Review

Salmonella Typhi: from a Human Pathogen to a Vaccine Vector

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***Salmonella (S.) typhi* is an important intracellular pathogen. Among the more than 2,300 closely-related *Salmonella* serovars bacteria recognized, *S. typhi* is the only one that is pathogenic exclusively for humans, in whom it causes typhoid or enteric fever. The pathogen has been around for many years and many studies have been done in an effort to combat it. Molecular and biologic features of *S. typhi* and host factors and immune responses involved in *Salmonella* invasion have been extensively studies. Vaccines that have been developed most notably are Vi polysaccharide and Ty21a. However, as the results show, there is still a long way to go. It is also shown that multi-drug resistance has occurred to the few available antibiotics. More and more studies have shown that *Salmonella* can be used as a vaccine vector carrying antigens of other pathogens. This has been promising in that the immune system can be elicited in response to both the *Salmonella* bacteria and the antigen of the pathogen in question. This review aims to highlight some of the milestones attained in the fight against the disease from the time *S. typhi* was seen as a pathogen causing typhoid fever to the use of *Salmonella* as a vaccine vector. *Cellular & Molecular Immunology*. 2008;5(2):91-97.**

Key Words: *Salmonella typhi*, vaccine vector, typhoid, Vi polysaccharide, Ty21a

Introduction

Salmonella is the causative agent of salmonellosis. It is a rod-shaped gram-negative facultative anaerobe bacterium belonging to the *Enterobacteriaceae* family. Among more than 2,300 closely-related *Salmonella* serovars recognized, *S. Typhi* and *Paratyphi* are pathogenic exclusively for humans, and cause systemic infections and typhoid fever, whereas others such as *S. Typhimurium* cause gastroenteritis (1).

Salmonellosis is more prevalent in developing parts of the world in Africa, Asia, and South America. South Asia are at highest risk for infections that are nalidixic acid-resistant or multidrug-resistant (i.e., resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole). In humans, salmonellosis is seen in two kinds of viz. enteric fever which can be typhoid or paratyphoid and gastroenteritis which is non-typhoidal. Typhoid fever is an acute, life-threatening febrile illness caused by the bacterium *S. Typhi* and *Paratyphi*, and there are estimated 20 million cases and 200,000 deaths worldwide each year (2).

There have been vaccines to salmonellosis as well. However, the immune system has not been able to mount a lasting immune response to *Salmonella* infections and the reason for this still remains elusive. This has called for extensive studies on new therapies. Indeed, new vaccines have been developed. Further, it has been found that a highly immunogenic live oral *Salmonella* vaccine would ideally be suited as a carrier of genes that express protective antigens cloned from other antigens (3-5) and such hybrid recombinant *Salmonella* vaccines are expected to invoke protective immunity against both the carrier strains as well as the foreign antigens (6). This is the basis of *Salmonella* vaccine vector and this review seeks to illustrate this milestone in the scientific research done to date.

We begin by highlighting the molecular and biologic features of *Salmonella*, host immune responses of *S. typhi* infections, therapeutic measures to salmonellosis and the challenges associated with them, and lastly but not least, the use of *Salmonella typhi* as a vaccine vector.

Molecular and biologic features of *Salmonella typhi*

The genome of *S. typhi* is approximately 5 million base pairs long and codes for some 4,000 genes of which more than 200 are functionally inactive. This is in line with other genomes of enteric bacteria sequenced so far which feature a single chromosome with 4.3-5.0 Mb in length (7-10). A comparison

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Received Nov 25, 2007. Accepted Feb 24, 2008.

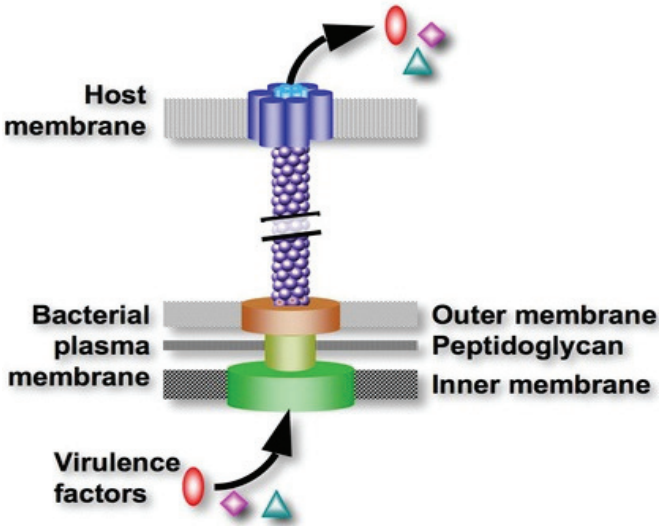

Figure 1. The TTSS is used by the bacteria to translocate virulence factors to the host during infections. Without it, invasion to host cells can not take place.

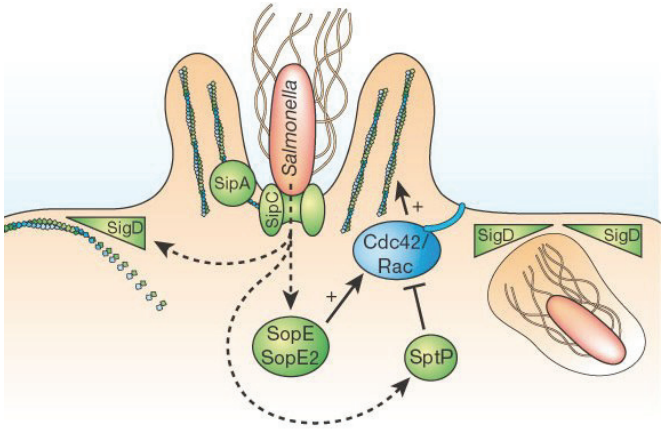

Figure 2. Entry into host cells is mediated by the TTSS and its effectors encoded by SPI-1. Membrane attachment to the cortical actin cytoskeleton is loosened by SigD/SopB. SopE and SopE2 enhance Cdc42 and Rac1 activity directly by acting as guanine-nucleotide-exchange factors. SipA and SipC alter cytoskeletal structure, SipC by nucleating actin and initiating polymerization and SipA by binding actin and modulating actin bundling. These cytoskeletal rearrangements are downregulated by the GAP (GTPase-activating protein) activity of SptP, which inactivates Cdc42 and Rac. SigD also is involved in sealing invaginating regions of the plasma membrane to form intracellular vacuoles.

of *S. typhi* isolates from around the world indicate that they are highly related and that they emerged from a single point of origin approximately 30,000 to 50,000 years ago (11). Different strains may also harbor extrachromosomal DNA in the form of plasmids which usually carry virulence or antibiotic resistance genes (11).

The genes for virulence factors cluster in pathogenicity islands (PI) after a foreign genome is integrated into the bacterial chromosome. Non-pathogenic related species of *Salmonella* do not have PIs. PI gene expression is generally limited to specific host compartments. They often "plug" in to endogenous two-component housekeeping regulators to "sense" where they are within a host and regulate expression accordingly (12). Two major PIs encode type three secretion system (TTSS) that translocates bacterial virulence proteins into host cells during infections (12) (Figure 1).

The TTSS and its effectors encoded by *Salmonella* pathogenesis island 1 (SPI-1) are required for invasion of epithelial cells (12) and is activated under conditions thought to be present in the intestinal lumen before host cell invasion (13). The SPI-1 secreted effectors SopE and SopE2 act as guanine-nucleotide-exchange-factors (GEFs) for the small GTPases Cdc42 and Rac (14) (Figure 2). Additional SPI-1-translocated effectors of *Salmonella* affect actin dynamics during the invasion process. SipA binds and stabilizes actin and SipC, which forms part of the TTSS delivery pore, nucleates and bundles actin while anchored in the host cell membrane (15). *Salmonella* also alters the actin cytoskeleton, through manipulation of phosphoinositides. The plasma membrane is intimately associated with the actin cyto-skeleton, and this interaction depends on phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (16). SigD/SopB is an SPI-1 translocated inositol phosphatase that induces the rapid disappearance of PtdIns(4,5)P_2 from invaginating regions of the membrane during *Salmonella* invasion. This increases elasticity to facilitate the remodelling of the plasma membrane associated with *Salmonella* entry (16). PtdIns(4,5)P_2 has also been implicated in vesicle fission during the creation of phagosomes and clathrin-coated vesicles, and accordingly, SigD also is involved in sealing plasma membrane invaginations to form bona fide vacuoles (17). After invasion, an additional SPI-1 effector, SptP, acts as a GTPase-activating protein (GAP) for Cdc42 and Rac1, thereby inactivating these G proteins and returning cell morphology to a relatively normal state (17). SptP is a bifunctional protein, with its GAP domain at the amino terminus, and a protein tyrosine phosphatase domain at the carboxy terminus (18). A potential target for the tyrosine phosphatase activity of SptP is the intermediate filament protein vimentin, which is recruited to the membrane ruffles stimulated by *Salmonella* (18). Other studies have also identified another intermediate filament protein involved in *Salmonella* entry, SipC, which binds cytokeratins and expression of dominant negative cytokeratin-18 inhibits *Salmonella* entry into HEp2 cells (19) (Figure 2). *Salmonella* pathogenesis island 2 (SPI-2) encodes a second TTSS, effector proteins, molecular chaperones, and a two component regulatory system that activates SPI-2 promoters (14). This pathogenicity island and related effectors are required for intracellular survival and replication at systemic sites of infection. Furthermore, studies by Coombes and others have shown that negative regulation of SPI-2 is required for full typhoid pathogenesis and they (Coombes and others) have also identified a protein in *Salmonella*

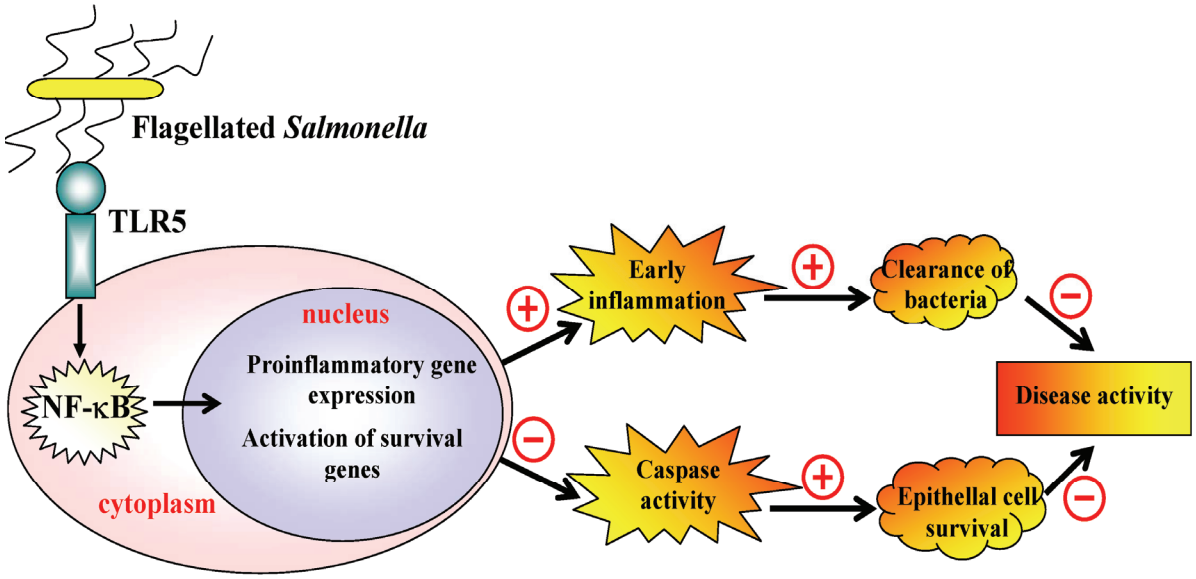

Figure 3. Flagellin of *Salmonella* bacteria stimulates the activations of TLR5 and NF-κB, suppresses epithelial apoptosis and limits disease during enteric infection.

enterica called YdgT that exerts a negative regulatory activity on SPI-2 (19).

Other than these two major PIs, there are other PIs that have been identified most of which are of utmost importance to the virulence and survival of the bacterium. Such islands are SPI-3, SPI-4 and SPI-5. It has been suggested that when genes associated with the SPI-3, SPI-4, and SPI-5 together with SPI-1 and SPI-2 are inactivated, *S. typhi* loses the ability to express several virulence-associated traits, a factor that could begin to explain the loss of host range of these serovars. Another important island is the SPI-7 which is responsible for the production of the Vi polysaccharide capsule (20). This island is 134 kb in length and encodes a variety of putative virulence-associated gene clusters, including the Vi locus, a phage encoding the SopE effector protein of PSI-1 (21-23), a type IV pilus, and a putative type IV secretion system (24-27). The type IV pilus is involved in aiding attachment to eukaryotic cells (24-27) and the SopE prophage harbors a gene encoding an effector protein secreted through the TTSS (14).

In addition, SPI-7 has a viaB operon which encodes the gene responsible for the synthesis and transport of the virulence antigen Vi (28, 29). Studies have shown that Vi antigen may be important in the survival of the bacterium within macrophages though this may not be in cellular invasion of the macrophage or the intestinal wall (14, 30). Unlike most other serovars, *Salmonella typhi* expresses the Vi polysaccharide capsule, an important virulent factor. However, its presence only increases the infectivity of *Salmonella typhi* and the severity of the disease but is not essential for infection since it has been shown that Vi negative mutants are able to cause a typhoid-like illness in human volunteers (7, 31). Supporting this view is the fact that there have been reports of outbreaks of typhoid fever caused by Vi-negative *Salmonella typhi* (32).

Host factors and immune responses involved in *Salmonella* invasion

Salmonella infections are usually acquired by ingestion of contaminated food or water. Once ingested, the organisms multiply in the small intestine over the period of 1-3 weeks, breech the intestinal wall, and spread to other organ systems and tissues.

Salmonella interactions with nonphagocytic cells and phagocytic cells. The primary site of invasion of *Salmonella* strains is thought to be the M cells in the Peyer's patches. Starting in the late 1990s, *Salmonella* flagella was highlighted in host early innate immunity against *Salmonella* beacause the flagella was identified by several independent groups as the mediator that causes intestinal epithelial or macrophage inflammation following infection. Ciacci-Woolwine et al. and Wyant et al. reported that *Salmonella enterica* serovar Typhi flagella induced cytokine release from human monocytes and impaired antigen presentation by human macrophages (33-35). Flagellin of *Salmonella* suppresses epithelial apoptosis and limits disease during enteric infection (Figure 3) (36). The molecular basis of these effects was revealed by Hiyashi et al., who demonstrated that flagellin was the component that activated TLR5, and subsequent work in many laboratories confirmed this finding for flagellins from various organisms (37, 38). Moreover, recent studies have identified Ipaf as an essential sensor for cytoplasmic flagellin (39, 40). Feuillet et al. generated TLR5-deficient mice and showed that TLR5 was crucial for

the *in vivo* recognition of flagellin but also may participate in the detection of systemic infection by *S. typhimurium* (41). After engagement, TLR5 activates NF-κB and MAPKs, leading to the secretion of many cytokines, including IL-6, IL-12, and TNF-α, whereas Ipaf permits the activation of caspase-1 and secretion of mature IL-1β.

It has been demonstrated that the activation of macrophages by LPS from *Salmonella* species also results in the release of a variety of inflammatory cytokines, such as IL-6 and IFN-β (42), which were not detected in macrophages of TLR4 knockout mice (43). After binding to LPS, in association with the proteins MD2 and CD14, TLR4 dimerizes and undergoes a conformational change required for the recruitment of downstream Toll/interleukin-1 receptor (TIR) domain-containing adaptor molecules to activate both NF-κB and the mitogen-activated protein kinases (MAPKs) (44). Other studies have shown that TLR4 triggers the early response to *Salmonella* and that TLR4 and TLR2 are required sequentially for efficient macrophage function in *Salmonella* infections (45). TLR2 can recognize *Salmonella* bacterial lipoproteins and lipoteichoic acid (46, 47), probably in cooperation with TLR6 and/or TLR1 (48-50). TLR9 is activated by bacterial DNA (detecting unmethylated CpG motifs) (51). Totemeyer et al. have demonstrated that TLR1, TLR2, and TLR9 are up-regulated while TLR6 is down-regulated which accounts for the plateau phase observed during sublethal *Salmonella enterica* serovar *Typhimurium* infection (51). Their results suggest that in addition to TLR4, the TLR2-TLR1 complex and TLR9 may play a role in controlling infection, particularly in the later stages when the bacterial growth is suppressed, possibly at the adaptive phase of the immune response (51).

The phase of early innate immunity is followed by activation of a complex host response that suppresses the growth of bacteria in tissues. Both macrophages and T cells may be involved in cell-mediated immunity to *Salmonella* infection, while antibody also plays a role. Central to the pathophysiology of all human salmonellosis is the induction of a strong host innate immune/inflammatory response (52). Both the host and pathogen have evolved mechanisms of triggering host responses. Induction of antimicrobial pathways during early-phase immune response to *Salmonella* spp. in murine macrophages: gamma interferon (IFN-γ) and macrophage migration inhibitory factor (MIF) play pivotal roles in immunity against *Salmonella* bacteria (53). Up-regulation of IFN-γ receptor α expression is required for NADPH phagocytic oxidase gp91-stimulated oxidative burst and control of virulent *Salmonella* spp. (54).

Therapeutic measures to *Salmonella typhi* infections and the challenges associated with them

Vaccination is one of the most important therapeutic means directed towards important antigens encoded by the bacterium. It can provide a near-term solution, as demonstrated in Thailand, where mass vaccination of school children with injectable inactivated, whole-cell vaccines in the 1970s and 1980s led to a sharp decrease in the incidence of typhoid fever which is credited for largely controlling the disease. However, because of their high rates of side effects, these older generation vaccines have generally been abandoned as public health tools (55). Fortunately, newer generation typhoid vaccines that have been available for approximately two decades have proved to be extremely safe. These are Vi polysaccharide and Ty21a vaccines (55). Both parental and live oral vaccines of Vi polysaccharide and Ty21a are clinically available for typhoid fever although there are some problems related to side effects and efficacy.

The development of a safe but highly immunogenic live oral typhoid vaccine against typhoid fever will have advantages over the currently available reactogenic whole-cell parenteral typhoid vaccines and the well tolerated but modestly immunogenic live oral vaccine strain Ty21a. However, development of these live oral serovar Typhi vaccines has been difficult, since little information is available regarding the mechanisms behind protective immunity and immunological memory for serovar Typhi or the interaction between the bacterium and the gut micro-environment. Another obstacle in the development of these live oral vaccines is the variety of typhoidal and nontyphoidal salmonelloses caused by various serovars and strains. Currently, the successes of clinical trials performed on the *△aroC/△ssaV* mutant to individuals in developing countries is a significant challenge. The new trials testing this vaccine strain in adults and children from developing countries are under way, and if successful, it could be the first single-dose oral vaccine for typhoid fever available worldwide (56).

Two vaccine strains harboring deletion mutations in *aroC* and *aroD* have been evaluated as candidate live oral vaccines in adult volunteers (57). One of these strains, CVD 908, a derivative of the wild-type strain *Salmonella typhi* Ty2, was well tolerated and highly immunogenic when given to volunteers in phase 1 studies. Another Δ*aroC*/Δ*aroD* strain, CVD 906, a derivative of wild-type strain ISP1820, was highly immunogenic but caused fever in a significant proportion of vaccines. Additional attenuating mutations in CVD 908 and CVD 906 were sought, a *htrA* gene locus, the resulting mutant is less virulent because of impaired ability to survive and/or replicate in host tissues (57).

Another Typhi vaccine candidate Ty800，is a derivative of *S. typhi* Ty2 and has a defined deletion mutation of the two-component virulence-regulating genes, *phoP/phoQ*. In human trials, Ty800 induced high-level immune responses with a single dose and without adverse side effects, except for diarrhea in a small number of individuals (58). Phase I/II clinical trials were undertaken by AVANT Immuno-therapeutics, Inc. which aimed at determining the safety and immunogenicity of the single-dose, oral Ty800 vaccine.

Antibiotics directed towards *Salmonella* have also been recorded as success stories in fighting typhoid fever. The first antibiotic used to treat typhoid was chloramphenicol. This has been met with resistance by the bacterium and more so in developing countries where chloramphenical was the most affordable drug to use for enteric fevers (59). Newer

antibiotics have been produced, an example of which is fluoroquinolones, which are better at treating the disease. But as the *Salmonella* bacterium continues to evolve, attaining new genes from the environments in forms of plasmids, prophages, and transposons *via* natural transformation, transduction and conjugation, these newer drugs will inevitablly be resisted sooner or later. Antibiotic resistance is notorious in developing countries where majority of the population is unable to afford full doses and in cases where people can afford, it may be the only drug affordable. Over use is also another factor contributing to drug resistance and this is seen in developed countries as well.

Salmonella: a vaccine vector

Typhoid fever affects millions of people worldwide and results in hundreds of thousands of death each year. This is to a large extent due to an increase in multi-drug resistance of *Salmonella typhi*. Consequently, the WHO has given a high priority to the improvement of vaccines against *Salmonella typhi* in an effort to attain greater control over the disease (60). The development of a safe but highly immunogenic live oral typhoid vaccine for use in such programs will have advantages over the currently available reactogenic whole-cell parenteral typhoid vaccines and the well tolerated but modestly immunogenic live oral vaccine strain Ty21a (31, 61, 62). *Salmonella typhi* penetrates the gastrointestinal epithelial barrier and infects phagocytes mainly macrophages within the lamina propia where they adapt to prolonged survival thereby giving the bacteria an opportunity to spread to other systemic organs. Because of the natural tendency of the *Salmonella typhi* pathogen to spread to systemic tissues and persist in macrophages, a hybrid *Salmonella typhi* vaccine strain could be developed to carry other antigens as well which would be directed at developing immunity to other diseases. *Salmonella* vaccines are also capable of stimulating other T-cell subsets such as Th-2, cytotoxic, and memory T cells.

Several attenuated *Salmonella typhi* oral vaccines have been constructed to serve as vectors for heterologous antigens, including but not limited to antigens of *Shigella sonnei*, *Escherichia coli*, *Vibrio cholerae*, *Bordetella pertussis*, *Plasmodium falciparum*, *B. anthracis*, *Helicobacter pylori*, and *Listeria monocytogens* (63-68). As a vaccine against typhoid fever, *Salmonella typhi* Ty21a is a widely used licensed live vaccine that is exceedingly safe but requires three to four immunizations to induce long-term (at least 6-7 years) protective immunity in two thirds of immunized individuals (69). More highly immunogenic live *S. typhi* vaccines, such as CVD908-*htrA* (59), are currently being clinically evaluated for the safety.

Strains of *S. Typhimurium* and *S. Typhi* were first used as recombinant vectors for antigen delivery (70). Compared to injectable vaccines, oral delivery of attenuated *Salmonella*-based vaccines should result in increased compliance, safety, and ease of administration. Eliciting mucosal, cellular, and humoral immunity is desirable for protection against many of the organisms that infect at mucosal surfaces, including gastrointestinal, genital, and respiratory pathogens. Salmonellae are the most commonly studied vectors, perhaps in part because of the ease with which they are genetically manipulated, the existence of the serovar Typhimurium mouse model for preclinical work, and favorable prior human experience with the live attenuated vaccine U.S. Food and Drug Administration approved vaccine for typhoid fever, Ty21a. Ty21a serves as an important safety benchmark for researchers undertaking clinical trials. However, there are other safety considerations. The foreign genes inserted into the chromosome or foreign plasmid vectors carried by *Salmonella*-based vaccines should possess some containment features to minimize the possibility of transfer to and maintenance in other bacterial species. The plasmid vectors should therefore be nonconjugative, preferably be nonmobilizable, possess a narrow replicon host range, and not specify resistance to any antibiotic (71).

Recently a type IV pilus operon promoter controlling nucleocapsid gene expression of severe acute respiratory syndrome associated coronavirus (SARS-CoV) in *S. typhi* elicits full immune response by intranasal vaccination has been reported (72). This provides insights that type IV pilus operon promoter controlling SARS-CoV viral gene expression in *S. typhi* might be an attractive live vector vaccine against both infections of SRAS-CoV and *S. typhi* for it could induce mucosal, humoral, and cellular immune responses. However, further clinical studies are needed to assert this.

Conclusion

Salmonella typhi is a host specific pathogen affecting humans. Its virulence is aided by SPIs. SPI-1 is required for invasion of the bacteria to epithelial cells while SPI-2 is essential for survival and replication in the host cells. *Salmonella* has been around for many years and a lot of studies have been done in an effort to combat its infections. However, formidable challenges have been met in the process. Some of these challenges are multi-drug resistance of the bacteria and failure of vaccines to induce a lasting protective effect. Other challenges are just sheer lack of affordability of the means of treatment and preventive measures in the developing world.

Recently, *Salmonella* has emerged as a vaccine vector carrying antigens of other disease causing organisms, making it possible for a double protective therapy. However, further studies are needed to ascertain prophylactic use of these new modes of treatment to infectious diseases.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (30670098, 30470087, and 30570070), the Major State Basic Research Development Program of China ("973" project 2005CB522901 and

2006CB504300), and a grant from Hubei Province Science Technology Department (2006ABD007).

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