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***Minireview***

# Immunological, Cellular and Molecular Events in Typhoid Fever

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*Salmonella*, a facultative intracellular Gram-negative bacterium infects a wide range of hosts causing several gastrointestinal diseases and enteric fever in humans and certain animal species. Typhoid caused by *Salmonella typhi* remains a major health concern in India and worldwide. Also, with emergence of multidrug resistant strains, *Salmonella* has acquired increased virulence, communicability and survivability, resulting in increased morbidity and mortality. Though a number of vaccines for typhoid are available against *S. typhi* (or also against *S. typhimurium*), these have certain undesirable side effects and the search for new immunogens suitable for vaccine formulation is still continuing. The immune response to primary *Salmonella* infection involves both humoral and cell-mediated responses. The protective immunity against *Salmonella* depends on host- parasite interaction, however; the detailed mechanism of virulence, innate resistance and susceptibility of host remains unclear. This review focuses on the molecular, immunological and cellular mechanisms of pathogenesis of *Salmonella* infection to provide an insight to counteract bacterial infections and allow a better understanding of its clinical manifestations. It also reviews better technological possibilities combined with increased knowledge in related fields such as immunology and molecular biology and allow for new vaccination strategies. Some new approaches such as subunit and nucleic acid vaccines and recombinant antigen which are becoming increasingly important for the development of potential vaccines have also been discussed. A significant progress has been made in our understanding of *Salmonella* pathogenesis. Despite these efforts, however, many challenges exist, especially for investigators who aim to understand how the pathogenic mechanisms operating *in vitro* apply to *in vivo* model systems. However, unyielding work and collaborations between *Salmonella* researchers and clinicians worldwide have made significant contributions to understanding the interaction between virulence determinants and immunity required to stop the spread of this pathogen.

**Keywords**: Outer membrane proteins, Pathogenesis, *Salmonella*, Typhoid, Vaccine, Virulence.

## Introduction

Typhoid caused by *Salmonella typhi*, a facultative gram-negative bacterium, remains a public health problem with an annual global burden of about 16 million cases, leading to 60,0000 deaths[1]. *Salmonella* species are an important group of pathogens, which infect a wide range of hosts causing a variety of syndromes. Other *Salmonella* serovars *(S. typhimurium, S. enteritis*) cause infections to domestic animals that can be transmitted to humans and also represent a serious concern for the food industry[2]. Typhoid is endemic in many developing countries, including areas of Africa, Asia and S. America[3] and children in endemic areas, travelers and microbiological laboratory technicians are particularly at risk of contracting the disease.

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*Abbreviations*: LPS, lipopolysaccharide; MDR, multidrug resistance; Nramp-1, natural resistance associated macrophage protein-1; OMP, outer membrane proteins; TTSS, type III secretion system.

Although many studies have been undertaken to understand the host response against *Salmonella* infection, precise events related to immune response against this microorganism are not fully understood. The development of effective vaccines for protection against diseases caused by *Salmonella* spp. continues to be an extensively studied field[6]. Live vaccines can effectively induce both humoral and cell-mediated immune response and are often preferred over vaccines derived from killed bacteria[7]. Though the later cannot replicate and are, therefore, non-infectious, these are less effective than live vaccines in inducing protective immunity. The ability to elicit antibodies, in addition to cell-mediated immunity is important for optimal protection conferred by *Salmonella* vaccines[8].

Two new vaccines, a parental capsular polysaccharide vaccine based on the *S. typhi* Vi antigen[9] and a live attenuated oral vaccine containing *S. typhi* strain Ty21a[10] have been licensed for use against typhoid fever during last 15 years. However, due to adverse reactions and less than desired

efficacy, a number of new genetically defined attenuated strains of *S. typhi* have been constructed as live candidate oral vaccines which have the potential as vector for delivery of foreign antigens also[11]. Although all clinical and field trials with *Salmonella* as delivery system have been limited to attenuated strains of *S. typhi*, the use of attenuated *S. typhimurium* for heterologous antigen delivery is a promising alternative to *S. typhi* as vaccine vector[12].

The development of an effective *Salmonella* vaccine is highly relevant and being pursued by a number of workers. A number of well-tolerated attenuated *S. typhi* strains have been found to be immunogenic in clinical trials[13] and show promise as candidates for new generation of typhoid vaccines. In addition, recombinant *Salmonella* vaccines have been shown to protect against a broad range of pathogens in animal models and preliminary results from clinical trials demonstrate that protective immunity against heterologous antigens is achievable[14]. A significant development involves the use of *Salmonella* for delivery of DNA vaccines. Use of bivalent *Salmonella* strains to deliver DNA vaccines allows the induction of immunity against the *Salmonella* carrier, the heterologous antigen(s) expressed by *Salmonella* and the antigen(s) encoded by the DNA vaccines. Further, it should be possible to simultaneously express the heterologous antigen integrated into *Salmonella* chromosome as well as cloned into the plasmid(s) carried by the *Salmonella*. It may even be possible to both 'prime' and 'boost' with a single oral immunization by delivering the DNA vaccine to appropriate antigen presenting cells (APCs) for expression of antigen and co-delivering an expressed antigen. This DNA vaccine delivery system is very promising and offers a new and exciting approach to mucosal vaccine delivery[15,16]. To date, however, there have been no reports of human trials of recombinant *Salmonella* for the delivery of DNA vaccines.

## Typhoid Fever: Molecular and Immunological Perspective

Classically typhoid fever is considered a multiple stage disease. The 1st week is characterized by progressive elevation of body temperature, followed by bacteremia, 2nd week with rose spots in the skin, abdominal pain and splenomegaly and the 3rd week with a more intensive intestinal inflammatory process, particularly in the Peyer's patches. Complications, such as digestive tract bleeding and intestinal perforation may also develop. The patients may have clinical recovery afterwards, however, some patients who are not responsive to the treatment with antibiotics against *S. typhi* may even die after a progressive clinical worsening[17].

The understanding of typhoid fever pathogenesis, especially the cellular and molecular phenomenon responsible for clinical manifestations has greatly increased with several important discoveries: a) Bacterial type III protein secretion system — Many virulence genes are clustered together on pathogenecity islands (PAIs) that encode structurally similar but functionally distinct type III secretion systems (TTSS) which translocate virulence proteins from bacterial to host cells during the infectious cycle. b) The five virulence genes of *Salmonella* spp. encoding *Salmonella* invasion proteins (Sips) A, B, C, D and E, which are capable of inducing apoptosis in macrophages. c) The function of Toll receptors R2 and R4 present in the macrophage surface (originally discovered in *Drosophila*) — The Toll family receptors are critical in cell signaling mediated through macrophages in association with LBP and CD14. d) The lines of immune defense between intestinal lumen and internal organs, and e) The fundamental role of endothelial cells in inflammatory deviation from bloodstream into infected tissues by bacteria[6,17-19].

The use of antimicrobials in typhoid therapy needs to be evaluated in light of increased risk of disease due to elevation of cellular concentration of LPS molecules. These molecules are responsible for mononuclear cell activation that releases cytokines, such as TNFα, IL-1, IL-6, IFNγ etc. They are considered as powerful agent for the inflammatory cell activation and act through CD14 and Toll R2 and R4 receptors. The signals are then transmitted to an intracellular protein p21 activated kinase[18]. Other proteins activated by *S. typhimurium* are the Rho-GTPases, which stimulate the membrane "ruffling" (rearrangement of cellular membrane), responsible for the entry of *Salmonella* spp. to the cell. At this stage, rearrangement of actin cytoskeleton and nuclear responses also occur. The contact of cytosol with proteins secreted by invasive bacteria (*S. typhi, S. typhimurium, S. cholerae-suis* etc.) is made through protein channels TTSS that translocate bacterial proteins inside the cells. The genetic acquisition of TTSS is a major evolutionary leap for gram-negative

bacterial pathogens. TTSS allows animal and plant pathogens to inject their own proteins (termed as effectors) directly into host cells that modulate specific cellular functions of the host. These 'molecular syringes' and their effectors are the essential virulence determinants. The protein Sop E (the substrate for this secretion system) stimulates the cytoskeleton reorganization, followed by the Jun N-terminal protein activation that are dependent on Rac-1 and CDC 4219. The role of mitogen activated protein kinases in nuclear response and production of cytokines induced by inflammatory cells and cultured epithelial cells are other phenomena that are responsible for the symptoms and clinical manifestation of the disease[19].

## Pathogenesis of Typhoid

Murine salmonellosis has extensively been used as the model for human typhoid fever. *S. typhimurium* causes a systemic infection in mice that is reminiscent of human typhoid and hence has been used to study clinically relevant mechanisms of anti-*Salmonella* host defense[20,21]. This model has also proven useful for the analysis of genetic basis of *Salmonella* virulence and for initial assessment of safety and immunogenicity of new generation of *Salmonella* vaccines.

## Molecular and Cellular Events in *Salmonella* Intracellular Proliferation

*Salmonella* proliferates within membrane bound vacuoles of eukaryotic cells. Recent work has shown that macrophages are the main cells that support bacterial growth *in vivo*. In contrast, tissue culture models have used epithelial cells as most permissive cells for bacterial growth[22]. Intracellular proliferation of *Salmonella* is fast and almost 100-fold increase in bacterial load can be seen within 24 h. In liver and spleen, *Salmonella* is located in CD18 containing phagocytes (polymorphonuclear cells and macrophages) that infiltrate to the infection loci[23]. If macrophages are immunodepleted, the severity of infection increases, resulting in macrophages becoming the permissive cells *in vivo* for proliferation of *Salmonella*[24].

## Intracellular Proliferation of *Salmonella in vivo*

### *Salmonella Determinants*

The products of the virulence plasmid-encoded *spv*-ABCD operon are the first pathogenic functions linked to *Salmonella* proliferation in liver and spleen. The *spv* genes are expressed efficiently in bacteria residing within epithelial or macrophage cells growing in a medium that mimics the eukaryotic intracellular environment[25]. The *spv*-ABCD operon is controlled by regulatory protein SPVR, encoded by a gene located upstream and in opposite orientation to the *spv* operon. Expression of SPVR, on the other hand, is regulated by the factors such as alternate sigma factor (RpoS), integration-host factor and leucine-responsive regulatory protein[26]. The expression of *spv* operon may also be controlled by the PhoP-PhoQ, the two-component regulatory system[27,28] and determinants encoded by *Salmonella* pathogenicity island 2 (SPI-2)[29,30]. The bacterial functions required for *Salmonella* proliferation in target organs include the cell envelope transporter (Toll B), a phosphoglycerol-transferase (Mdo B), a methionyl-tRNA-formyl-transferase-homologous protein (Fmt) and a putative malate oxidoreductase (Mdh)[31,32].

### *Host Controlling Determinants*

The eukaryotic natural resistance associated macrophage protein-1 (Nramp 1) is a membrane protein present exclusively in professional phagocytes and essential for resistance to *Salmonella*[33]. INF-γ is another host factor that impairs intracellular proliferation of *Salmonella.*

## Intracellular Proliferation of *Salmonella* in Cultured Cells

Infection of cultured phagocytic or epithelial cells mimics relevant bacteria-host interaction taking place in *in vivo*, bacterial invasion and intracellular proliferation. In phagocytic cells, diversity of phenotypes in cultured macrophages ranges from active bacterial killing to pathogen proliferation at rates comparable to those of epithelial cells[34]. *Salmonella* must express and deploy a TTSS located in SPI-2 to survive the host phagocytic vacuoles and to cause systemic infection in mouse models of typhoid fever. The screening of *Salmonella* genes that are transcriptionally co-regulated *in vitro* with SPI-2 genes has been used to identify bacterial loci that might function in a mouse model of systemic disease. Strains with mutations in three SPI-2 co-expressed genes have been constructed and tested for their ability to cause disease in mice[35]. The SPI-2 encoded TTSS plays a role in macrophage growth and SPI-2 determinants are involved in inhibition of phagosome-lysosome fusion and host NADPH oxidase delivery to the *Salmonella* containing vacuoles and both these activities are essential for bacterial growth[36]. Other

factors include PhoP-PhoQ system, ompR/EnvZ and transcription regulators RpoS, SlyA and RpoE[37].

In cell culture models, epithelial cells are the most permissive cells for bacterial growth[22] and massive bacterial growth is observed in cultured epithelial cells. The onset of bacterial growth correlates to Sif formation though these structures are dispensable for intra-epithelial growth[38]. The contribution by both the host and the pathogen for fine adjustment of the intracellular growth rate has been suggested.

The severity and outcome of *Salmonella* infections in mice depend on several variables, such as virulence of infecting strain, infectious dose, route of infection, genetic background and immunological status of the host[1]. *S. typhimurium* encounters a diversity of environments throughout the course of systemic infection.

***Intestinal-mucosal Immunity* (*First line of defense*)**

The infectious dose of *S. typhi* in volunteers varies widely, ranging between 1,000 to 1,000,000 organisms[39]. The bacteria must survive the gastric acid barrier to reach the small intestine and the low gastric *p*H is an important defense mechanism. In small intestine, *S. typhi* moves across the intestinal epithelial cell and reaches the M cells, thus penetrating the Peyer's patches. The M cells are specialized epithelial cells overlying Peyer's patches (probably originated from intestinal epithelial cells) and are present as small pockets in the mucosal surface. After contact with M cells, the bacteria are rapidly internalized and interact with antigen-presenting cells, get partially phagocytized and neutralized[22]. The infected phagocytes are organized in their discrete foci that become pathological lesions surrounded by normal tissue. The formation of these lesions is likely to prevent the uncontrolled spread of bacteria in the body, thus confining the bacteria to localized foci of infection.

Lesion formation is a dynamic process that requires the presence of adhesion molecules such as ICAM1 and the balanced action of cytokines (TNFα, IL12, IL18, IL14, IL15 and IFNγ). Failure to form pathological lesions results in abnormal growth and dissemination of bacteria in infected tissue and some bacteria escaping this barrier reach Peyer's patches. The dendritic cells help in antigen presentation that provokes T and B lymphocyte activation[21].

***Dissemination from Intestinal Mucosa's Lamina Propria***

The T and B lymphocytes released from the lymphatic nodules reach liver and spleen via reticulo-endothelial system, where the bacteria are destroyed mainly by phagocytosis through macrophage system. However, *Salmonella* are able to survive and multiply within the mononuclear phagocytic cells[40]. At a threshold level determined by the number of bacteria, bacterial virulence and host immune response, the bacteria are released from their sequestered intracellular habitat into the bloodstream. Hence, this bacteremic phase of disease is characterized by dissemination of the organisms to secondary sites of infection. The most common sites of secondary infection are the liver, spleen, bone marrow, gallbladder and Peyer's patches in the terminal ileum. In the liver, *S. typhi* provokes Kupffer cell activation and neutralize the bacteria with oxidative free radicals, nitric oxide as well as enzymes while the survived bacteria invade hepatocytes resulting into cellular death.

***Virulence***

*Salmonella* pathogenesis is a complex, multi-factorial process that results from activity of many bacterial gene products and about 4% of its genome is involved in virulence[41]. In *S. typhimurium*, many of virulence genes cluster together on pathogenecity islands (PAIs). SPI-1 and SPI-2 are two PAIs that encode structurally similar, but functionally distinct TTSS encoded by all serovars of *S. enterica*, which translocate virulence proteins from bacteria to host cells, stimulating cellular functions of the host during the infectious cycle[19]. SPI-1 plays an important role in invasion of epithelial cells, whereas SPI-2 is required for bacterial replication within macrophages and systemic growth in the mouse[42]. In addition, a number of other genes have also been found to be necessary for *S. typhimurium* virulence[43] and the biochemical functions of some of these genes have been elucidated, though little is known about their regulation *in vivo* and mechanism of their interaction during infection. Recently, *Salmonella* invasion proteins coded by a new group of five virulence genes Sip A to E have been identified. The Sip B protein is involved in protein translocation via TTSS and has the potential to induce apoptosis in macrophages through caspase-1 activation[17].

***Invasion of Salmonella***

The invasive mechanism of *Salmonella* has been studied extensively in *S. typhimurium* and several loci important for invasion have been identified in various *Salmonella* spp. using molecular genetic approaches.

It has been suggested that invasion system in *S. typhi* is genetically distinct from *S. typhimurium*. A large chromosomal fragment (100 kb) around the *Salmonella* inv locus may be responsible for the cell invasion[44]. Sip proteins secreted through TTSS apparatus play a key role in the invasion of *S. typhimurium* and a similar mechanism is expected to work for *S. typhi* also[45]. *S. typhimurium* infection results in release of a set of effector proteins that induce actin cytoskeleton rearrangement, membrane ruffling and macropinocytosis. The effector proteins include an exchange factor for Rho GTPases (Sop E), an inositol phosphate phosphatase (Sop B) and an actin-binding protein (Sip A)[46]. Several factors including anaerobic growth state, calcium concentration and osmolarity regulate *S. typhimurium* invasiveness, whereas osmolarity and growth phase regulate the adherence and invasion of cultured human epithelial cells by *S. typhi.*

***Apoptosis and Human Toll Receptor Activation in Typhoid Fever***

Typhoid is an important example of severe sepsis, as it causes high degree of cellular death (necroapoptosis) besides severe toxemia. The innate immune system uses Toll family receptors to sign microbe's presence and initiate host defense. Bacterial lipoproteins (BLP) expressed in all bacteria species are potent activators of Toll R2. The innate immune system includes macrophages and natural killer (NK) cells that act directly on pathogens through cytokines and other stimulatory molecules and activate the adaptive immune responses (cellular and molecular) through T and B lymphocytes. It identifies the pathogen by standard recognition receptors, which attach to microbial macromolecules. CD14 and Toll R2 receptors were considered as fundamental in recognition of LPS/endotoxin on the surface of gram-negative bacteria such as *Salmonella*. These receptors differ in the composition. CD14 is a GPI (glycosyl-phosphatidylinositol) receptor that does not cross cellular membranes and does not transmit signals for cytoplasm or activate protein chains of macrophages, while Toll R2 molecule crosses cellular membranes and transmits signals for the intracellular protein pathways. Activation of these protein pathways spreads to transcriptional factor NF-kB that migrates from cytoplasm to nucleus and recognizes and expresses genes encoding the adhesion molecules TNFα and other Th1 cytokines (IFNγ, IL-2, etc.). The patients with failure of immune response fail to combat the infection by macrophage activation that may lead to septic shock. The pathogens have antigens such as LPS of gram-negative bacteria, glycolipids of mycobacteria, lipoteichoic acid of gram-positive bacteria, mannans of yeast, RNAs of virus etc that are recognized by the receptors.

Two LPS-binding proteins BPI (bactericide permeability increasing protein) and LBP (lipopolysaccharide binding protein) have distinct effects. BPI (mol. mass 55 kD) is present in neutrophils, has an antimicrobial role with selective toxicity against gram-negative bacteria and is more effective when acting on neutrophil-phagocytosis in synergism with defensins (intestinal mucosa's antimicrobial factors). LBP increases the sensitivity to LPS, allowing the effector cell activation by subpicomolar LPS concentrations. It recognizes lipid A and also carries out an important role in the bacterial clearance of peripheral blood through CD14. Some recent studies indicate that Toll family receptors are critical in LPS mediated signaling in association with LBP and CD1423 and discovery of a Toll-like human receptor4 (hTLR4) has enhanced the understanding of binding of receptors with LPS-34[47-49].

***Host Immune Defense***

The early growth of *Salmonella* in mouse tissues is controlled by the innate resistance autosomal dominant gene Nramp 1, located on chromosome 1 and expressed mainly in macrophages and cells of granulocyte lineage[50]. Nramp1 codes for an integral membrane phosphoglycoprotein that is recruited to the bacteria-containing phagosome, where it functions as a divalent metal ion pump. The main host defense against *Salmonella* spp. occurs through the neutrophils, followed by mononuclear cells. These inflammatory cells produce cytokines such as TNFα (produced mainly by Kupffer cells in liver), IFNγ, IL-1, IL-2, IL-6 and IL-8. Clearance of bacteria from tissues requires the CD28-dependent activation of CD4+ and TCR-α β T cells and is controlled by MHC class II genes[51-53]. Dendritic cells and B-cells are involved in the initiation and development of T-cell immunity to *Salmonella*[54,55]. Interaction between B and T-cells is needed for development of antibody responses to *Salmonella* proteins and for isotype switching of antibody response against LPS antigens[56]. Resistance to reinfection with virulent *Salmonella* microorganisms (secondary infection) in immunized mice requires the presence of CD4+

dependent Th1 type immunological memory, CD8+ T cells and anti-*Salmonella* antibodies[8].

Epithelial cells play a key role in the inflammatory response to intestinal pathogens. Their interaction with *Salmonella* spp. leads to the generation of a great number of biochemical signals including the basolateral release of chemokines (including IL-8) and apical secretion of `pathogen-elicited epithelial chemoattractant`. These substances are partially responsible for guiding the recruitment and traffic of polymorphonuclear cells across intestinal epithelial cells. After initial localization in resident phagocytes (macrophages), the bacteria are associated mainly with PMNs in early phase of infection. However, evidence suggests a predominant role of mononuclear cells in early resistance to the disease[57]. In a recent study, *S. typhimurium* infection has been shown to induce IL-8 secretion by intestinal epithelium mediated through increase in intracellular calcium and this phenomenon is found to be NF-κ B dependent[58].

The inflammatory deviation on migration of blood leukocytes across the endothelial cells into hepatic and spleen tissues is another important event during *Salmonella* infection that occurs through the action of adhesion molecules such as integrins present in inflammatory cells and selectins in endothelial cells. The inflammatory microenvironment is completed by chemokines that are capable of stimulating leukocyte motility (chemokinesis) and directed movement (chemotaxis) of neutrophils and mononuclear cells. The chemokines help the blood leukocyte migration straight for host cells infected by bacteria. TNF-α is produced by macrophages and other mononuclear cells. Besides the macrophage phagocytosis, TNF-α causes neutralization of these invasive bacteria in association with IFN-γ, IL-2 and other cytokines[47]. Bacteria-infested Peyer's patches produce strong inflammatory reaction with the recruitment of leukocytes. The potent inflammatory reaction against *Salmonella* spp. provokes host cell death, as well as apoptosis of both inflammatory and epithelial cells resulting in the appearance of several clinical signs such as fever, jaundice (due to hepatocyte death and cholangiocyte activation) and increase of levels of enzymes AST, ALT, glutamyltranspeptidase, alkaline phosphatase, etc. The inflammatory response of Th1-dominant type is destructive for host cells and bacteria; it attenuates progressively and coincides with increase of Th2-immune response[54]. Th2 cells produce IL-4, IL-10, IL-13 and tissue growth factor that confer powerful protective effect on host cells (hepatocytes, CEIs, inflammatory cells etc.) through partial inhibition of cytokines associated with Th1 response. The predominant assessment of Th1 immune response can be detected by immunohisto-chemistry techniques using specific antibodies, bioassays or immunoassays and detection of cyto-kines in peripheral mononuclear cells by the flow cytometer or ELISPOT[17,54].

Extensive knowledge about molecular and cellular mechanism of pathogenesis of *Salmonella* infection and typhoid has allowed a better understanding of its clinical phases and more rational approaches for clinical manifestations. In future, it should be possible to decrease the intensity of inflammatory phenomenon using therapeutic maneuvers intended to disable inflammatory cells (with cytokines decrease), as well as to inhibit the cellular death (hepatocytes, inflammatory cells, etc.) related to *Salmonella* invasion.

## Outer Membrane and Proteins of *Salmonella* as Candidate Antigens for Typhoid Vaccine

*Salmonella* have an inner cell membrane, an outer membrane and in between these a peptidoglycan cell wall structure. The inner membrane is the typical phospholipid bi-layer structure, enclosing cytoplasm and other cell inclusions. The outer membrane consisting of two leaflets is constituted by lipopolysaccharides (LPS), proteins, phospholipids (PLs) and enterobacterial common antigen (ECA); the inner leaflet is composed of PLs and outer leaflet mainly of LPS[59]. As the exposed cell wall components of gram-negative bacteria interact directly with host cells and antibodies, an immune response evoked against these would be able to recognize the invading pathogens and confer protection. Of the surface components, LPS and protein components of outer membranes have gained attention because of the immune response evoked by them.

The outer membrane proteins (OMPs) of gram-negative bacteria account for approximately 50% of outer membrane mass and include integral membrane as well as anchored proteins[60]. OMPs differ from other membrane spanning proteins as the spanning structure of OMPs is mainly anti-parallel β barrel structure[59]. Integral OMPs are essential for maintaining the integrity and selective permeability of bacterial membrane. OMPs represent important

virulence factors and play important role in bacterial adaptation to host niches, which are usually hostile to invading pathogens. Some of these roles have been enumerated in Table 1.

The emergence of multi-drug resistance (MDR) strains of *Salmonella* has complicated the management of typhoid due to their acquired increased virulence, communicability and survivability, leading to increased morbidity and mortality. Though a number of vaccines for typhoid are currently available, none of these is without side effects. Thus, it is pertinent to search for new immunogen molecules suitable for vaccine formulation. The outer membrane proteins of *Salmonella* have been implicated as possible candidates for conferring protection against typhoid. Although significant advances have been made regarding the structure and function of OMPs, the number of OMPs that have been characterized represents only a small portion of the total OMPs revealed by bacterial genome sequences[59].

Humoral and cell-mediated immune responses against OMPs have been studied in control, infected and immunized-infected mice and the humoral immune response to crude OMPs of *S. typhimurium* in mice has been characterized. The crude OMPs of *S. typhimurium* (smooth C5 strain) evoke antibody response to both LPS and proteins. The crude OMPs from rough mutants (lacking *O*-specific chain of LPS) of *S. typhimurium* produce antibodies to proteins, but not to LPS[61].

The *O*-antigen of LPS is a virulence factor in enterobacterial infections. *S. enterica* expresses β-barrel surface proteases of the omptin family that activate human plasminogen. The presence of *O*-antigen repeats on wild-type or recombinant *S. enterica* prevents plasminogen activation by PgtE of *S. enterica* and does not affect incorporation of omptins into bacterial outer membranes. However, its presence prevents PgtE-mediated bacterial adhesion to basement membranes. Expression of LPS, on the other hand, prevents Pla-mediated adhesion of recombinant *E. coli* to basement membranes as well as invasion into human endothelial cells. Pla and PgtE require LPS for their activity and *O*-antigen sterically prevents recognition of large-molecular weight substrates. Loss of *O*-antigen facilitates Pla functions and LPS renders plasminogen activator cryptic in *S. enterica*[62].

The expression and deployment of TTSS by *Salmonella* is essential for causing systemic infection in mouse models of typhoid fever[63]. A genome-wide approach for screening of *Salmonella* genes that are transcriptionally co-regulated *in vitro* with SPI-2 genes has been used to identify bacterial loci that might function in a mouse model of systemic disease. VirK, a homologue of a *Shigella* virulence determinant and RcsC, a sensor kinase are important at late stages of infection. SomA, another *Salmonella* gene having homology with VirK is also important for systemic infection in mice. Expression of both VirK and SomA requires the transcription factor

Table 1—Various mechanisms of OMP-mediated bacterial adaptive responses to host environment[60]

| Adaptive response(s) | Mechanism(s) | OMP(s) involved |
|---|---|---|
| Iron uptake | Expression of OMPs directly binding to host proteins such as transferrin, lactoferrin or hemoprotein. | Tbp, Lbp, HemR |
| | Synthesis of high-affinity iron-siderophores and expression of OMPs and their binding to siderophore complexes. | FhuA, FepA |
| Antimicrobial peptide resistance | Direct degradation of antimicrobial peptides through production of outer membrane associated proteases. | OmpT, PgtE |
| | Modification of bacterial surface through production of OMPs with enzymatic activities. | PagP |
| Serum resistance | Prevent the activation of complement cascades by binding to factor H or C4bp, down-regulators of complement activation. | Por1A, Por1B, OspE |
| | Unknown | OmpX |
| Multi-drug resistance | Key roles in multi-drug efflux systems. | TolC, OprM |
| Bile resistance | Modulate membrane permeability by regulating the production of specific porins. | OmpC, OmpU |
| | Key roles in multi-drug efflux systems. | TolC |

PhoP, whereas RcsC expression is independent of PhoP. Further, transcription factor OmpR is needed for the expression of YajN RcsB which is a target of RcsC, even though expression of RcsC itself does not require OmpR. VirK, SomA and RcsC are important during late stages of enteric fever and probably contribute to the modulation of bacterial outer membranes in response to the host environment[35].

Penetration of intestinal epithelial cells is an important step in the pathogenesis of *Salmonella.* A gene InvE necessary for *Salmonella* invasion of cultured epithelial cells has been characterized and its predicted amino acid sequence shows significant homology to *Yersinia* outer membrane protein YopN (LcrE). Unlike wild-type *S. typhimurium*, InvE mutants fail to change intracellular free calcium levels or distribution of polymerized actin in cultured epithelial cells or the normal architecture of microvilli of polarized Madin-Darby canine kidney cells. Wild-type *S. typhimurium* can rescue the invasive phenotype of InvE mutants in simultaneous infections of cultured epithelial cells. Hence InvE mutants are deficient in triggering the intracellular events that lead to bacterial internalization. Also, the expression of ShdA, a surface-localized fibronectin-binding protein is induced *in vivo* in the murine caecum, a tissue having cognate receptors for this OMP. Expression of cloned ShdA gene from T7 promoter *in vitro* results in detection of ShdA in the outer membrane of *S. typhimurium* and binding of fibronectin to the bacterial surface. Deletion of ShdA gene results in decreased colonization of cecum and Peyer`s patches of terminal ileum to a lesser degree than that of mesenteric lymph nodes and spleen 5 days post-oral inoculation of mice[64].

Porins are the major class of OMPs that form non-selective pores for small hydrophobic molecules and have been extensively documented in relation to immunogenecity and pathogenecity of typhoid fever. They are excellent antigens that interact efficiently with both arms of the host immune systems and can play a role in providing protection against the disease[61,65]. However, they are not specific to *Salmonella* and their efficacy as protective immunogen depends upon their association with LPS[66,67]. In addition to major OMPs viz. OmpC, OmpF and OmpA, many minor classes of OMPs have been identified[68]. Some of these minor species are expressed only at very low levels. Two-dimensional electrophoresis of *S. typhimurium* displays a greater number of higher molecular mass proteins (>80 kDa), majority of which appear to be localized in the outer membranes[69].

Beside porins, many medium and high molecular mass proteins are present in the outer membrane of gram-negative bacteria including *Salmonella,* some of which are species-specific[70-72]. Four non-porin OMPs (molecular masses 15 kDa, 33 kDa, 37 kDa and 49 kDa) have been selected from the OM profile of *S. typhimurium*[73]. These proteins confer varying degree of protection against bacterial challenge with experimentally induced murine salmonellosis and also decrease the number of bacteria reaching liver. Immunization with 49 kDa protein provides 100% protection against *Salmonella* infection, causing an enhanced phagocytic capacity and index in the reticulo-endothelial system. However, the role of these non-porin OMPs in pathogenecity and immmunogenicity has not been explored and biological role of majority of bacterial OMPs is still unknown.

## Conclusion

Salmonellosis (and typhoid fever) continues to be an important health problem in developing countries. The preventive measures against salmonellosis are not adequate and its management is becoming increasingly difficult due to appearance of new drug resistant strains. Presently a number of vaccines against typhoid are available; however, there is a need for better and improved new generation vaccines against *Salmonellae*. For successful development of a defined vaccine, a clear understanding of both the cellular and humoral components of the immune responses elicited during infection and identification of antigens that trigger protective response is essential. Many studies have been undertaken to understand the genetics as well as physiological responses of *Salmonella* under different conditions during infection. Antigens that are currently being exploited include the outer membrane proteins (OMPs), the heat shock proteins and the Vi capsular antigen.

OMPs of *Salmonella* are being looked upon as new immunizing agents that can confer protection against typhoid. With the aid of the newly developed technologies such as functional genomics and DNA microarrays, characterization of bacterial OMPs can now be performed at a previously unprecedented large scale. These systems in conjunction with other

strategies, such as signature-tagged mutagenesis, subtractive and differential hybridization, *in vivo* expression technology etc would reveal more OMPs that are essential for bacterial virulence and adaptation during in vivo infection. These OMPs will be promising targets for the design of antimicrobial drugs and vaccines. To circumvent some of the adversities, various approaches of immunological manipulations, either alone or in combination with chemotherapy and vaccination have been explored.

The development of effective vaccines for protection against diseases caused by *Salmonella* spp. continues to be an extensively studied field. Use of well-defined recombinant protein in combination with appropriate adjuvant is more likely to overcome many limitations and provide species or strain specificity. The nucleic acids have strong potential and may become the vaccines of future. Better technological possibilities combined with increased knowledge in related fields such as immunology and molecular biology allow for new vaccination strategies.

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