can protect against multiple zoonotic pathogens. Live vectored viral vaccines containing heterologous antigens of *Salmonella* could be easily achieved to protect food animals against salmonellosis. Adenovirus from food animals is being examined for their efficiency to deliver antigens from zoonotic bacterial pathogens, including *Salmonella* [342]. Innovative vaccine technologies can improve the immunogenicity and protection efficacy of non-typhoidal vaccines to reduce the risk of zoonotic infection in humans via consumption of the contaminated meat, egg and milk products by *Salmonella*.

CONCLUSION

The genome projects of *Salmonella* have hastened the identification of the majority of *Salmonella* virulence genes. It is evident that NTS can affect its host in many ways, including invasion and colonization of the gastrointestinal tract and subsequent systemic spreading to the RES. *Salmonella* has evolved a series of strategies to survive inside the harsh milieu of phagolysosomal compartments of phagocytic cells in the host, which leads to the homogeneous distribution of different subpopulations within an organ. However, an increase in the number of infection foci in organs is likely to require lysis of infected cells. By inducing cell death, *Salmonella* can effectively escape from the host cell and re-infect new cells, and also remove host effector cells, which could weaken the host immune response. Alternatively, a delay in cell death would allow the bacteria to replicate intracellularly, and would allow for alterations in both the host and viral gene expression. Despite ample information, our understanding of the intricacies of the host-pathogen interactions associated with NTS remains rudimentary. The substantial impact of drug-resistant non-typhoidal *Salmonella* in the developing world is a formidable challenge. However, *Salmonella* researchers and clinicians have made significant contributions to understanding the interaction between virulence determinants and immunity required to control the spread of this pathogen.

In modern science, vaccines are developed to protect animals and humans against NTS diseases. Veterinary vaccines have already made progressive impacts not only on animal health and welfare, but also on human health. As highlighted in this review, much progress has been made in developing a range of veterinary and human NTS vaccines to increase safety and efficacy. There is ample scope to incorporate new knowledge and technologies into vaccine design to overcome current unsolved problems in vaccine development. Apart from the scientific

challenges, the development of a commercially successful veterinary vaccine should also fulfill the regulatory guidelines that pave the route to the vaccine industry. However, veterinary vaccines are at the forefront of the testing and commercialization of innovative technologies due to their less stringent regulatory requirements and quicker route to market.

In particular, most vaccines are based on live, attenuated pathogen strains. An attenuated live vaccine approach is not generally desirable for commercial companies, as it exposes them to risks of mitigation, and the short shelf-life and strain/region specificity of many vaccines make them uneconomical to produce. Although several variably defined killed and subunit vaccines are available in the veterinary vaccine market, they have been implicated in a series of immune-deficiencies that increase susceptibility to *Salmonella* infection. A deep understanding of the molecular and immunological disease processes of *Salmonella* is likely to be required to improve the effectiveness of killed or subunit vaccines. The use of an adjuvant and antigen delivery system may improve the effectiveness of killed and subunit vaccines. Currently, the development of safe and effective mucosal and ghost vaccines remains a particular priority to overcome the problems associated with live, killed and subunit vaccine strategies against NTS. It is apparent that considerably more research is required to develop safe and efficient NTS vaccines. The aim of developing a potent veterinary NTS vaccine is likely to be achieved in the near future by use of biogenetic engineering methods. Different immune modulator technologies and vaccine delivery vehicles are being the subject of innovative research to improve NTS vaccines in the future. Developed veterinary and human non-typhoidal vaccines can prevent human salmonellosis by minimizing foodborne zoonotic NT-*Salmonella* infection via consumption of contaminated meat, egg and dairy products.

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