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Live attenuated vaccines for invasive *Salmonella* infections

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Abstract

Salmonella enterica serovar Typhi produces significant morbidity and mortality worldwide despite the fact that there are licensed *S*. Typhi vaccines available. This is primarily due to the fact that these vaccines are not used in the countries that most need them. There is growing recognition that an effective invasive *Salmonella* vaccine formulation must also prevent infection due to other *Salmonella* serovars. We anticipate that a multivalent vaccine that targets the following serovars will be needed to control invasive *Salmonella* infections worldwide: *S*. Typhi, *S*. Paratyphi A, *S*. Paratyphi B (currently uncommon but may become dominant again), *S*. Typhimurium, *S*. Enteritidis and *S*. Choleraesuis (as well as other Group C *Salmonella*). Live attenuated vaccines are an attractive vaccine formulation for use in developing as well as developed countries. Here, we describe the methods of attenuation that have been used to date to create live attenuated *Salmonella* vaccines and provide an update on the progress that has been made on these vaccines.

Keywords

Salmonella; vaccine; live; attenuated; invasive

1. Introduction

The first vaccines against typhoid fever consisting of heat-inactivated typhoid bacilli preserved in phenol administered parenterally, were developed in the late 19th century.[1] Experiences with implementation of typhoid vaccines in the British and US military in the early 20th century and subsequent large-scale controlled field trials sponsored by the World Health Organization documented that the inactivated whole cell vaccines were efficacious

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but were highly reactogenic.[1] Whole-cell vaccines against *Salmonella enterica* serovars Paratyphi A and B were also developed in the early 20th century and used by the U.S. military as a trivalent "TAB" vaccine against enteric fever.[2] However, these whole-cell vaccines lost favor due to their propensity to produce high fever, severe headache and malaise and gave way to the development of better tolerated *Salmonella* vaccines using other approaches such as parenteral polysaccharide and polysaccharide-protein conjugate vaccines and live attenuated oral vaccines. There are currently three types of licensed *Salmonella* vaccines: the live attenuated vaccine Ty21a marketed as Vivotif® (PaxVax Corporation); unconjugated Vi polysaccharide vaccine commercialized as Typhim Vi® (Sanofi Pasteur), Typherix® (GSK) and Typbar Vi® (Bharat Biotech), amongst others; and Vi polysaccharide conjugated to tetanus toxoid (Typbar TCV®, Bharat Biotech and Peda Typh™, Biomed).

Currently, licensed vaccines exist against no *Salmonella* serovars other than *S*. Typhi (although *S*. Typhi vaccine strain Ty21a confers moderate cross protection against *S*. Paratyphi B as well as *S*. Typhi).[3] There is growing recognition that other invasive *Salmonella* serovars also cause a notable disease burden.[4] *S*. Paratyphi A is emerging as a pathogen in Asia;[5] the non-typhoidal *Salmonella* serovars *S*. Typhimurium and *S*. Enteritidis cause invasive disease throughout sub-Saharan Africa,[6] and *Salmonella* Group C serovars such as *S*. Choleraesuis are associated with invasive disease in certain countries such as Taiwan.[7] As such, a multivalent vaccine that targets the following serovars is needed to control invasive *Salmonella* infections worldwide: *S*. Typhi, *S*. Paratyphi A, *S*. Paratyphi B (currently uncommon), *S*. Typhimurium, *S*. Enteritidis and *S*. Choleraesuis (as well as other Group C *Salmonella*).

At the Center for Vaccine Development, University of Maryland School of Medicine, we have developed and evaluated a variety of *Salmonella* live attenuated vaccines. There are several advantages of live oral attenuated vaccines over other vaccine formulations: 1) they can induce local immune responses at mucosal surfaces; 2) they are economical to produce; 3) they induce *Salmonella*-specific B and T cell immunity; 4) they are practical to administer to a large population, and 5) they do not generate hazardous waste (e.g., needles and syringes) that needs to be discarded appropriately.[8, 9] However, there are several limitations to live attenuated vaccines. First, one needs to balance immunity and reactogenicity, particularly if the vaccine is to be used as a live vaccine vector.[10] The vaccine may also need to be formulated differently for infants. For example, Ty21a at times has been available in both a sachet formulation for use in young children as well as enteric-coated capsules for use in older children and adults.[11–13] Finally, safety of live attenuated vaccines needs to be determined in immunocompromised subjects and also the very young prior to widespread use.

Here, we describe the methods of attenuation that have been used to date to create live attenuated *Salmonella* vaccines and provide an update on the progress that has been made on these vaccines.

2. Methods of attenuation

The first method used to mutate bacteria to create live attenuated vaccines was chemical mutagenesis. However, with the advent of molecular biology, live attenuated vaccines are now constructed by making focused site-directed mutations using genetic engineering.

a. Chemical mutagenesis

Here, bacteria are exposed to a mutagen and spontaneous mutants are selected and passaged. The licensed typhoid vaccine Ty21a was constructed in the early 1970's using chemical mutagenesis.[14] Spontaneous *galE* mutants were selected and shown to lack UDP-galactose-4-upimerase activity. In the absence of galactose, these mutants produce rough LPS whereas when galactose is supplied exogenously, smooth LPS is produced. Chemical mutagenesis is a simple procedure and highly effective if the mutation is not lethal to the bacteria. However, one disadvantage of this method is that additional mutations may occur in several locations in the genome and as such the mutations are not fully defined. For example, Ty21a has more than two dozen mutations in addition to *galE*, the sought mutation.[15] Interestingly, the *galE* mutation alone is not responsible for the attenuation of Ty21a.[16] Instead, attenuation is most likely due to a combination of the *galE* mutation and one or more of the other mutations.

b. Genetically engineered mutagenesis

With the introduction of recombinant DNA technology, bacteriologists were able to genetically engineer defined mutations in bacteria. This meant that researchers were able to accurately characterize the mutations in attenuated vaccine strains. Mutations can be introduced into the *Salmonella* genome using homologous recombination such that the final live attenuated vaccine is free of antibiotic resistance genes.[17, 18] Presently, regulatory agencies such as the U.S. Food and Drug Administration require a live attenuated vaccine strain to possess two independently attenuating mutations. Interestingly, the choice of background strain also plays a role in generation of effective live attenuated vaccine strains. In some backgrounds, certain mutations were fully attenuating whereas in other strains, the effect on virulence was not as profound.[19, 20]

Below, we describe some the most commonly mutated genes in live attenuated *Salmonella* vaccines which have been evaluated in human volunteer studies.

i. Aromatic acid biosynthesis pathway—The first live attenuated *Salmonella* vaccines contained mutations in aromatic acid biosynthesis pathway genes.[21] Deletion of genes involved in aromatic amino acid synthesis (e.g., *aroA*, *aroC* and *aroD*) produces bacteria that are auxotrophic for para-aminobenzoic acid (PABA) and 2,3-dihydrobenzoate. When administered to mice, *Salmonella aro* mutants are unable to scavenge enough PABA and dihydrobenzoate to replicate.[21] Multiple pre-clinical studies have shown that *Salmonella aro* mutants elicit robust immune responses which can protect animals against lethal challenge.[22–25]

ii. htrA—HtrA (also known as DegP) is a serine protease that is induced by heat shock in *E. coli* and other *Enterobacteriaeceae*.[26] This protein degrades misfolded proteins in the bacterial periplasm. *S.* Typhimurium Δ*htrA* mutants show decreased survival within macrophages, decreased virulence in mice and are protective.[27–31]

iii. ssaV—The *ssaV* gene has been used as an attenuating mutation in *S.* Typhi and *S.* Typhimurium vaccines.[32] This gene is encoded on *Salmonella* Pathogenicity Island 2 (SPI-2) a Type 3 Secretion System (TTSS) which is required for virulence of *S.* Typhimurium in mice.[33] SPI-2 mutants show decreased survival within macrophages.[34–36] This pathogenicity island translocates *Salmonella* effector proteins across the bacterial inner and outer membranes to the host cell cytoplasm. SsaV forms part of the TTSS needle apparatus. *Salmonella ssaV* mutants are unable to secrete SPI-2 effector proteins. [37]

iv. PhoP-PhoQ virulence regulon—The PhoP/PhoQ regulon is a two component regulatory system which controls the transcription of multiple genes.[38–40] PhoP is a cytoplasmic transcriptional regulator and PhoQ is a membrane associated sensor kinase. This operon contributes to survival within macrophages and resistance to antimicrobial peptides.[38, 41] *S.* Typhimurium *phoP* mutants are avirulent and can induce a protective immune response in mice.[42, 43]

v. Adenylate cyclase and cyclic AMP receptor protein—Cyclic AMP (cAMP) and cAMP receptor protein (CRP) are required for multiple essential cellular processes including transport of metabolites.[44] The *cya* gene is required for adenylate cyclase synthesis and *crp* encodes cAMP receptor protein. *S.* Typhimurium *cya* and *crp* mutants are attenuated in mice and protective in various animal models.[45–48]

vi. clpPX—At the CVD, we have deleted *clpPX* in several live attenuated *Salmonella* vaccines.[49] This is an attenuating mutation in *S.* Typhimurium and other *Salmonella* serovars and also has an added benefit. The *clpPX* genes encode a protease that degrades the master flagella regulator FlhD/FlhC.[50, 51] The FlhD/FlhC complex is a transcriptional activator of the flagella synthesis pathway. When ClpPX is absent, FlhD/FlhC accumulates and large amounts of flagellin are produced. We have used this phenotype to our advantage to enable economical purification of flagellin from recombinant *Salmonella* strains for use as a carrier protein in conjugate vaccines.[49]

vii. Other genes—Many other mutations have been shown to produce effective live attenuated *Salmonella* vaccines in preclinical studies. For example, *S.* Typhimurium DNA adenine methylase (Dam) mutants are avirulent and can protect mice against lethal challenge.[52–54] Dam methylates adenine in GATC sequences and controls the expression of multiple *Salmonella* virulence genes.[55] A *S.* Typhimurium Δ*relA* Δ*spoT* mutant which is unable to produce ppGpp, a signal important for *Salmonella* pathogenicity island (SPI) virulence gene-encoded expression, was also effective as a live attenuated vaccine in a murine challenge model.[56, 57] Other genes which have been deleted to create live attenuated *Salmonella* strains include *cdt* (colonization of deep tissue), *fur* (ferric uptake regulator), *gidA* (encodes a glucose-inhibited division gene), *wecA* (encodes a UDP-N-

acetylglucosamine-1-phosphate transferase gene required for production of enterobacterial common antigen [ECA]) and *rpoS* (encodes the alternative sigman factor RpoS).[58–64] Several groups have also shown that modifications of *Salmonella* LPS can produce effective live vaccine strains.[65, 66] Interestingly, instead of deleting genes, some investigators have attenuated bacteria by overexpressing bacterial surface appendages such as flagella and pili using a method termed Attenuated Gene Expression (AGE).[67]

3. Live attenuated vaccines against invasive *Salmonella* serovars

Since the majority of invasive *Salmonella* disease burden has traditionally been attributed to *S*. Typhi, multiple typhoid vaccine candidates have been evaluated in clinical trials whereas vaccines against other *Salmonella* serovars have been neglected. Here, we describe some of the live attenuated invasive *Salmonella* vaccines that have been developed to date including vaccines that are currently in development (summarized in Table 1).

a. *S*. Typhi

Ty21a, a licensed *S*. Typhi live attenuated vaccine, was derived from *S*. Typhi Ty2 by chemical mutagenesis.[14] This vaccine is well-tolerated and shown to be immunogenic and protective against *S*. Typhi in several large-scale, randomized placebo-controlled field trials.[11, 68, 69] Ty21a also confers significant protection against *S*. Paratyphi B disease.[70] However, the vaccine needs to be administered in 3 – 4 doses every other day. Therefore, new candidate live attenuated *S*. Typhi vaccine strains have been developed which elicit higher immunogenicity and only require a single oral dose.

In the 1990's, the CVD developed live attenuated *S*. Typhi vaccines that possessed mutations in the aromatic acid biosynthesis pathway.[71] The *aroC* and *aroD* genes were deleted from *S*. Typhi Ty2 to produce CVD 908.[72] This vaccine was well-tolerated at doses of 5×10^4 CFU and 5×10^5 CFU and also immunogenic.[19, 73] However, upon subsequent testing CVD 908 produced a clinically silent bacteremia at higher doses (5×10^7 CFU and 5×10^8 CFU).[74] Interestingly, CVD 906 which is another *S*. Typhi candidate vaccine strain with *aroC* and *aroC* deletions in the wild-type strain ISP1820 also produced asymptomatic vaccinemia in volunteers at 5×10^7 CFU.[75] To further attenuate CVD 906 and CVD 908, an additional mutation was introduced. The *htrA* gene was deleted from CVD 906 to produce CVD 906-*htrA* and from CVD 908 to produce CVD 908-*htrA*. Incorporation of this mutation had the desired effect and no vaccine bacteremias were observed at doses up to 5×10^9 CFU with no reduction in immunogenicity.[20, 74] CVD 908-*htrA* was subsequently tested in a Phase 2 study as a lyophilized formulation (in contrast to freshly harvested bacteria as was used for the Phase 1 studies).[76] At the two doses tested, 5×10^7 CFU (low dose) and 4.5×10^8 CFU (high dose), no bacteremias were observed. Even after only one dose of vaccine, 100% of high-dose recipients and 92% of low-dose recipients possessed antibody secreting cells (ASCs) producing IgA against LPS.

To further improve on the live attenuated vaccine CVD 908-*htrA*, this strain was genetically engineered to constitutively express the Vi polysaccharide. Generally, live attenuated *S*. Typhi vaccines elicit poor anti-Vi responses presumably due to down regulation of the genes that express Vi *in vivo*. The native P*tviA* promoter which regulates Vi expression in CVD

908-*htrA* was replaced with the strong constitutive promoter P*tac* to produce CVD 909.[77] Vi-specific IgA ASCs were detected in 80% of volunteers given 10^8 – 10^9 CFU CVD 909.[78] Although impressive ASC responses were produced, only 2 out of 32 volunteers generated anti-Vi serum IgG antibodies.

Another *aro*-based *S*. Typhi vaccine is M01ZH09 (*S*. Typhi Ty2 Δ*aroC* Δ*ssaV*). This vaccine has been evaluated as a single dose vaccine in Phase 1 and Phase 2 clinical trials and shown to be safe and well-tolerated in adults and children including in Vietnam, a typhoid-endemic country.[79–81] This vaccine, now called Typhella®, is licensed by Prokarium and is also being investigated for use as a vaccine delivery vector.

Two other live attenuated *S*. Typhi vaccines, Ty800 and χ3927, have been evaluated in human volunteers but have not progressed past Phase 1 studies. Ty800 (*S*. Typhi Ty2 Δ*phoP*/*phoQ)* was evaluated in 11 volunteers.[82] Ty800 was safe and immunogenic as a single dose. χ3927 (*S*. Typhi Ty2 Δ*cya* Δ*crp*) was well tolerated and immunogenic in a Phase 1 study but produced vaccinemia in 2 of 12 volunteers and fever in an additional volunteer.[19]

b. *S*. Paratyphi A and B

There is growing recognition that *S*. Paratyphi A should be targeted in addition to *S*. Typhi. Roland et al. [83] have constructed a *S*. Paratyphi A Δ*phoPQ* vaccine strain which was well tolerated and immunogenic in an oral rabbit model. The CVD has developed a live attenuated *S*. Paratyphi A vaccine, CVD 1902, which harbors Δ*guaBA* Δ*clpX* deletions in the ATCC9150 parental strain. This vaccine has been tested in a Phase 1 clinical trial (NCT01129452; ClinicalTrials.gov) at the CVD. The vaccine was well-tolerated at doses ranging from 10^6 – 10^{10} CFU and was immunogenic (K. Kotloff, personal communication).

Little *S*. Paratyphi B vaccine development has been performed to date. This is partly due to the fact that currently, *S*. Typhi and *S*. Paratyphi A are the dominant typhoid disease causing serovars. However, in anticipation that *S*. Paratyphi B could potentially resurface in the future, we are developing a candidate live attenuated *S*. Paratyphi B vaccine with mutations in *guaBA* and *clpX*.

c. *S*. Typhimurium and *S*. Enteritidis

Invasive non-typhoidal *Salmonella* are increasingly being recognized as a significant cause of morbidity and mortality in sub-Saharan Africa. In particular, *S*. Typhimuium and *S*. Enteritidis are responsible for 80–95% of invasive NTS infections.[6]

One of the early *S*. Typhimurium live attenuated vaccines that has been tested in a Phase 1 clinical trial was *S*. Typhimurium Δ*aroC* Δ*ssaV*. This vaccine was well-tolerated by volunteers but when ingested at 10^8 and 10^9 CFU, was shed in stools for up to 23 days. In contrast, a *S*. Typhi vaccine with the same gene deletions was well tolerated and not persistently excreted in stool.[32]

At the CVD, we have created live attenuated *S*. Typhimurium and *S*. Enteritidis vaccines with mutations in the *guaBA* and *clpPX* genes.[49] With further genetic modifications, these

strains also serve as reagent strains for economical purification of components of a bivalent conjugate vaccine that is also in development.[84, 85] The live attenuated vaccine strains CVD 1921 (*S*. Typhimurium I77 Δ*guaBA* Δ*clpP*) and CVD 1941 (*S*. Enteritidis R11 Δ*guaBA* Δ*clpP*) were safe and immunogenic in BALB/c mice.[49] Importantly, they were able to protect against a lethal challenge. Furthermore, the *S*. Typhimurium vaccine CVD 1921 was also safe in SIV-infected rhesus macaques.[86] We have also created another candidate *S*. Typhimurium live attenuated vaccine CVD 1931 (*S*. Typhimurium D65 Δ*guaBA* Δ*clpX*). The parent of this vaccine wild-type strain D65 was isolated from the blood of an infant in Mali, West Africa. This isolate is multi-locus sequence type 313, the dominant genotype of *S*. Typhimurium that is circulating in sub-Saharan Africa. We have recently shown that *S*. Typhimurium ST313 strains are phenotypically different from ST19 isolates (the most common genotype found throughout the world and which causes gastroenteritis).[87] *S*. Typhimurium ST313 isolates from sub-Saharan Africa are highly resistant to killing by macrophages and elicit reduced inflammation compared to *S*. Typhimurium ST19 isolates.[87] Carden *et al*. have also shown that ST313 isolates produce less caspase 1 dependent macrophage cell death and IL-1β release compared to ST19 strains.[88] We anticipate that *S*. Typhimurium live attenuated vaccines of the ST313 backbone may manifest different effects in human volunteers compared to ST19-derived strains. We hypothesize that the ST313 vaccine strain CVD 1931 will not be shed in stool for an extended period of time as was seen for the *S*. Typhimurium Δ*aroC* Δ*ssaV* vaccine that was constructed in the gastroenteritis-causing strain TML.[32] This is primarily based on genomics analyses which showed that *S*. Typhimurium ST313 are lacking *pipD* which is required for fluid secretion in bovine ileal loops.[89, 90] Okoro *et al*. provide evidence to support this hypothesis and found that ST313 isolates exhibit reduced enteropathogenicity in streptomycin-treated C57BL/6 mice and in bovine ligated loops.[91]

d. *Salmonella* Group C

Several *S*. Choleraesuis (Group C1) vaccines have been developed for use in pigs.[92– 96] A *S*. Bovismorbificans (Group C2) live attenuated vaccine has also been developed with the aim of reducing Salmonellosis in sheep. This live attenuated Δ*aroA* vaccine was able to protect mice against a lethal challenge with wild-type *S*. Bovismorbificans.[97] A *S*. Choleraesuis Δ*aro* mutant was also able to partially protect mice against a lethal dose of *S*. Choleraesuis delivered intraperitoneally.[98] To date, no *Salmonella* Group C vaccines have been evaluated in humans. At the CVD, we are developing live attenuated vaccines against *Salmonella* Group C1 and C2 infections with the view to combine these strains with live attenuated *S*. Typhi, *S*. Paratyphi A, *S*. Paratyphi B, *S*. Typhimurium and *S*. Enteritidis vaccines to create a multivalent vaccine that protects against the major causes of invasive *Salmonella* disease worldwide. It is unclear how many vaccine strains would need to be included to provide adequate protection against all of these serovars. Studies that have examined cross-protection elicited by *Salmonella* vaccines have shown mixed results with some reports describing cross-protection against heterologous challenge organisms and others reporting no protection.[24, 99– 103] Similarly, volunteer studies have shown that immune responses generated by live *S*. Typhi vaccines are cross-reactive with other *Salmonella* serovars but it is not yet known whether these responses would be protective.[104–106]

4. Conclusions

Despite the first *Salmonella* vaccines being developed over a century ago, invasive *Salmonella* disease is still a significant cause of mortality and morbidity worldwide. Due to improved surveillance efforts, there is a growing realization that in addition to preventing *S.* Typhi, other invasive *Salmonella* serovars, particularly *S.* Paratyphi A in Asia and *S.* Typhimurium and *S.* Enteritidis in sub-Saharan Africa should also be targeted. Live attenuated vaccines are an attractive vaccine platform given that they are economical, provide long lived protection and easy to implement.

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Table 1

Live attenuated *Salmonella* vaccines

Serovar	Vaccine	Parent	Genotype	Developer	Stage	References
Typhi	CVD 906	ISP1820	Δ*aroC* Δ*aroD*	CVD	Phase 1	[75]
	CVD 908	Ty2	Δ*aroC* Δ*aroD*	CVD	Phase 1	[19, 20, 72–74]
	CVD 906-*htrA*	ISP1820	Δ*aroC* Δ*aroD* Δ*htrA*	CVD	Phase 1	[20]
	CVD 908-*htrA*	Ty2	Δ*aroC* Δ*aroD* Δ*htrA*	CVD	Phase 1 and 2	[20]
	CVD 909	Ty2	Δ*aroC* Δ*aroD* P_{tac}-*tviA*	CVD	Phase 1	[78]
	Typhella (M01ZH09)	Ty2	Δ*aroC* Δ*ssaV*	Prokarium	Phase 1 and 2	[79–81, 107–109]
	χ3927	Ty2	Δ*cya* Δ*crp*	Curtiss, R. 3rd	Phase 1	[19]
	Ty800	Ty2	Δ*phoPQ*	Celldex Therapeutics	Phase 1 and 2	[82]
Paratyphi A	CVD 1902	ATCC 9150	Δ*guaBA* Δ*clpX*	CVD	Phase 1	ClinicalTrials.gov: NCT01129453
	MGN10028	MGN9772	Δ*phoPQ*	Celldex Therapeutics	Pre-clinical	[83]
Paratyphi B	CVD 2005	CMF 6999	Δ*guaBA* Δ*clpX*	CVD	Pre-clinical	Higginson E., unpublished data
Typhimurium	CVD 1921	I77	Δ*guaBA* Δ*clpP*	CVD	Pre-clinical	[49]
	CVD 1931	D65	Δ*guaBA* Δ*clpX*	CVD	Pre-clinical	Tennant, S.M., unpublished data
Enteritidis	CVD 1941	R11	Δ*guaBA* Δ*clpP*	CVD	Pre-clinical	[49]
	CVD 1944	R11	Δ*guaBA* Δ*clpX*	CVD	Pre-clinical	Tennant, S.M., unpublished data
Paratyphi C (C1)	TBD	TBD	TBD	CVD	Pre-clinical	Fuche, F., unpublished data
Newport (C2)	TBD	TBD	TBD	CVD	Pre-clinical	Fuche, F., unpublished data

TBD, To be determined