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Review Article

Microbiology Section

Vaccine Development Against *Salmonella* Typhi: The Search is Still On

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ABSTRACT

Salmonella serovar typhi infection still remains a serious problem in South Asia, South-East Asia, and sub-Saharan Africa. The emergence of multidrug resistance, lack of proper diagnosis and chronic typhoid carrier are the main causes of such a high level of morbidity and mortality. Presently, three vaccines have been licensed for typhoid infection but none of them is optimum for complete eradication for want of safety and long lasting immunity. Several subunit and attenuated candidate vaccines are under trial. Recently, in India Typbar TCV conjugate vaccine has been licensed. However, this vaccine being subunit may not induce appropriate immune response and also it cannot be given to infants. We need a multivalent attenuated vaccine which can induce both cellular as well as humoral immunity against different pathogenic serovar of *Salmonella*. Live attenuated vaccine could be an attractive choice taking care of all the above points. In this review, we focus on the pathogenesis of *Salmonella* serovar typhi, role of humoral as well as Cell-Mediated Immune (CMI) response, different licensed vaccines with their pros and cons, and also the targets which are already been put on clinical trials. We have discussed the attenuation of the candidates by modification of certain structural and functional genes especially looking for induction of CMI.

Keywords: Candidate vaccine, Cell-mediated immune response, Live attenuated

INTRODUCTION

Enteric fever is an acute generalised infection caused by human restricted *Salmonella enterica* subspecies *enterica* serovar Typhi, Paratyphi A and B. Majority (90%) of enteric fever cases are caused by Typhi serotypes. Enteric fever is a very important public health problem in countries with poor sanitation and inadequate hygiene. The recent global burden of typhoid fever is estimated to be ranging between 11 and 21 million. This burden is associated with 128000 to 161000 deaths annually [1]. The most affected age group for enteric fever is less than 14 years [2]. The most disturbing issue is that about 27% of the infections occur in the age group <4 years of which 10% are in infants <1 year of age. Typhoid is a major threat for the Indian subcontinent due to its high prevalence, severity, development of Multidrug Resistant (MDR) and persistence of carrier state in our population. Approximately, 2-5% of cases as well as subclinical infections develop carrier state irrespective of preexisting gall bladder disease [3]. The carrier state has been implicated in biliary tract cancer [4]. Chronic carriers are the reservoir of infection and are responsible for endemicity of the fever. Following ingestion, the organisms apart from resisting the low pH of the stomach, they get signals for induce multiplication before reaching the ileum [5]. In the small intestine, the bacteria multiply in the submucosa and Peyer's patches [6]. During systemic infection, bacteria reach the phagocytes of spleen, liver and bone marrow. Later, the infection manifests as bacteraemia. Death due to typhoid fever occurs usually due to perforation of the intestine or haemorrhage.

Chloramphenicol was introduced in 1948 and their resistance started to develop within two years of introduction of the drug and until 1972 chloramphenicol resistant *S.* typhi became a major problem [7]. Outbreaks of chloramphenicol resistant *Salmonella* occurred in Mexico, India, Vietnam, Thailand, Korea and Peru. The case fatality rate without antibiotic therapy was 10-20%. However, with antibiotics, it has come down to 1-4%. This mortality rate is usually due to delay in start of therapy. After the suggestion of Threfall JE et al., that *S.* typhi with decreased sensitivity to ciprofloxacin is endemic in several Asian countries [8], there has been several reports indicating that quinolones have almost lost their ground as a magic drug [9]. A gradual increase in mean Minimum Inhibitory Concentration (MIC) of ceftriaxone has already been reported by our group recently [9]. Emergence of resistance against the two second line drugs; ciprofloxacin and ceftriaxone has become a serious problem in the recent time. Recently, azithromycin is also showing the resistance as 20% of the recent isolates of *S.* typhi was showed unresponsive [10]. Then what can be done? Bacteriophage therapy (still under preliminary phase of development) or newer antibiotic molecules (which takes long time to reach clinical application) may be an alternative to the existing antibiotics. Immunotherapy is not practicable at the community level. So logically, a perfect or near perfect vaccine may be able to eradicate this human restricted bacteria globally.

It is important to understand the host immune response against the bacteria before addressing the issue of vaccine development. The subclinical, clinical and chronic carrier states basically depend on the balance between the virulence of the invader and the immune response of the host. The immunity against the bacterium is divided in two parts:

Innate Immunity against *Salmonella*

Components of innate immune system viz., complement and opsonising serum antibodies facilitate the uptake of the bacteria by phagocytes (macrophage, neutrophils and dendritic cells) [11] and epithelial cells and help to identifying specific Pathogen-Associated Molecular-Patterns (PAMPs) of the pathogen. In Salmonellosis, TLR4, TLR5 and TLR9 signaling systems are activated by bacterial DNA, flagella and Lipopolysaccharide (LPS). *Salmonella enterica* species is adapted to mammals including humans and has a complex cross-talk system resulting ultimately in induction of host immune response. The bacteria are seeded into different organs of the body viz., liver, gallbladder, spleen, bone marrow etc., during sustained bacteraemia. A cycle of infection continues and bacteria either reinvade epithelial cells of the intestinal wall or shed in the faeces. However, after a certain period, the symptoms of Salmonellosis resolve. A small population of hosts may become chronic carriers [3,12]. Proinflammatory cytokine (IL-1β, IL-6, IFN-γ and TNF-α) are recruited to promote systemic inflammation [13,14]. IFN-γ is a

known macrophage activating factor. It plays an important role in persistence of infection. Macrophage activity and different type of cytokine secretion is primarily involved in the induction of innate as well as adaptive immune responses. Further, equilibrium between pro-inflammatory and anti-inflammatory cytokines is maintained which controls the infection along with ensuring minimal damage to the host. Bone marrow derived macrophages and primary cell line have shown that *Salmonella* promote chemokine and cytokine synthesis in macrophages, dendritic and epithelial cells [15]. In typhoid fever cases, an aggressive pro-inflammatory response against *Salmonella* is not a common occurrence. However, in patients suffering from typhoid fever, distinct peripheral blood metabolite profile has been elucidated by microarray and transcription profiling techniques [14,16]. It is interesting to note that if the hosts are incapable of mounting an appropriate response, they are more prone to relapse, re-infection and occasionally becoming chronic carriers [16]. There is scarcity of data of host immune defence in human beings against *Salmonella* infection. In mouse tissue, early invasion of *Salmonella* is controlled by the innate resistance by autosomal gene *Nramp1* (Natural resistance associated macrophage protein 1) which functions as a divalent metal ion pump. This gene is expressed mainly in macrophages and cells of granulocyte lineage [17]. It has been stated that the main host defence against *Salmonella* is exhibited through neutrophils followed by mononuclear cells. Clearance of bacteria from tissue requires activation of CD4+ and TCR-αβ T-cells. This activation depends on CD28 and is controlled by MHC class II genes [18,19]. Dendritic cells and B-cells are involved in initiation and development of T-cell immunity to *Salmonella* [20]. *Salmonella* is a facultative intracellular organism; therefore, apart from antibody, cell mediated immunity through B and T-cell cooperation, Th1 type immunological memory and CD8+ T-cells may essentially be required.

Humoral Immune Response

There are studies showing antibody response to LPS and protein determinants in mice [21], rabbits [22] and humans [23] following exposure to live or killed *Salmonella*. Serum and mucosal IgA has usually been found to be induced after vaccination and also by some of the killed preparations [21]. However, it has been speculated that antibodies alone may not provide immunity against re-infection and recurrence.

Cell Mediated Immune Response

Delayed Type of Hypersensitivity (DTH) response not only against proteinaceous antigens, porins but also to LPS antigen determinants and Vi surface polysaccharide has been observed in mice [24] after immunisation with live vaccine. However, DTH could not be detected after immunisation with killed vaccine. Intracellular bacteria have a challenge to maintain a balance of weakened host to prevent clearance along with the healthy enough to establish a suitable niche for their existence and intracellular replication. This is the basic essential requirement for human restricted pathogen like *Salmonella* typhi serotypes. Such pathogens modulate host intracellular signaling in their own favour to consume resources and neutralise the host defence mechanisms. Moreover, different bacteria have developed individualised strategies to target the same host cell. The killing of intracellular bacteria is by phagocytosis in phagosomes which is followed by fusion with the lysosome to give phagolysosomes. The pH of phagolysosome is 3.5-4.0 which is toxic to bacteria. The killing is further enhanced by acid proteases. The surviving facultative *Salmonella* bacteria inside the *Salmonella*-Containing Vacuoles (SCV) express certain genes (*spiC, sseB*) which inhibit the fusion of phagosome to lysosome.

PAST AND CURRENTLY AVAILABLE VACCINES

Vaccination is a powerful technique for mitigating *Salmonella* related problems. In the past, two methods were used for vaccine development: attenuation (heat, oxygenation, chemical agents or old culture) and inactivation by killing the bacteria. Subunit vaccines have also been developed and used against *Salmonella* with variable success. Killed vaccine failed due to some reactogenicity and poor immunity. Attenuated vaccine has also been tried with variable success. The two vaccines are available in clinical practice against typhoid. However, they are not satisfactory in prevention of the disease [25]. These two vaccines are, live attenuated Ty21a and Vi capsular polysaccharide derived [26]. Recently, a new vaccine has been approved by WHO (World Health Organisation) which recommended for use in areas with a high burden of typhoid and higher antibiotic resistance [1].

Whole Cell Inactivated Vaccine

Initially, whole cell inactivated vaccine was used for prevention of typhoid fever which provided 73% protection for three years. Unfortunately, this vaccine was reactogenic. Therefore, this vaccine is no longer recommended. This was a heat killed phenol preserved or acetone killed lyophilized vaccine derived from whole bacteria. Heat killed vaccine is no better than acetone killed vaccine because heat may destroy protein conformation as well as the Vi polysaccharide which are immunogenic otherwise [27]. However, inactivated whole cell vaccines can cause inflammation, pain fever and other side effects which are not suitable for mass vaccination [28]. Killed vaccine can induce sufficient humoral immunity but they are unable to induce Th1 or in some case even, Th2 mediated immune response [21,29]. In case of humans, killed vaccine gave better protection when given parenterally than orally.

Vi-Vaccine and Other Subunit Vaccines

Vi antigen on *Salmonella* serovar Typhi gives protection from complement-mediated bacterial lysis and also inhibits alternative complement activation pathway. Vi polysaccharide is purified from the Ty21 strain of serovar Typhi. Vi vaccine is an unconjugated purified capsular polysaccharide and it has provided 77% protection for 20 months and it is prohibited for children below two years of age. A single dose of Vi vaccine is sufficient to induce a high titre of anti-Vi antibody. Felix A et al., (1935) for the first time showed that Vi polysaccharide of *Salmonella* typhi induces humoral immune response against typhoid [30]. It is a linear homopolymer of galacturonic acid isolated from *Salmonella* typhi cetavlon detergent treatment. This vaccine was first licensed in USA in 1994 and is available globally. Vi antigen does not induce a T-cell response because it is a polysaccharide and therefore no affinity maturation and memory cells development. Further Vi vaccine does not provide protection against Vi negative *S.* typhi and Paratyphi serotypes of *Salmonella enterica* and it needs re-vaccination two years after the initial dose. The Vi vaccine gives 55% to 75% protection in typhoid endemic areas. Their protection rate varied in different age groups and countries such as 75% protection rate in five to seven-year-old for 20 months in Nepal, 64% in 5-16-year-old in South Africa for 21 months whereas it came down to 55% after 36 months, while in China 69% protection in 6-9 year old children [31,32]. In children below 2 years of age even after booster, it does not induce immunity. Another drawback is that all strains of *Salmonella* typhi do not express Vi polysaccharide or hide their Vi polysaccharide on their surface and so it is unable to prevent typhoid [33]. Immunogenicity was found to increase when it was conjugated with a carrier protein such as tetanus toxoid, diphtheria toxin, porin or recombinant exoprotein of *Pseudomonas aeruginosa* [34].

Live Attenuated Ty21a Vaccine

Due to the presence of adverse effects of heat killed vaccine, a live attenuated vaccine was required which could induce prolonged immunity against typhoid. Ty21a, the first oral live attenuated vaccine against typhoid was developed in a wild type strain by chemical mutagenesis [35]. In case of Ty21a, two genes *galE* and *Vi* were mutated randomly yielding highly attenuated strain

of *Salmonella* typhi. The *galE* gene encodes UDP galactose-4-epimerase and *Vi* gene encodes surface polysaccharide. Once this gene is mutated, the mutant produces rough LPS in place of smooth LPS. When galactose is exogenously supplied the mutant was able to make smooth LPS till it was the available. This smooth LPS can protect the bacterium from phagocytosis. This vaccine gives protection via induction of anti-O and anti-H antibody (mucosal and serum) and also up to some extent of cellular immunity. Cellular immunity is induced in the form of T-cell activation and cytokine secretion. Currently this vaccine has been licensed in 56 countries of Africa, USA, Europe and Asia [36]. However, the immunity induced by this vaccine diminishes with increasing duration after vaccination. The protection rate differs from region to region viz., in Egypt it has given 96% protection for three years whereas in Chile, only 67% protection for three years. Later the protection rate decreased to 62% for 7 years. The protection rate also varies in different age groups i.e. 59% in 5-9 years, 67% in 10-14 years and 85% in more than 15 years of age. Interestingly this chemically mutated vaccine also gives cross-protection against paratyphoid fever (Paratyphi B) causing serotypes but their protection level was only 42-56%. This vaccine is also able to develop herd immunity in endemic settings.

Typbar-TCV (Typhoid Conjugate Vaccine)

It is developed by conjugation of tetanus toxoid protein with purified Vi polysaccharide (Vi-TT). It was licensed for the first time in India in 2013. It is given to children more than 6 months of age. Typbar TCV induces a high titre of anti-Vi antibody as well as higher avidity in comparison to Vi–unconjugated vaccine. Protection persisted in 84% infants (6-23-month-old) for 5 years. The efficacy in human volunteers after one month has been reported to be 87.1% and sero-efficacy 85% [37]. [Table/Fig-1] shows the status of current typhoid vaccines [25,26,38-43].

Newer Vaccine Development

What should be the criteria for ideal *Salmonella* vaccine?

For an ideal vaccine against enteric fever the recommended criterion are:

1. Vaccine strain should not be resistant to any antibiotics.
2. It should be highly protective against intestinal as well as systemic infection of *Salmonella* typhi and should also protect against other serotypes of *Salmonella*.
3. It should be attenuated to protect humans.
4. Live attenuated vaccine should not cause any harm or inhibit the growth of the vaccine.
5. Vaccine strain should not interfere with *Salmonella* detection methods.
6. Vaccine should have a marker to differentiate it from wild type of strains.
7. It should easily be administered.
8. Live attenuated vaccine should inhibit the colonisation of other *Salmonella* serovars in the intestine.

Vaccine characteristics	Ty21a vaccine	Vi polysaccharide vaccine (ViPS)	Typhoid conjugate vaccine (TCV)
Type	Live, attenuated (Chemically induced mutagenesis affecting several genes)	Subunit (Purified Vi polysaccharide from Ty2 *S.* typhi strain)	Conjugated subunit vaccine (purified Vi polysaccharide conjugate with tetanus toxoid, recombinant Exo Protein A and recombinant Diphtheria Toxin) [41,43]
WHO recommended	(2008) endemic and epidemic settings	(2008) endemic and epidemic settings	(2013) High typhoid burden or high antimicrobial resistance
Storage and preservative	2-8° C to for 3 years	2-8° C for 3 years Phenol (1.250 mg) per dose	2-8° C 2-Phenoxyethanol (5 mg)
Type of immune induction	It induces serum and mucosal immunity against both O (somatic) and H (flagellar) antigens	Single dose induces higher anti-Vi antibody titre in serum.	Single dose elicited higher anti-Vi antibody titre in serum
Licensed	1983 in Europe,1989 in USA	1994 in USA	2013 in India
Age of vaccination	Enteric coated capsules >6 years as per manufacturer	≥2 years	≥6 months
Schedule	Enteric coated capsules: 3-4 doses on alternate days	Single dose	Single dose
Administration	Oral	0.5ml injection (Intramuscularly or subcutaneously	0.5 ml injection (Intramuscularly)
Efficacy	33-77% [25].	55-72% [25].	85-87.1% [41,43].
Duration of protection	5-7 years	2-3 years	Up to 5 years
Cross protection against NTS	Partial protection against Paratyphi B (49%) [39].	No evidence of cross protection	Not studied
Co-administration	It is given with Live vaccine of polio, cholera, yellow fever and MMR	- It is given as routine vaccine in children. - Hepatitis A and yellow fever in traveler.	Vaccine of Mumps, Rubella and Measles show no interference with TCV [43].
Herd immunity	Herd immunity develops	Not applicable	Not applicable
Persistence of immunity	Protection from 7th day to 7-years	Protection from 7th day to 3-years	Not clear
Immunity in immuno-compromised	Not recommended specially CMI deficient	Safe	No data available
Immunity in pregnancy	No data from pregnant women regarding these three vaccines		
Disadvantage	Not licensed for infants, Require 3-4 doses, Lack of anti Vi antibody	Not licensed for infants, lacks memory response, lacks affinity maturation, Only protects against *S.* typhi, revaccination after every 3 year.	Protection only against *S.* typhi
Adverse effects	Transient fever and gastrointestinal problems.	No serious adverse effects known.	Fever, pain, swelling reported in 10% of cases.
References	Fraser A, et al., 2007 [25]; Poolman J, et al., 2011 [38]	Khan MI, et al 2010 [26]; Kantele A, et al., 2012 [39]; Sur D, et al., 2009 [40]	Mai NL, et al., 2003 [41]; Thiem VD, et al.2011 [42]; Szu SC, et al., 2013 [43]

[Table/Fig-1]: Status of current typhoid vaccines [25,26,38-43].

In the light of above mentioned parameters, candidate vaccines are being searched in the following three directions:

i. **Subunit vaccine (protein derived vaccine)**

ii. **Carbohydrate conjugated vaccine**

iii. **Live attenuated vaccine**

i. **Subunit vaccine (protein derived vaccine)**

Subunit vaccine consists of purified recombinant protein from the targeted bacteria and, are an alternative to carbohydrate conjugated and live attenuated vaccine. The subunit vaccines are designed with bioinformatics tools that should be targeted at selection of proteins that can induce both humoral and CMI and also provide cross protection against other enteric fever causing serotypes. For designing better subunit vaccines, we must know the *Salmonella* epitopes on B and T-cells to enhance the potency of the protein derived subunit vaccines [44]. Protein based subunit vaccines that are under development are given below:

Flagellin and Porins, OmpC, OmpF and OmpD

OmpR-envZ is a two-component system of *Salmonella* in which *OmpR* has a response regulator of *OmpC*, *OmpF* and Vi antigen expression while *EnvZ* is membrane sensor protein which senses different environmental condition such as osmolarity in the surrounding [45]. The *OmpC* and *OmpF* proteins can induce a long lasting humoral response against *Salmonella* in a mouse model and in humans. It has been found safe and immunogenic in phase-1 clinical trials. Since these are membrane proteins, their conformation is very important for vaccine development. Protein based vaccines are non-infectious while attenuated vaccines have the possibility of reverting back to virulent form.

ii) Carbohydrate Conjugated Vaccine

Conjugated carbohydrate vaccine has more beneficial effect over pure Vi capsular polysaccharide vaccine. Polysaccharide antigen attached with carrier protein by covalent linkage can activate both arms of immune system i.e., T-cell mediated CMI and B-cell mediated antibody response. If we conjugate LPS O- antigen O: 1,2,12 with carrier protein it gives protection against *S.* Paratyphi-A, *S.* Typhimurium and *S*. Enteritidis O: 1,9,12 sharing O-12 antigen [46]. Generally none of the *Salmonella* proteins are used as a carrier. The Tetanus toxoid (TT), Diphtheria toxoid (DT) and recombinant diphtheria toxin (CRM197) are nontoxic and commonly used. Sometime rEPA (recombinant exoprotein A from *Pseudomonas* sp.) is also used as carrier protein for glycoconjugate vaccine. This carbohydrate conjugate carrier protein converts the antigen processing from T-independent to T-dependent antigen which may induce both humoral immunity as well as CMI. TT covalently attached with Vi antigen and rEPA carrier protein attached with Vi antigen has already been used in China and India.

If the *Salmonella* protein is used as carrier protein instead of exogenous protein which are shared amongst different serovars, it may provide immunity against many of them [44]. It has been reported that *Salmonella* O:4 conjugate with porin and O:4,5,9 conjugate with flagellin are more effective in comparison to lone porin or O:4 DT or flagellin [44,47]. Vi-CRM 197 glycoconjugate vaccine developed by Novartis Vaccine Institute for Global Health and NVGH has been tested in phase-2 clinical trials in children, infants and adults in India, Pakistan and the Philippines and phase-1 and phase-2 in adults in Europe [48]. Drawbacks of this glycoconjugate vaccine (Vi-DT or Vi-TT) are that they do not protect from enteric fever caused by *Salmonella* Paratyphi A or other strains because they lack the Vi antigen on their surface. On the contrary, the O:2 TT glycoconjugate vaccine is safe as well as immunogenic and target *S*. Paratyphi A [49]. Other O:2 DT and O:2 CRM197 glycoconjugate vaccine are under preclinical trial at the IVI (International Vaccine Institute, South Korea) and NVGH [50,51]. GelSite-OAcTM is a new derivative vaccine of Vi. In this vaccine O-acetylated heavy molecular weight polygalactouronic acid residue is attached to induce better immunity as well as memory response. Their boosting effect is effective for children under 2 years of age [52].

iii) Live attenuated Vaccine under Development

When molecular biology was not evolved to the present level, all vaccines were developed through chemical mutagenesis. However, with the development of molecular biology, specific genes can be mutated by homologous recombination as well as one step gene inactivation (site-directed mutagenesis) and selected by antibiotic resistance and checked for their live attenuated status and potential to prevent the disease. Later, the antibiotic resistance gene may be deleted from the mutant strain [53]. Live attenuated vaccine are potentially superior to killed vaccines because they.

- Induce humoral immunity along with CMI.
- Are more effective after single dose administration.
- Can be given orally with no risk of prick, pain and needle contamination.
- Induce immune response at various mucosal sites.
- Are cost-effective, easily stored without the need for cold chain, and can be lyophilised.

Understanding of *Salmonella* genetics, growth and survival in the host are very helpful for developing a rational attenuated vaccine. Attenuation should produce an organism with diminished ability to grow inside the host. Attenuated strain persists in the host to induce protective immunity than the killed vaccine and their growth should be limited even in the immune-deficient hosts. Various genes of *Salmonella* are already identified for virulence and survival. These genes either belong to housekeeping such as bacterial structural components (LPS, OMP), synthesis of essential metabolites such as purine or pyrimidine biosynthesis or virulence gene involved in bacterial resistance to host defence system [54]. Live attenuated oral vaccines have the good potential to activate lymphocyte expression of the mucosal receptor. Live attenuated vaccine of *Salmonella* expresses their all antigens which induce broad-spectrum immunity to protect the host from *Salmonella* serovars. The optimal level of attenuation which does not affect the expression of immunogenicity is a major problem in developing a live attenuated vaccine. We need a live attenuated vaccine which is heat stable and requires few doses. Currently the following attenuated vaccines against typhoid are under different phases of trials.

CVD 909

Live attenuated CVD909 is triple gene mutant of *aroC*, *aroD* and *htrA* along with replacement of P_{tviA} promoter (regulator of Vi antigen expression) with constitutive P_{tac} (recombinant promoter made by fusion of lac promoter with trp promoter) promoter. P_{tac} promoter with Vi genes leads to strong and constitutive expression of Vi polysaccharide. The P_{tviA} promoter has an inhibitory effect on Vi expression. This candidate vaccine has the advantage of constitutive expression of Vi antigen which is generally not expressed in Ty21a or inhibited expression or switching off Vi gene expression in the host [55,56].

Ty800

The phoQ is a membrane associated sensor kinase which senses the external environment and induces signaling activation by phosphorylation of PhoP which is a cytoplasmic transcriptional regulator. After induction of PhoP, it can activate and deactivate several genes to combat unfavorable condition. This two-component system is activated inside host macrophage and protect *Salmonella* from phagocytic activity and from antimicrobial peptides which are secreted by host macrophages for their survival [57-59] The PhoP is crucial for survival inside the mononuclear cell

and their mutants in *Salmonella* typhimurium is highly attenuated and induce immune response in mice but hypersensitive to the microbicidal defensins [60,61]. The live attenuated vaccine of *S.* typhi is developed for oral administration by mutating global regulator phoP-phoQ gene. This vaccine is based on parent Ty2 strain and developed by Avant Immunotherapeutics. This candidate vaccine has good immunogenicity to induce mucosal and systemic antibodies. The phoP/Q mutants are avirulent inside macrophages. The phoP mutant or constitutive expression of phoP gene was attenuated in mice because it is able to establish infection in Peyer's patches but unable to infect or reaching spleen. Thus, these mutants are highly suitable for induction of CMI measured by DTH and may be used as an oral candidate vaccine [59-61]. The phoP/Q mutant of *Salmonella* typhi is safe and immunogenic in humans [62].

clpPX

In *Salmonella* flhD/flhC is a transcriptional regulator of flagellar gene synthesis. This regulator is degraded by *clpPX* gene which encodes a protein digesting enzyme. If the *clpPX* gene is mutated, flagellar gene synthesis is enhanced as the inhibitory action of *clpPX* is lost. However, *clpPX* mutated CVD strain of *Salmonella* is under vaccine trial and outcome is awaited [63].

htrA mutant

The *htrA* gene encodes heat stress protein of *E.coli*, and their mutants are avirulent in mice model. *HtrA* is a serine protease which can cleave misfolded protein in response to heat shock. This protease is induced in heat shock condition and mostly digests periplasmic proteins which are misfolded during the heat stress. The *htrA* mutant is not heat sensitive but their susceptibility to oxidative stress is increased in macrophages as compared to wild type strain. It protects the mice from re-infection [64]. These gene mutant are safe in normal mice as well as immune-compromised mice such as x-ray irradiated, anti TNFα treated (XID) etc., but it showed some virulence in T-cell deficient mice [65].

ssaV

It is a part of type III secretion system (T3SS) which are encoded by SPI-2. It is required by both the serovars of Typhi and Typhimurium for virulence in human and mice models [66]. SPI-2 encoded genes playing major role in survival of the bacteria inside macrophages of the host [67]. The SsaV protein makes a part of injectosome (T3SS) which secretes various effector proteins outside as well as inside of cell membrane of the host. The ssaV gene mutants are thus made not to secrete effectors protein inside the host cells to cause the disease.

Aromatic Amino Acid Biosynthetic Pathway

Aromatic amino acid biosynthetic genes are needed for bacterial growth. In general three genes are involved in aromatic amino acid biosynthesis viz., *aroA*, *aroC* and *aroD*. Aromatic amino acid biosynthesis gene mutant is a type of auxotrophic mutant which needs a specific metabolite for their growth. Human host is deficient in such metabolites. Therefore, the strain containing these mutations is attenuated in the absence of metabolite and cannot grow in vivo. The bacteria are easily killed but can induce an immune response. If one or more genes were mutated in this operon, it causes irreversible loss of virulence and it also transform from prototroph to auxotroph for Dihydrobenzoate (DHB) and Para-aminobenzoic acid (PABA) [68]. These mutant strains were able to induce an immune response and also protect from the lethal dose of bacterial challenge [69]. These mutants were able to induce both types of immunity viz., cellular as well as humoral immunity [68]. More than one mutation in aro operon (Aromatic amino acid biosynthesis) ensures lack of virulence reversion in bacteria but it does not affect immune inducing potential. A double mutant of *aroC* and *aroD* were also used in human volunteers and they showed good immune response but reported causing bacteraemia in some cases [70].

cya (Adenylate cyclase) and *crp* (cyclic AMP receptor protein)

The *cya* gene encodes adenylate cyclase enzyme which synthesises cAMP from ATP for various cellular activities. Mostly cAMP is needed for transport of various carbohydrates and amino acids. It also plays a role in the synthesis of flagella, fimbriae and outer membrane protein of bacteria [70]. Oral or intraperitoneal vaccination of single mutant *cya* or *crp* gene or double mutant of *cya* and *crp* were highly attenuated. Oral vaccine provides protection from oral challenge with wild type strains. When given orally, mutant bacteria invade the Peyer's patches and mesenteric lymph nodes but unable to invade and survive in spleen in mouse models [71]. In humans, it has been reported that it induces immune response but also gives some side-effects such as fever and bacteraemia.

DNA Vaccines

Injection of naked DNA induces both humoral immunity and CMI. In fact bacterial DNA encodes such a gene which after expression induces Th1 and Th2 responses. For DNA vaccine against typhoid, five peptides were screened that showed high expression in vivo. Those genes are *mig14*, *iicA*, *sseB*, *ssaJ*, or *sifB*. Of these five, *sseB* and *mig14* have been observed to be exceptionally efficacious antigens providing specific immunity and protection as compared to other antigens used. Other than high expression level, there are certain antigenic parameters which can influence protective efficacy and show the different immune response for different antigen [72]. For intracellular pathogens, expression of a selected antigen during infection may be more important for the candidate vaccines [Table/Fig-2]. Other vaccine candidate against iNTS (Non Typhoidal *Salmonella*) and *Salmonella* Paratyphi A are explained in [Table/Fig-2] [50,51,72-74].

	Name	Summary	Stage of development	Developer	References
1.	**Vaccine against *Salmonella* Paratyphi A**				
a.	O:2-TT	O:2 Conjugate	Phase 2 and under clinical trial	-Technology transfer from NIH to Chengdu Institute (China). -Changchun Institute of Biological Products.	Konadu E et al., 1996 [50]
b.	O:2-CRM197	O:2 Conjugate	Preclinical	NVGH (Novartis Vaccine Institute for Global Health)	Micoli F, et al., 2012 [51]
2.	**Vaccine against iNTS**				
a.	O:4,5/O:9-CRM	O:4,5/O:9 Conjugate	Preclinical	NVGH (Novartis Vaccine Institute for Global Health)	Micoli F et al., 2012 [51]
b.	WT05	Live attenuated	Phase 1	Microscience, Wokingham Berkshire	Hindle Z, et al., 2002 [73]
c.	*Salmonella hfq* mutant	mutant Live attenuated	Preclinical	Indian Institute of Science, Bangalore	Allam US, et al., 2011 [74]
d.	DNA vaccine	*AroQ, IicA, Mig-14, SsaJ, SsaV, SseB, SifA, SifB, Stm4065*, and *VirK*	Mice Study	Max Planck Institute for Infection Biology, Germany	Rollenhagen C, et al., 2004 [72]

[Table/Fig-2]: A glimpse of developing vaccines against *Salmonella serotypes* [50,51,72-74].

S. No.	Gene	Function	Protection in model animal	Reference
1	*relA, spoT*	Play a role in stringent response	Protective in murine model	Na HS et al., 2006 [75]
2.	*dam*	DNA adenine methylase (it controls virulence genes)	Protective in murine model	Heithoff DM et al, 2001 [76]
3.	*recA, recBC*	DNA recombination and repair	NA	Buchmier NA et al., 1993 [77]
4.	*cdt*	Colonisation as deep regulator	NA	Zhang X et al., 1997 [78]
5.	*wecA*	UDP-N acetylglucosamine 1-phosphate transferase	persistent infection provide protection from lethal challenge	Gilbreath JJ et al., 2012 [79]
6.	*gidA*	Glucose inhibited division	Induce immunity	Shippy DC et al., 2012 [80]
7.	*rpoS*	Alternative sigma factor for stress response	Immune to lethal infection	Coynault C et al., 1996 [81]
8.	*surA*	Biosynthesis of a peptidylprolyl-cis, trans-isomerase	Immune to virulent strain in mice model	Sydenham M et al., 2000 [82]

[Table/Fig-3]: List of new targets for vaccine development [75-82].

Different types of attenuated new candidates are under research such as recA, recB, dam, waaN, cdt, rpoS etc [Table/Fig-3]. All the other new targets for vaccine development are explained in [Table/Fig-3] [75-82].

CONCLUSION

Despite the currently available two vaccines (Ty21a and Vi) *Salmonella* serotype Typhi mediated problem is still existing in developing countries and causing significant morbidity and mortality. The search of an ideal vaccine against human restricted *Salmonella* serotypes viz., Typhi and Paratyphi-A, B, C is going on. There is a need to develop satisfactorily attenuated and multivalent subunit candidate vaccine against different serotypes and capable of inducing cell mediated, long lasting immune response. The issue of an efficacious and potent vaccine against typhoid fever inducing strong humoral as well as cellular immunity which can be given to children below 2 years of age still remains to be solved.

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