Chapter 2

Review of Literature
2.1 SHIGELLOSIS

2.1.1 Shigella

*Shigellae* are 0.5-0.7 μm, gram-negative, non-motile, facultatively anaerobic, non-sporulating bacilli which belong to the family *Enterobacteriaceae*. They are the causative agent of acute invasive enteric infection known as Shigellosis or Bacillary dysentery. *Shigella* was named after its discoverer Kiyoshi Shiga who identified the bacilli as the etiology agent in 1898 during an outbreak in Japan in 1897. Later, the genus *Shigella* was divided into four species viz., *Shigella dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C) and *Shigella sonnei* (serogroup D). Based on the variations in their O-polysaccharide portion of their LPS, the species were further classified into several serotypes, as *S. dysenteriae* known to have 15 serotypes, *S. flexneri* have 14 serotypes and subserotypes, *S. boydii* 20 serotypes and *S. sonnei* with a single serotype (Kotloff *et al.*, 1999; Niyogi, 2005).

*Shigellae* are transmitted mainly by faeco-oral route or by ingestion of the contaminated food and also by poor hygienic practices. Since the organism is more resistant to acid, it can easily pass through the gastric acid barrier. So, a very low infectious dose as few as 10-100 microorganisms are adequate to cause the disease. Its viability in stool stays for 2-3 days, in soil for 6-10 days, in water for several months and in ice for 6-8 weeks. They are able to grow at temperatures ranging from 12°C to 48°C (optimum 37°C) at a pH range of 5.0 to 7.5. The common selective or differential agar media used for the culturing of *Shigella* are MacConkey (MAC),
Xylose Lysine Deoxycholate (XLD), Hektoen (HEK), *Salmonella-\textit{Shigella} (SS), Deoxycholate Citrate Agar (DCA). On the lactose enriched media such as on MAC, DCA and SS agar, nonlactose fermenting characteristic colonies was observed. *\textit{Shigella}* is resistant to bile salts and this typical feature is usually useful in the selective media. Colonies on the MAC and DCA agar appear to be large, 2 to 3 mm in diameter, translucent and colourless (non-lactose fermenting). Whereas, on the XLD agar, colonies appear to be much smaller (1 to 2 mm diameter) and red in colour as lysine is decarboxylated producing alkaline end products which raises the pH and cause the agar to turn into deep red colour. On the XLD, HEK and SS agar, *\textit{Shigella}* does not produce hydrogen sulphide (H$_2$S). *\textit{Shigella}* utilizes glucose and other carbohydrates, producing acid but not gas (with the exception of *\textit{S. flexneri} 6*) and is oxidase negative. Neither citrate nor malonate is used as the sole carbon source for growth and the organisms are inhibited by potassium cyanide.

Earlier *\textit{Shigella} and Escherichia coli* were classified in the same genus because of their genetic similarity. Recent studies reveal that 175 of the total 3235 open reading frames were exclusive for *\textit{S. flexneri} as the result of the comparative genomic study between the two organisms (Wei \textit{et al.}, 2003). Phylogenetic groups obtained from the chromosomal and plasmid genes were similar, suggesting that the virulence plasmid and the chromosome share similar evolutionary histories. However, *\textit{Shigella} strains were put in a different genus from *\textit{E. coli} because of their medical significance, human host interactions, pathogenicity, physiology (failure to ferment lactose or decarboxylate lysine) and serological characteristics (Escobar-Paramo \textit{et al.}, 2003). It has been reported that *\textit{Shigellae} have evolved from different
strains of *E.coli* and have become highly specific human pathogens through extensive convergent evolution involving gain and loss of functions (Yang *et al.*, 2005).

### 2.1.2 Clinical Manifestations

*Shigella* spp. is the causative agent of the disease shigellosis. This acute intestinal infection is also called as Bacillary dysentery. World wide, this disease is recognized as a major burden in public health care (Kotloff *et al.*, 1999). It is primarily a disease of humans. The disease is characterized by the damage of the colonic epithelium followed by intracellular and intercellular spread, infection in the neighboring cells and the host’s acute inflammatory response leads to colitis of the mucosa. It results in the leakage of blood and mucous in the intestinal lumen (Schroeder and Hilbi, 2008). Since the disease is acquired via the fecal-oral route, the severity and the range of symptoms may be dependent upon the number of organisms ingested. The symptoms vary from mild watery diarrhea to severe inflammatory dysentery with the passage of frequent bloody and mucoid stools. The other symptoms include fever, malaise, abdominal cramping and convulsions. The onset of symptoms usually occurs within 24 to 48 hours of ingestion of the etiologic agent. Possible other complications of shigellosis include bacteremia, septicemia, hypoglycemia, dehydration, hemolytic-uremic syndrome, reactive arthritis, toxic megacolon and other neurological problems (Phalipon and Sansonetti, 2007). The persons infected with *S. flexneri* subsequently develop pain in their joints, irritation of the eyes and painful urination. This condition is called Reiter’s syndrome. It is the late complication of *S. flexneri* infection and lasts for months which leads to chronic arthritis. Shiga toxin of *S. dysenteriae* type I is responsible for the Hemolytic-uremic
syndrome. Usually, shigellosis is a self-limiting disease. Life-threatening are often seen in malnourished infants and young children under 5 years of age, also in elderly people who have weak immune system (Kotloff et al., 1999; Ashkenazi, 2004; Seidlein et al., 2006).

2.1.3 Epidemiology

Kotloff and colleagues reviewed *Shigella* infection worldwide between 1966 to 1997 in the developed and developing countries in different age groups. *Shigella* cases were estimated to be 164.7 million per year, of which 163.2 million were in the developing countries with 1.1 million death. Annually, 1.5 million cases were reported in the industrialized countries. Both endemic and epidemic shigellosis are present in developing countries. Among 69% infections, 61% of all deaths were reported in the children under 5 years of age. *S. flexneri* and *S. sonnei* were responsible for increased percentage of infections in the developing as well as in the industrialized countries. It has been reported that annually around 58 million travelers from industrialized countries were affected. Bacillary dysentery was also reported among the military troops (Kotloff et al., 1999). A study in Bangladesh demonstrated the existence of *S. dysenteriae* type I and *S. flexneri* in the surface water where the rural and semiurban population consume surface water for daily needs (Faruque et al., 2002). A report reviewed the epidemic outbreak of *S. dysenteriae* type I between 1993 to 1995 in the refugee settlement of Central Africa (Kerneis et al., 2009). New serotypes of *S. dysenteriae* were isolated from the patients in Bangladesh (Ansaruzzaman et al., 1993). Recent studies reported the existence of new serovar of *S. dysenteriae* isolates from the patients with diarrhea at Dhaka hospital in
Bangladesh (Talukder et al., 2007). It was reported that *S. dysenteriae* type I and *S. flexneri* was responsible for the epidemics in Bangladesh and India (Kotloff et al., 1999). During a sporadic outbreak of dysentery in Kolkata, *S. dysenteriae* type I and *S. flexneri* were the commonest serotypes found in the tested stool samples of the patients (Dutta et al., 2003; Pazhani et al., 2004; Taneja, 2007; Pazhani et al., 2008). A surveillance report of *Shigella* infections in Indonesia states that *Shigella* was isolated from 9.3% of diarrhea patients in the health centers. *S. flexneri* was found in 5.9% of patients, and was the most frequent species isolated, comprising 63.2% (36/57) of all *Shigella* species isolated. *Shigella* species were found significantly more often among children over 2 years old, and the rate of isolation increased with age. Stool with mucus and/or blood were the main characteristics of *Shigella* infection in these patients (Herwana et al., 2010). Meta-analysis from Pubmed and Chinese biomedical literature database showed the prevalence of *S. flexneri* and *S. sonnei* in mainland China from 2001 until 2012 (Chang et al., 2012). In a multicentre study of *Shigella* infection in six Asian countries, *S. flexneri* was found to be predominant in Bangladesh, China, Pakistan, Indonesia and Vietnam site whereas *S.sonnei* was common in Thailand, *S. boydii* was responsible for the series of infection in Bangladesh (Seidlein et al., 2006). Kimura et al., reported about *S. sonnei* outbreak in United States through the commercially prepared food (Kimura et al., 2004). *S. boydii* was generally found only in the Indian subcontinent (Niyogi, 2005). Imported iceberg lettuce caused *S. sonnei* outbreak in the Norwegian population (Kapperud et al., 1995). *Shigella* spp. was isolated from the patients with acute gastroenteritis in western Nepal (Wilson et al., 2006). *S. sonnei* outbreak among the school students has
been reported in China where diarrhea, fever and abdominal pain were the three common symptoms found in the infected patients (Xiao et al., 2012).

2.1.4 Pathogenesis of *Shigella*

Infection is initiated by ingestion of *Shigellae* (usually via fecal-oral contamination). The organism enters the host and travels through the digestive system until it reaches the large intestine. In general the infection is limited to the intestinal mucosa. Pathogenesis is initiated by the invasion through the basal face of the intestinal epithelium by *Shigella*. Thus, upon reaching the large intestine, *Shigella* is taken up in vacuoles by microfold cells (M cells) which are specialized structure of the follicle associated epithelium which covers the mucosal lymphoid follicles, the stimulating site of the mucosal immune system (Wassef et al., 1989, Perdomo et al., 1994). The organism escapes from the vacuole and finally travels to underlying macrophages that are associated with M cell-associated lymphoid follicles (Sansonetti et al., 1999). *Shigella* is phagocytized by macrophages in the dome area of these follicles, which then induces apoptosis which results in the escape of the pathogen to the basal side of the colonic epithelium. Studies also report that virulent *S. flexneri* causes destruction to the host cell mitochondria and triggers necrosis in the infected human monocyte derived macrophages (Koterski et al., 2005). Another study indicated that *Shigella* induced mitochondrial dysfunction in nonmyleoid cells, result in caspase-independent necrotic cell death through a new pathway during oxidative cell stress in epithelial cells (Carneiro et al., 2009). Death of the macrophage results in the release of proinflammatory cytokine IL-1β which eventually results in the
recruitment of polymorphonuclear (PMN) cells to the site of infection and the onset of inflammation (Sansonetti et al., 1999; Hathaway et al., 2002; Sansonetti 2006).

Figure 2.1: Differential expression of the Shigella invasive phenotype depending on the cellular target. a) Entry into the epithelial cells by macropinocytosis and escape onto the host cytoplasm. Intracellular and intercellular spread through actin filament formation. b) Phagocytosis by monocytes/macrophages followed by apoptosis and the release of IL-1β & IL-18. c) Phagocytosis by PMN leucocytes and the release of granular content. The overall process leads to the rupture, invasion and inflammatory destruction of the intestinal barrier (Sansonetti, 2001).

Meanwhile, the bacteria induce their own uptake into colonic epithelial cells and spread laterally through the cells of the epithelium by a process known as actin based motility (ABM) through the comet tail formation (Reutz et al., 2012). This actin based filamentous tail propels the *Shigella* into the protrusions on the contiguous enterocytes. During cell-cell spread the plasma membrane envelops the bacteria are lysed which results in the intracellular replication and intercellular bacterial spread (Sansonetti et al., 1999; Philpott et al., 2000). As the inflammation persists and expands, the infiltration of the PMNs facilitates the entry of additional bacteria onto the epithelium. Ultimately, it is the cells of the host’s immune system that cause inflammation and ulceration of the mucosa to the colon and develops the symptoms associated with shigellosis (Wassef et al., 1989; Islam et al., 1997; Sansonetti 2001; Jennison et al., 2004; Torres et al., 2004). It has been reported that the destruction of epithelial cells in the experimental models of shigellosis is due to the host inflammatory response and probably not by the intracellular multiplication of the pathogen (Mantis et al., 1996).
2.1.5 Virulence of Shigella

2.1.5.1 Virulence associated genes

Virulence plasmid

The pathogenesis and virulence of Shigella is attributed to the presence of 214-220 Kb large invasion plasmid (Sansonetti *et al*., 1983; Sansonetti *et al*., 1986; Hale 1991). The complete DNA sequence of the large virulence plasmid was sequenced and analysed (Venkatesan *et al*., 2001; Nie *et al*., 2006). The loss of the plasmid leads to loss of invasive phenotype of the pathogen (Sansonetti, 1981). It was reported that *S. flexneri* virulence plasmid was necessary for rapid and efficient killing of the macrophages whereas noninvasive bacteria remain trapped inside the phagocytic vacuole leading to bacterial destruction (Clerc *et al*., 1987). The plasmid DNA was reported to be the potential mediator for the downregulation of antibacterial peptides in the host cell thereby promoting the survival of bacteria (Islam *et al*., 2001). All the genes that are required for the pathogen entry into the host cells are
located in a 31 kb region of the invasion plasmid which is known as the pathogenicity island (PAI) (Sansonetti et al., 2001). Figure 2.3 shows the map of the virulence plasmid of *S. flexneri* 5a.

31 kb PAI region of the invasion plasmid includes the ipa (invasion plasmid antigen), mxi (membrane expression of ipa antigens) and spa (surface presentation of ipa antigens) genes which encode a type III secretion system (T3SS). The VirF (Virulence protein) induces the expression of VirB protein. The VirB protein then activates the ipa, mxi and spa promoters leading to the expression of the mxi and spa operons. This results in the synthesis and assembly of the protein complex mxi-spa translocon. This region is necessary to cause the entry into epithelial cells through macropinocytosis, apoptosis and activation of PMNs. The T3SS acts like a molecular syringe that translocates the effector proteins from the bacterial cytoplasm into the host cell (Tamano et al., 2000). The ipa genes are primarily the effector and/or translocator proteins responsible for inducing cytoskeletal rearrangements, membrane ruffling and pathogen uptake. The *mxi/spa* proteins assemble to form the structural components of the TTSS apparatus including the basal body and the needle complex. *MxiE* and *virB* proteins are the transcriptional activators of the TTSS genes in the entry region. It has been reported that *MxiE* mediates the intracellular expression of effector proteins when the organism reaches the eukaryotic cytosol (Kane et al., 2002). The genes necessary for the intracellular and intercellular spreading are *virG (icsA)* and *icsB*. The intercellular spread (*IcsA*) protein elicit the polymerization of the filamentous actin. Formation of the actin tail provides a motive force for the bacteria impinging on the plasma membrane of the infected host cell. The *IcsB* protein then lyses the plasma membrane which results in the intercellular spread. In the TTSS
molecular chaperones are found that aid to retain the stability of the effector proteins while they are in the bacterial cytoplasm (Sansonetti and Egile, 1998; Tamano et al., 2000; Schroeder and Hilbi, 2007; Sasakawa, 2010). The table below illustrates the role of virulence associated genes in the invasion plasmid of *Shigella* (Hale, 1991).

![Circular map of the large virulence plasmid of Shigella](image)

**Figure 2.3.** Circular map of the large virulence plasmid of *Shigella*. Outer ring depicts ORFs and their orientations, color coded according to functional category: 1. Identical or essentially identical to known virulence-associated proteins (red); 2. Homologous to known pathogenesis-associated proteins (pink); 3. Highly homologous to IS elements or transposases (blue); 4. weakly homologous to IS elements or transposases (light blue); 5. Homologous to proteins involved in replication, plasmid maintenance, or other DNA metabolic functions (yellow); 6. No significant similarity to any protein or ORF in the database (brown); 7. Homologous or identical to conserved hypothetical ORFs, i.e., proteins of unknown function (orange); and 8. Tn501 insertion-associated genes (green). The second ring shows complete IS elements. The third ring graphs G+C content, calculated for each ORF and plotted around the mean value for all ORFs, with each value color coded for the corresponding ORF. Scale is in base pairs (Venkatesan et al., 2001).
**Table 2.1**: Virulence-associated genes and functions encoded by the large Shigella virulence plasmid.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Product MW</th>
<th>Regulatory or Effector function</th>
</tr>
</thead>
<tbody>
<tr>
<td>virF</td>
<td>30 kDa</td>
<td>Positive regulators of the virG and ipa-mxi-spa loci</td>
</tr>
<tr>
<td>invA(mxiB)</td>
<td>38 kDa</td>
<td>Necessary for invasion (orients ipa gene products in outer membrane)</td>
</tr>
<tr>
<td>mxiA</td>
<td>76 kDa</td>
<td>Same as above</td>
</tr>
<tr>
<td>ippI</td>
<td>18 kDa</td>
<td>Same as above</td>
</tr>
<tr>
<td>ipaB</td>
<td>62 kDa</td>
<td>Necessary for invasion: mediates endocytic uptake of Shigellae</td>
</tr>
<tr>
<td>ipaC</td>
<td>43 kDa</td>
<td>Same as above</td>
</tr>
<tr>
<td>ipaA</td>
<td>38 kDa</td>
<td>Same as above</td>
</tr>
<tr>
<td>ipaD</td>
<td>78 kDa</td>
<td>May mediate adherence of Shigellae to host cells</td>
</tr>
<tr>
<td>virB</td>
<td>33 kDa</td>
<td>Positive regulator of the virG and ipa-mxi-spa loci</td>
</tr>
<tr>
<td>virG (icsA)</td>
<td>120 kDa</td>
<td>Assembles actin tails that propel the bacteria through the cell cytoplasm and into adjacent cells</td>
</tr>
<tr>
<td>ipaH</td>
<td>60 kDa</td>
<td>Has 5 alleles; IpaH7.8 facilitates the escape of Shigella from phagocytic vacuoles</td>
</tr>
</tbody>
</table>

**Invasion plasmid antigens**

In *Shigella*, the entry region of the virulence plasmid contains a cluster of genes encoding the type III secreted effector molecules which includes the invasion plasmid antigens IpaA, IpaB, IpaC, and IpaD required for host cell invasion and escape from the phagosome (Menard *et al*., 1996; Menard *et al*., 1993). After the initial uptake by epithelial cells, as well as within macrophages, the Mxi-Spa T3SS is required for further interactions of *S. flexneri* with its host cells. During cell-to-cell spread the contact with the protrusion membrane likely triggers the type III secretion (Schroeder *et al*., 2007). Contact of pathogen with host cell activates the release of
Ipa’s onto the surface of host during pathogenesis (Watarai et al., 1995). It has been reported that addition of chemical compounds such as Congo red, Evans blue and direct orange induced the secretion of Ipa proteins in the absence of host cell contact (Bahrani et al., 1997). The secreted Ipa proteins (IpaA, IpaB, IpaC, IpaD) together with Lipopolysaccharide (LPS) are the dominant antigens in the humoral immune response during shigellosis (Hale 1991, Jennison 2004). Furthermore, ipgC which encodes a molecular chaperone that associates with IpaB and IpaC in the bacterial cytoplasm, is found immediately upstream of the ipa genes in this region. While not secreted, IpgC is required for bacterial entry by stabilizing IpaB and IpaC and preventing their premature association (Menard et al., 1994). It has been reported that IpaB, IpaC, and IpaD elicit actin rearrangements at the site of bacterial attachment that are required for entry, whereas IpaA targets β1 Integrins and Rho to promote the reorganization of these actin-rich structures (High et al., 1992; Demali et al., 2006). Studies shows that the IpaD carboxyl-terminal region may be involved in transport modulation, and the amino-terminal region would be involved in lysis and escape from host cell phagosomes (Turbfill et al., 1998).

It has been reviewed that Shigella IpaB is a specific inhibitor of the eukaryotic cell division cycle and colonizes the epithelial cells (Alto, 2007). IpaB is required for type III secretion as it localizes to the needle tip and forms pores in the host plasma membrane (Blocker et al., 1999). Guichon and colleagues previously investigated the regions of IpaB that are necessary for invasion, phagosome escape, caspase-1 binding and cytotoxicity. The S. flexneri IpaB gene was divided into three regions, the amino-terminal, the carboxyl-terminal, and the middle region, which possesses two important domains (α-helix and hydrophobic). The amino-terminal region is very important for the
expression and secretion of IpaB, while mutation in the carboxyl-terminal region do not affect these phenomena (Guichon et al., 2001). Hilbi et al., reported that Shigella invasin IpaB specifically binds to Caspase-1 and that this caspase is absolutely required for Shigella-induced apoptosis thus initiating the massive inflammation characteristic of shigellosis (Thirumalai et al., 1997; Hilbi et al., 1998). IpaB and IpaC are secreted at the host-pathogen interface following bacterial contact with a host cell. Studies reported that IpaB is the primary effector of host cell invasion by S. flexneri and that this protein is responsible for lysis of the resulting phagosomal membrane (High et al., 1992) Figure 2.4 describes the molecular pathogenesis of Shigella.

![Figure 2.4. Current scheme of Shigella pathogens](image)

**Figure 2.4. Current scheme of Shigella pathogens.** Rupture, invasion, and inflammatory destruction of the intestinal barrier through effector proteins (Sansonetti, 2001).

In this role, IpaB alone would provide S. flexneri with a direct route into the cytoplasm of host cells where it could divide and subsequently invade neighboring
cells. It has been also studied that the effector protein IpaB with its ion channel activity causes phagosomal destabilization and induces macrophage death thus escaping from phagosome and kill the host cells during infection (Senerovic et al., 2012). Two amino acids regions of IpaB aa 227 to 236 and aa 297 to 306 has been reported to be necessary for the maintenance of IpaB at the needle tip, secretion regulation, and normal pore formation regulating the type III secretion and removal of either of these regions leads to an inability to block secretion prior to reception of the activation signal and a defect in host cell sensing (Shen et al., 2010).

**Chromosomal Genes**

Various chromosomal loci are also involved in the pathogenesis of *Shigella* by complementing the core virulence of the invasion plasmid genes and are, therefore, needed for full virulence (Hale, 1991). A group of genes regulates the expression of the plasmid virulence genes. The virR gene encodes a histone-like molecule controlling the temperature-dependent expression of the Ipa and Mxi-Spa proteins, and the kerato -conjunctivitis provocation (kcpA) gene positively regulates virG (Maurelli and Sansonetti 1988; Sansonetti 2001). Another group of genes needed for bacterial survival in the intestine and for resisting host defense mechanisms, such as those encoding lipopolysaccharides (LPS) and siderophores. *Shigella* lacking complete LPS (“rough colonies”) are avirulent. Shiga toxin (Stx) is encoded chromosomally and found mainly in *S. dysenteriae* serotype 1, is a potent protein-synthesis inhibitor that targets primarily the vascular endothelium (Sandvig, 2001; Bitzan and Loo, 2003). This toxin mediates the severe complication of hemolytic-uremic syndrome and increases the severity of the diarrhea caused by *S. dysenteriae*
serotype 1 (Ray and Liu, 2001). *Shigella* spp. also produce two enterotoxins (60 kDa), ShET-1 and ShET-2. ShET-1 is produced mainly by *S. flexneri* serotype 2a, whereas ShET-2 is produced by all *Shigella* spp. (Vargas *et al*., 1999). These enterotoxins may contribute to the high-volume, watery diarrhea often seen in the initial stages of the disease. For the invasion of the epithelium the presence of all these regions of both the invasion plasmid and chromosomal regions are required (Hale, 1991; Sansonetti *et al*., 1983).

2.1.6. **Immune responses**

The ability to cross the epithelial lining, induce apoptosis of phagocytic cells, escape into the cytoplasm of epithelial cells and followed by cell-to-cell spread is the key determinant of the disease that elicited acute inflammatory reaction (Islam *et al*., 1997; Sansonetti *et al*., 1998; Sansonetti *et al*., 1999; Sansonetti *et al*., 2006; Phalipon & Sansonetti, 2007). In *Shigella* the humoral immunity response is largely due to its extracellular activity. IgA is secreted by IgA secreting plasma cells in the lamina propria of submucosal layers. It is then endocytosed through the polymeric immunoglobulin receptor (pIgR), which is then transported through the mucosal epithelial cells into the mucosal secretions as secretory IgA (sIgA). In serum, IgA exist predominantly in monomer form, whereas in mucosal secretions it is mostly in dimers form (two monomeric IgA units linked by a J chain), mainly in the form of sIgA (present with a secretory component) (Ogra *et al*., 2001). Studies have reported that the circulating IgA antibody secreting-cells (ASC) were significantly higher in the *S. flexneri* infected patients 5 to 7 days after the onset of the disease (Rasolofo-Razanamparany *et al*., 2001). Several studies have suggested that serum IgA
reactivity reflected that of intestinal IgA since these antibodies were derived from the same source (Oberhelman et al., 1991; Van De Verg et al., 1995).

LPS (O-antigen) is an important virulence factor of *Shigella*, as it is an endotoxin stimulating a strong inflammatory response in the host by activating immune cells, in particular macrophages, to release pro-inflammatory cytokines. It also enables *Shigella* to resist host defence systems such as opsonisation and phagocytosis (Lindberg et al., 1991). As the immune response to *Shigella* is O-antigen specific, after infection with a particular *Shigella* strain, the host then has protective immunity to all strains of the same serotype, but not to infection with other serotypes; therefore, the ability to change serotypes would confer a selective advantage to the invading organism (Schmidt and Hensel, 2004). A study showed that patients infected with *S. flexneri* had significantly higher serum IgA and IgG titres against LPS and Ipa proteins compared to the healthy controls (Minh et al., 1996). The highest serum IgA and IgG titres were seen on day 7 and day 14 after the onset of diarrhea, respectively (Oberhelman et al., 1991). Anti-LPS antibody response was observed following natural infection by *S. flexneri* 2a and 1a (Rasolofo-Razanamparany et al., 2001).

The involvement of the invasion plasmid antigens with the invasive phenotype indicates them as key antigens eliciting a protective immune response in primates and humans. Immunoblot analysis of convalescent serum from a rhesus monkey which had been challenged with *Shigellae* indicates that antibody recognizing invasion plasmid antigens viz., IpaA, IpaB, IpaC, IpaD were present after infection and recognized by serum IgG in this animal (Hale et al., 1985). Studies also characterized
the immune response against plasmid-coded antigens by ELISA and immunoblot analysis of sera from a large group of monkeys challenged with *S. flexneri* serotype 2a and also from acute and convalescent sera of humans infected with either *S. flexneri* or *S. sonnei*. These studies indicate that invasion plasmid antigens as the major antigens inducing a serum immune response during *Shigella* infection in the natural host (Oaks *et al.*, 1986). Studies described the increase of serum IgG and IgA subclass responses against *Shigella* LPS, Ipa, and Shiga toxin in *S. dysenteriae* and *S. flexneri* infected patients (Islam *et al.*, 1995).

A critical mediator of the proinflammatory response to *S. flexneri* is the cytokine interferon γ (IFN γ) which acts on a wide variety of cells types to regulate the expression of over 2,000 genes. The immunocompetent mice challenged with *S. flexneri* were able to clear the infection by 5 days post infection, while IFN γ deficient mice were unable to inhibit *S. flexneri* replication and eventually succumbed to the infection. Also primary mouse macrophages or rat L2 fibroblasts pre-treated with IFN γ prior to infection significantly inhibited *S. flexneri* growth compared to the untreated cells (Way *et al.*, 1998). It has been reported that IFN γ inhibited the cytosolic replication of *S. flexneri* through the cytoplasmic RNA sensor RIG-I (retinoic acid-inducible gene I) thus restricting the pathogen in the host cells (Jehl *et al.*, 2012). It has been studied that the children with shigellosis have a persistent activation of the innate immune response in the convalescent phase, indicating delayed elimination of *Shigella* antigens compared to adults (Raqib *et al.*, 2003). Increased production of IFN γ and IL-10 in response to Ipa antigens was observed in the volunteers orally inoculated with shiga toxin deleted *S. dysenteriae* type I strain and suggested that the type 1 responses play an important role in systemic and mucosal immunity to *S.*
*dysenteriae* type I (Samandari *et al.*, 2000; Sellge *et al.*, 2010). Studies revealed that mice after intranasal challenge with *Shigellae* developed pulmonary and serum IgG and IgA antibody recognizing both LPS and invasion plasmid antigens IpaB and IpaC and also developed significantly elevated levels of pulmonary IFN-γ and IL-4 indicating the local B lymphocyte activity, as well as Th1 and Th2 to the survival of immune mice (Van de verg *et al.*, 1995).

2.1.7. Diagnosis of Shigellosis

Clinically *Shigella* infection was identified by the presence of bloody and mucoid stool, but the differential diagnosis should include infection caused by enteroinvasive *Escherichia coli* (EIEC), *Salmonella enteritidis*, *Campylobacter* species and *Entamoeba histolytica* (Goodman & Segreti, 1999; Sur *et al.*, 2004). Usually, fresh and bright red blood is present in the stools of patients infected with *Shigella* (Niyogi, 2005). But in the laboratory diagnosis, stool culturing is the ideal method to detect the *Shigella* infection followed by biochemical and serological tests for further confirmation. *Shigella* was isolated from the fresh stool samples by culturing them in the MAC, DCA, XLD, HEK agar by incubating at 37°C for 18-24 hrs. The characteristics of the *Shigella* colonies were described in the section 3.1.1. Suspected colonies are subjected to biochemical screening medium such as MR test medium, Motility medium, Triple sugar iron agar, Kligler iron agar, Citrate agar and Urea agar. *Shigella* produces an alkaline slant and an acid but due to the inability to ferment lactose aerobically in the slope and the anaerobic fermentation of glucose in the butt and fail to produce H₂S gas. *Shigella* is negative for motility, citrate and urea test, and positive reaction is observed in the MR test. Serological identification was
done by slide agglutination test. Agglutination test is done using the polyvalent or monovalent O antigen grouping sera. Since *Shigella* contains distinct type of O antigen, *Shigella* polyvalent antiserum will agglutinate strains of the same serogroup and monovalent antiserum will agglutinate the specific serotype or sub-serotype.

Other diagnosis techniques include DNA based PCR techniques and immunological assays for rapid detection of infection. A multiplex PCR assays were developed by Aranda and colleagues using IpaH as probe. These PCRs were reported as specific and sensitive for rapid detection of target isolates in stools (Aranda et al., 2004; Na-Ubol *et al*., 2006; Gomez-Duarte *et al*., 2010). Studies also used DNA hybridization techniques, restriction endonuclease analysis for detecting *Shigella* (Boileau *et al*., 1984; Venkatesan *et al*., 1988; Litwin *et al*., 1991). Molecular epidemiology of multidrug resistant *S. sonnei* outbreak in a day care center has been studied using pulse field gel electrophoresis (PFGE) and plasmid DNA analysis (Brian *et al*., 1993). Immunoassays were employed for most rapid detection of the *Shigella* infection. Several immunoassays include latex agglutination, immunodiffusion and enzyme immunoassays (EIA) Mostly different methods of EIA were used. Monoclonal antibody-based ELISA has been developed using a 43 kDa invasion plasmid-coded protein antigen (IpaC) to identify EIEC and *Shigella* strains in faecal samples from children in Kuwait (Pal *et al*., 1997).

### 2.1.8. Treatment, prevention and control

*Treatment with antibiotics, antibiotic resistance*

In Shigellosis, dehydration is the major problem which is caused by loss of water in stools, increased evaporation through the skin due to fever and decreased
fluid intake due to anorexia. Oral rehydration therapy with a solution containing salts is sufficient and only severe cases need intravenous therapy. Continued feeding is rather encouraged preventing hypoglycaemia and weight loss because the disease does not affect the small intestine much where most of the absorption of nutrients takes place. Shigellosis can usually be treated with antibiotics which shortens the duration of diarrhoea, fever, and period of communicability. The antibiotics commonly used for such treatment are: ampicillin, trimethoprim/sulfamethoxazole (TMP-SMX, also known as Bactrim or Septra), nalidixic acid, or ciprofloxacin. The first choice of antimicrobial agent is ciprofloxacin and azithromycin (Khan et al, 1997), alternatively TMP/SMX and ampicillin (resistance is common in Middle East, Latin America) (Gilbert et al, 2001). Existence of multidrug resistant Shigella strains made the disease more worse. Shigella acquired resistance to sulfa drugs in the 1940s, to tetracycline and chloramphenicol in the 1950s, to ampicillin in the 1970s and to trimethoprim-sulfamethoxazole in the 1980s (WHO, 1997). Antibiotic resistance emerging among the S. dysenteriae 1 strains, which cause the most severe clinical features, mainly in Africa, South-Eastern Asia and South America is a major problem (Jamal et al 1998; Materu et al 1997; Taylor et al 1989; Hoge et al 1998). A study reports that strains of Shigella isolated during periods of infectious diarrhea in Abidjan from 2004 to 2005 showed resistance to ampicillin and tetracyclin (90%), sulphamamide (85%) and to a lesser extent to cotrimoxazole (65%) (Antoine et al., 2010). Also a multidrug resistant S. dysenteriae type I strain has been reported to cause sporadic outbreak in and around Kolkata in India (Dutta et al., 2003; Pazhani et al., 2004; Pazhani et al., 2008). Study from six asian countries shows that S. flexneri isolates were resistant to amoxicillin and cotrimoxazole. Ciprofloxacin-resistant S.
flexneri isolates were identified in China [6%], Pakistan [3%] and Vietnam [2%] (Seidlein et al., 2006). Emergence of ciprofloxacin resistance Shigella strains has been reported in India (Taneja, 2007). Therefore using antibiotics can actually make the bacteria more resistant.

The only way to prevent the spread of shigellosis is the sanitary handling of food. Special measures are required, like cooking food thoroughly rather than eating raw food, protecting food from flies, avoiding preparing food when ill with diarrhea or vomiting.

Vaccine development

There is an urgent need for a safe and efficacious vaccine against shigellosis due to severity of the disease, its fast spread and high mortality and morbidity rates with emerging antibiotic resistant strains. But still there is no reliable vaccines against this disease. The development of vaccine has been hampered by the ineffectiveness of parenterally injected inactivated whole-cell vaccines which led researchers to believe that serum antibodies do not confer immunity, the lack of a suitable animal model and the fact that there is only indirect evidence of immune mechanisms in humans (Passwell et al 2001; Lindberg and Pal 1993). The ideal vaccine against shigellosis would be multivalent, orally administered in a single dose, well tolerated, inducing high level and long term protection and would also be easy to manufacture. Candidate vaccines were under development and have now reached phase I, II or III clinical trials to evaluate their efficacy. At the meeting organised at the WHO in Geneva in 1996, priority was given to vaccine development against S. dysenteriae type 1, which is associated with the most severe cases of bacillary dysentery (WHO 1997).
Mel et al. attempted first to develop a live attenuated vaccine against shigellosis (Mel et al., 1965). The recent vaccine candidate strains contain genetically engineered deletions in key enzymes in the metabolic pathway, or in virulence genes or their combination. Two live oral candidate vaccines developed in the Centre for Vaccines Development in Baltimore, USA. S. flexneri 2a and S. sonnei vaccines have been evaluated in phase I clinical study (Kotloff et al., 2000; Kotloff et al., 2002). Another candidate using S. dysenteriae 1 WRSd1 showed protection against challenge in animal models (Venkatesan et al., 2002). One live oral S. flexneri 2a SC602 candidate attenuated in its intra and intercellular spread showed promising results in a phase I study (Phalipon and Sansonetti 1995; Coster et al., 1999). As a different approach, the O antigen gene clusters of LPS of S. dysenteriae 1 expressed in a live attenuated vector has been used in Germany (Tzschaschel et al., 1996). WRSs2 and WRSs3, a second-generation live, oral S. sonnei vaccine candidates primarily attenuated by the loss of the virG (icsA) gene has also been characterized (Kotloff et al., 2002; Barnoy et al., 2010). A live attenuated vaccine was reported to protect against disease caused by S. dysenteriae 1 against the toxic effects of other Shiga toxin 1-expressing pathogens (Wu et al., 2011). Recently, subunit vaccines have been constructed, in the form of conjugate vaccines, proteosomes or nucleoprotein vaccines. Parenteral conjugate vaccine based on S. sonnei developed by NIH in Israel was under phase II study (Passwell et al., 2001). A trivalent conjugate vaccine composed of the O-specific polysaccharide from S. flexneri 2a, S. sonnei and S. dysenteriae type-1 covalently bound to carrier proteins was also developed (Taylor et al., 1993).

Broad spectrum vaccines protecting against Shigella serotypes and subserotypes has also been developed by scientist at Center for Vaccine Development, University of Maryland (Noriega et al., 1999; Levine et al., 2007). Intranasal

Investigators also studied on the development of \textit{Shigella} invasion complex based vaccines. Intranasal administration of \textit{Shigella} invasion complex (Invaplex) containing the invasion plasmid antigens B and C (IpaB, IpaC) and LPS protected mice from pulmonary pneumonia and guinea pigs from keratoconjunctivitis after challenge with either \textit{S. flexneri} 2a or \textit{S. sonnei} (Turbyfill \textit{et al}., 2000; Oaks \textit{et al}., 2006; Turbyfill \textit{et al}., 2008). In a trial \textit{S. flexneri} 2a Invaplex 50 vaccine was reported to be safe, well-tolerated and induced robust levels of antigen-specific intestinal IgA and ASC responses in the volunteers (Riddle \textit{et al}., 2011). Recently, a broadly protective vaccine molecule consisting of dmLT with IpaB alone or IpaB combined with IpaD reported to fully protective against lethal pulmonary infection with \textit{S. flexneri} and \textit{S. sonnei} (Martinez Becerra \textit{et al}., 2012).

\section*{2.2. \textbf{HEAT SHOCK PROTEINS (HSPs)}}

\subsection*{2.2.1. HSPs as molecular chaperones}

Heat shock proteins (HSPs) are the phylogenetically conserved molecular chaperones in all prokaryotes and eukaryotes. Since they are chaperones, they maintain cell homeostasis and are essential for life (Kaufmann, 1990; Zugel and Kaufmann, 1999; Feder and Hofmann, 1999). These antigenic proteins are expressed
under various stressful conditions including pathological, environmental and physiological insults (Wu et al., 2006; Jaattela 1999). Major classes of HSPs include the small HSPs, HSP40, 60, 70, 90, and 110 families. HSPs are regarded as intracellular molecules, however upon necrotic but not apoptotic cell death, HSPs are released extracellularly in response to various stress conditions (Basu et al., 2000; Zugel and Kaufmann, 1999).

However in recent years, HSPs attracted the biologists due to their implication in stimulating both innate and adaptive immunity (Min-Fu Tsan et al., 2004; Basu and Srivastava, 2000). HSPs such as Hsp 60, Hsp 70 and Hsp 90 families were reported to be a potent activators of the innate immune system (Tsan et al., 2004 a, b; Basu and Srivastava, 2000; Srivastava et al., 2001). During bacterial infections HSPs, particularly Hsp 60 families are the most common target of humoral and cell-mediated immune responses in mammals (Kaufmann, 1990; Zugel and Kaufmann, 1999). HSPs of microbial pathogens appear to be involved in pathogenesis and host immune responses.

High degree of sequence homology of Hsps results in the presence of cross-reactive epitopes on different Hsps. For the host, frequent interaction with microbes results in the generation of an immunological memory for these cross-reactive determinants. As a result, an immune response to the conserved determinants shared by HSP is developed which will prevent further colonization of host by the microbes. These cross-reactivity to shared epitopes may cross-protect against different pathogens (Zugel and Kaufmann, 1999).
Figure 2.5: HSPs released from the necrotic cells.

Three factors explain the immunogenicity of HSPs, (i) they are highly conserved molecules, therefore the immune response against conserved epitopes of pathogens; (ii) easily recognized by the host’s immune system; (iii) during infection, their association to the released antigens forming the antigen-HSPs complex which is captured, processed and presented to the immune system resulting in an efficient response against the pathogen (Van Eden et al., 2005) (Figure 2.5).

2.2.2. HSPs as vaccine molecule

Although many vaccines have been highly successful in preventing several bacterial infections, still there exist an unmet need for a novel vaccine by its cost,
stability and efficacy. Many vaccines were effective, immunogenic and some are still under trials (Roy Curtiss, 2002). HSPs especially Hsp 60 and Hsp 70 act as immunodominant antigens of many pathogenic microorganism. In mycobacterial infections, Hsp 60 specific antibodies have been detected in the patients with tuberculosis and leprosy and also in mice after infection with *Mycobacterium tuberculosis* (Shinnick, 1991; Young *et al*., 1988). In Yersiniosis, development of Hsp 60 specific T cells immune response has been studied in mice (Noll *et al*., 1996). Increased antibodies to Hsp 70 has been studied in the sera of patients suffering from malaria, leishmaniasis, schistosomiasis, filariasis and candidiasis (Shinnick, 1991). Hsp 90 antibodies gave protection against candida albicans infection (Matthews and Burnie, 1992). Vaccination with GroES and GroEL homolog of *Helicobacter pylori* protects mice against gastroduodenal disease (Ferrero *et al*., 1995).

Studies also report the recombinant bacterial Hsps as protective and immunogenic molecules. Immunization of recombinant GroEL, DnaK and DnaJ of *S.Typhi* protected mice against *Salmonella* infection inducing both cellular and humoral immunity conferring 70-90% protection (Paliwal, *et al*., 2008; Sagi *et al*., 2006; Recombinant DnaJ of *Streptococcus pneumoniae* protected mice from the lethal infection *S. pneumoniae* (Khan *et al*., 2006). Recombinant GroEL of *Porphyromonas gingivalis* protected and reduced alveolar bone loss induced by multiple periodonto pathogenic bacteria in the rats (Lee *et al*., 2006). Also recombinant GroEL of *H. capsulatum*, GroEL and DnaK of *Piscirickettsia salmonis* were found to be immunogenic and protective in mice (Pinho *et al*., 2009; Gomez *et al*., 1995; Wilhelm *et al*., 2005). Intraperitonial immunization of recombinant Hsp 70 (DnaK) of *Salmonella* Typhi induced a predominant Th2 response and protective immunity in mice against lethal *Salmonella* infection (Paliwal *et al*., 2011). Our
previous study evaluated the immunogenicity and protective efficacy of recombinant Hsp60 of *Salmonella enterica* serovar Typhi against lethal challenge by *S. Typhi Ty2* and *Salmonella enterica* serovar *Typhimurium* in mice (Paliwal *et al.*, 2008). Due to the homology of HSPs between the species, these were considered as candidates causing autoimmune diseases and hence were thought to be a poor vaccine candidate (Elias *et al.*, 1990; Boog *et al.*, 1992; Abulafia-Lapid *et al.*, 2003; Puga yung *et al.*, 2009). However, most people don’t develop dangerous autoimmune responses to self-HSPs, although they do possess T-cells which recognize these self HSPs, suggesting that these cells are highly regulated (Rees *et al.*, 1988; Munk *et al.*, 1989; Schawartz, 1989). HSPs represent unique targets for γδ T cells and these cells are considered to contribute to the first line of defence. A minimal peptide of mycobacterial Hsp60 which is not homologous to the mammalian Hsp60 allows recognition by Hsp60 reactive γδT cells (Fu *et al.*, 1994). Further, detailed analysis of HSP induced immune responses in experimental model shows that reactivity to self-HSPs can down regulate the disease process rather than promoting the disease (Fu *et al.*, 1994; Graeff-Meeder *et al.*, 1995). Several other researchers have also reported the protective efficacy of microbial HSPs (Hsp60, Hsp70, Hsp90, small HSP) against respective pathogens *Yerisinia enterocolitica*, *Paracoccidioides brasiliensis*, *Leishmania infantum*, *Mycobacterium tuberculosis*, *Brugia malayi* inducing both arms of immunity, thus fulfilling the requirement of traditional vaccine (Noll *et al.*, 1996; Soares *et al.*, 2008; Carrillo *et al.*, 2008; Lowrei *et al.*, 1997; Dakshinamoorthy *et al.*, 2012).

### 2.2.3. HSPs as Adjuvant Molecule

A adjuvant molecule is used to enhance or modulate the immunogenicity of a less immunogenic vaccine antigen. It increases the immune response by delivering the
antigen presenting cells (APCs), promotes and enhances the activation of APCs by upregulating the costimulatory signals or MHC expression by inducing cytokine release, stimulates both humoral and cell mediated immunity (Gupta et al., 1993). Adjuvants currently licenced for human use includes alum, squalane oil/water emulsion, influenza virosomes, cytokines as IFN-γ, IL-2. Also some adjuvants were under study viz., DNA motifs, monophosphoryl lipid A, cholera toxin (CT), E.coli heat labile toxin (LT), Flt3 ligand, a glycoprotein, immunostimulating complexes (ISCOMs), liposomes, saponins and non-ionic block copolymers (Marx et al., 1993; Vogel et al., 1995).

HSPs are reported as the potent inducers of innate and adaptive immunity. HSPs function as danger signals priming multiple host defence pathways and these properties augment their use in vaccine development for infections (Todryk et al., 2000). HSPs play dual role in vaccine development against infectious diseases, pathogen-derived HSPs as vaccine antigens and host-pathogen derived HSPs as adjuvants (Zugel and Kaufmann, 1999). The immunomodulatory of HSPs are based on their interaction with APCs thereby stimulating chemokines production which attracts immunological cells, initiates innate immune response by activating dendritic cells (DC) and delivering the peptides to the MHC molecules to prime the adaptive immunity, therefore integrating innate and adaptive immune responses (Srivastava, 2002; Basu et al., 2002). It has also been reported that Hsp70 Like protein 1 activated Dendritic cells (DCs) indicating its potential as a novel adjuvant for use with peptide immunizations (Wan et al., 2004). Due to these above properties, HSPs has been exploited as a promising vaccine adjuvant for a broad spectrum of infections (Segal et al., 2006; Quintana and Cohen, 2005; Kurella et al., 2000). mHsp70 when fused or
co-delivered with protein antigen enhanced its potency (Li et al., 2006). Also kinceto plasmid membrane protein-11 fused to Hsp 70 elicited CTL response (Maranon et al., 2001). Many studies also reports the adjuvant property of HSPs (Rico et al., 1998; Wang et al., 2002; Lehner et al., 2004).