Cross-protective potential of live *Shigella* mutants lacking immunodominant antigens

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Abbreviations

AIDS	– acquired immunodeficiency syndrome
AR1 and AR2	– acid-resistance pathway 1 and 2
BAL	– bronchoalveolar lavage
CFU	– colony forming unit
Chr	- chromosome
CR TSA	– Congo red tryptic soy broth agar
CRN	– Congo red negative
CRP	– Congo red positive
ESBL	– extended spectrum β-lactamase
FAE	– follicular associated epithelium
FCS	– foetal calf serum
GEMS	– Global Enteric Multicenter Study
HIV	– human immunodeficiency virus
HUS	– haemolytic uremic syndrome
Ip	- invasion plasmid
Ipa	– invasion plasmid antigen
Kdo	– 2-keto-3-deoxy-D-mannooctulosonic acid
LB	– Luria Bertani broth
LD_{50}	– 50 % lethal dose
LPS	– lipopolysaccharide
Lpt pathway	- LPS transport pathway
M cells	– membranous epithelial cells
MDR	– multidrug resistant
MOI	– multiplicity of infection
MS	– mass spectrometry
MSD	– moderate-to-severe diarrhoea
NETs	– neutrophil extracellular traps
NHP	– non-human primates
OD	– optical density
OmpA	- outer membrane protein A
OmpC	- outer membrane protein C
PBS	– phosphate buffered saline

PEtN	– 2-aminoethylphosphate
p.i.	- post infection
PMN	– polymorphonuclear cell
R form	– rough LPS form
rEPA	– recombinant exoprotein A
S form	– smooth LPS form
SEM	– standard error of the mean
ShET-1 and ShET-2	– *Shigella* enterotoxin-1 and enterotoxin-2
SR form	– semi-rough form
ST-ETEC	– stable toxin producing enterotoxigenic *E. coli*
T3SS	– type 3 secretion system
TMP-SMX	– trimethoprim sulfomethoxazol
TSA	– tryptic soy broth agar
TSI	– triple sugar iron
WHO	– World Health Organization

1. Introduction

1.1. *Shigella* genus

1.1.1. Microbiological features

The genus *Shigella* belongs to the class of Gammaproteobacteria in the family Enterobacteriaceae. The bacterium was first identified as the causative agent of non-amoebic dysentery by Dr. Kiyoshi Shiga, during an outbreak in Japan, in 1898 and named as *Bacillus dysentery*. After several revision of the nomenclature the genus was termed as *Shigella* in the 3rd edition of Bergey's Manual of Determinative Bacteriology in 1930. *Shigella* has always been considered as closely related to *Escherichia coli*, traditionally the microbiological differentiation based only on the non-motility and the lack of lactose fermentation. However, current genomic analysis revealed that *Shigella* strains are forms of *E. coli*, and they do not even form a subgroup (1). Therefore there are attempts to reclassify *Shigella* as a pathotype of *E. coli* species (2).

The genus is traditionally divided into 4 species; *S. dysenteriae* (group A), *S. flexneri* (group B), *S. boydii* (group C) and *S. sonnei* (group D). The groups can be further classified into nearly 50 (sub)serotypes based on the structure of the O-antigen of the lipopolysaccharide (LPS) molecule.

The members of the genus are uncapsulated Gram-negative non-motile, non-sporulating rod-shaped facultative anaerobic bacteria, which do not produce H_2S in triple-sugar-iron (TSI) medium and are urease negative. *S. flexneri*, *S. dysenteriae* and *S. boydii* strains do not ferment lactose and except for *S. dysenteriae* they ferment mannitol.

1.1.2. Clinical manifestation

Shigella bacteria are highly human-adapted pathogens causing an invasive infection of the colon termed shigellosis or bacillary dysentery. The symptoms range from a short watery diarrhoea to the classical triad of dysentery with fever, tenesmus (intestinal cramps) and mucopurulent, bloody diarrhoea (3). The acute complications are often life-threatening, especially in children and in severe cases can lead to death due to dehydration, hypoglycaemia, intestinal perforation, toxic megacolon, peritonitis and Gram-negative sepsis (4). The chronic effect of the infection can be prolonged malnutrition and autoimmune

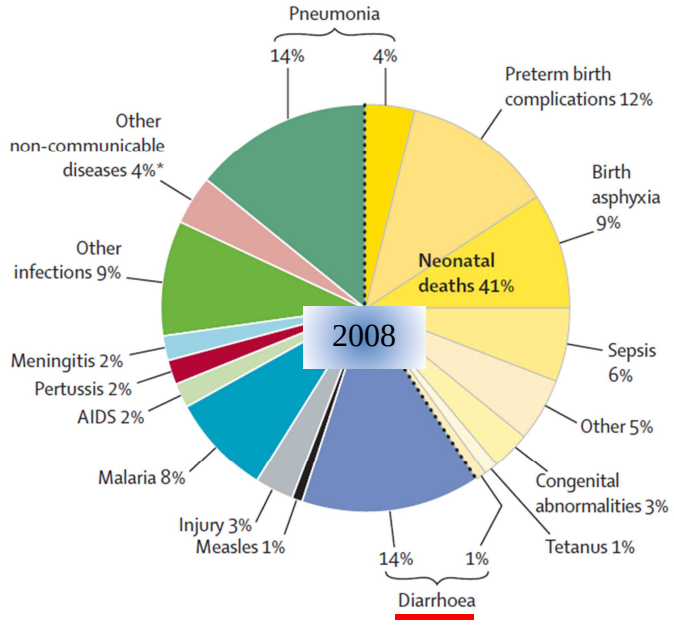
inflammatory bowel disease or irritable bowel syndrome, however the exact mechanism leading to the long term symptoms is not fully elucidated (5).

A distinct clinical manifestation is the haemolytic uremic syndrome (HUS) which occurs in 13% of infections due to the Shiga-toxin producing *S. dysenteriae* type 1. The symptoms cover the classical signs of haemolytic anaemia, thrombocytopenia and renal insufficiency. With a case-fatality rate of over 35%, it is the most severe complication of *S. dysenteriae* type 1 infections (6).

1.2. Epidemiology of bacillary dysentery

Diarrhoea is a leading cause of death among children under the age of five. According to the WHO 8.8 million children under five died in 2008, ~1.5 million of them because of diarrheal disease; more, than of TB and HIV/AIDS together (Fig. 1a) (7;8). The mortality of children younger than 5 years decreased to 7.6 million by 2010 (mainly due to reduction of lethal infections), however diarrheal diseases remained the second leading cause of children's mortality (Fig. 1b) (9).

a)



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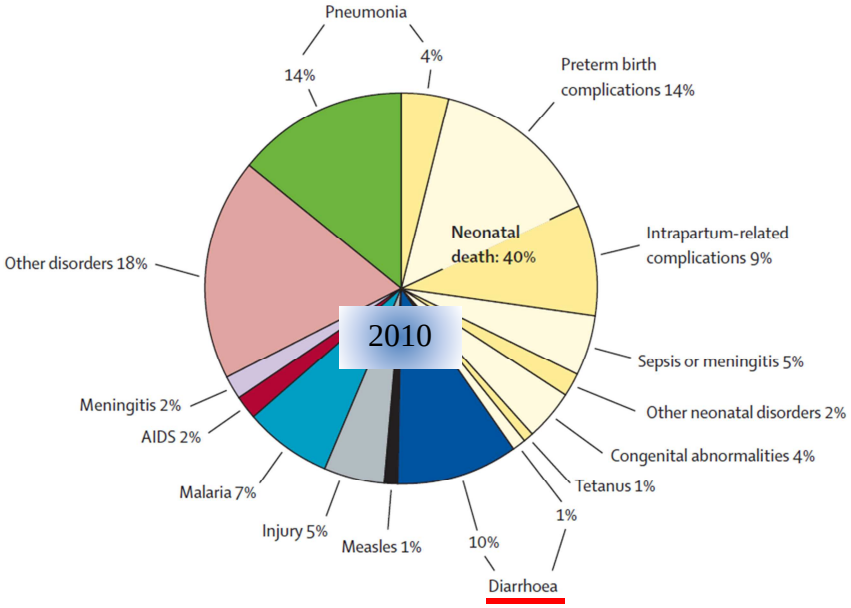


Figure 1 Distribution of mortality causes in children under 5 in a) 2008 (8) and b) 2010 (9) according to the WHO. Graph adapted from Black et al. and Hope et al. (8;9)

In 1999, *Shigella* was estimated to be responsible for ~113 million cases and approximately 1 million deaths annually, mostly under the age of five (10). Consequently roughly every second lethal diarrheal episode was caused by *Shigella* pathogens in children.

The latest study about the aetiology of moderate-to-severe diarrhoea (MSD) among children younger than 5 years in sub-Saharan Africa and south Asia (where 80% of the mortality occurs) was performed between 2007 and 2011 called the Global Enteric Multicenter Study, GEMS (11). The study enrolled close to 10,000 children with moderate-to-severe diarrhoea and revealed, that four pathogens are mainly associated with MSD; rotavirus,

Cryptosporidium, *Shigella* and stable toxin producing enterotoxigenic *E. coli* (ST-ETEC), with rotavirus causing the most cases in infants (0-11months) and toddlers (12-23 months), and *Shigella* being the second and first attributable factor among toddlers and 2-5-year-old children respectively. In this study *Shigella* was not associated with higher risk of dying in contrast to the results of previous studies in Kenya in 2005-2007, where children, with lethal diarrhoea were more likely to be infected with *Shigella* (12).

Although over 90% of shigellosis occurs in developing countries, *Shigella* infections are present in industrialized countries as well; children in day-care centres, immigrants, homosexual men and travellers to developing countries are infected mostly (13). Furthermore, due to the fact that minute inoculum is enough to cause disease, *Shigella* is considered as a potential biological weapon.

The distribution of *Shigella* groups responsible for the majority of shigellosis shows different pattern in developing and developed countries (Fig. 2).

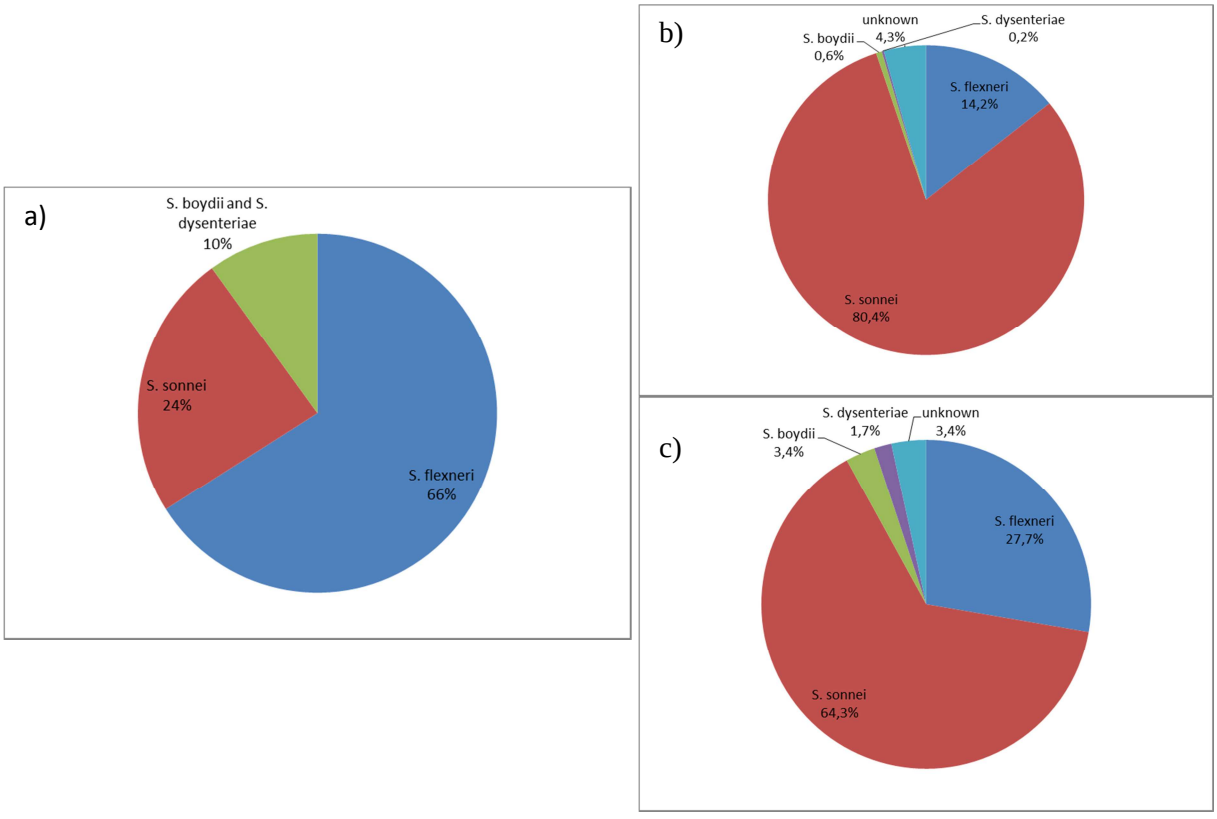


Figure 2 Distribution of serotypes among *Shigella* strains isolated in a) developed countries from children under the age of five (14) b) developed countries from non-travel-associated cases (15) c) developed countries from travel-associated cases (15)

S. flexneri is responsible for most endemic cases among children, as confirmed in the GEMS study, where *S. flexneri* was identified in 66% of MSD due to *Shigella*, while *S. sonnei* in 24% and *S. boydii* and *S. dysenteriae* collectively in 10% (Fig. 2 a). Four serotypes accounted for 65% of all dysenteric cases: *S. flexneri* 2a, 3a, 6 and *S. sonnei* (14). In contrast in industrialized countries, *S. sonnei* is by far the most commonly isolated species followed by *S. flexneri* (Fig. 2 b) (10;11;16). Among travellers, *S. sonnei* was identified in 64% of the cases, *S. flexneri* accouting for 27.7% of the imported *Shigella* infections in the United States in 2004-2009 (Fig. 2 c) (15). In this study *Shigella* was identified in 13% of all travel-associated enteric infections, as third major pathogen after *Campylobacter* (41.7%) and *Salmonella* (36.7%).

Interestingly, although *S. sonnei* was responsible for 80% of non-travel associated shigellosis in the United States and is in general the leading causative agent of the disease in industrialized countries, there is a shift towards *S. flexneri* in case of infections among homosexual men worldwide (17), with *S. flexneri* 3a responsible for an ongoing outbreak in the UK (18).

The geographically different and changing distribution of the dominant serotypes, as well as the lack of a single major O-type implies the necessity of a multivalent vaccine approach tailored to the target population (traveller's vaccine vs. vaccine for endemic regions) or preferentially a cross-protection eliciting vaccine protective against multiple serotypes and groups.

1.3. Pathomechanism

Shigellae are strictly human-adapted bacteria. Although non-human primates (NHP) can also develop bacillary dysentery, the infectious dose required for eliciting disease in NHP is multiple logs higher than that for humans. Shigellosis is feco-orally transmitted, the bacteria enter the host with contaminated water or food, however there are case reports about rare transmission routes, such as intrauterine infection (19). As little as 10-100 bacteria are sufficient for the manifestation of the disease (20) making dysentery an exceptionally contagious disease.

The low infectious dose is mainly due to the survival of the bacterium at the acidic pH of the stomach (21). There are three acid-resistance pathways described in *Shigella*, the acid-

resistance pathway 1 (AR1) is an acid–induced, glucose-repressed oxidative pathway, regulated by the RNA polymerase sigma factor RpoS (22). The AP2 is a glutamate-dependent pathway, induced by mild acid conditions. In *S. flexneri* strain 2457T a third oxidative mechanism was described recently, which is not repressed by the presence of glucose in the medium (23). The gene expression profile under acidic conditions revealed, that there are several acid-resistance genes and outer membrane porin proteins upregulated (ie. RpoS and OmpF) however most virulence genes are supressed at low pH (24).

By reaching the colon *Shigella* is proposed to disrupt the mucus layer on the luminal surface of the epithelium. Surface expressed serine proteases play an important role in this process (25). The invasion of the epithelial cells occurs through their basolateral surface (26). To cross the epithelial layer, *Shigella* can disrupt the tight-junctions (Fig. 3 route 1) (27) or exploit the transcytotic property of the membranous epithelial cells (M cells) (Fig. 3 route 2) (28) as well as the inflammatory response provoked (Fig. 3 route 3) (29).

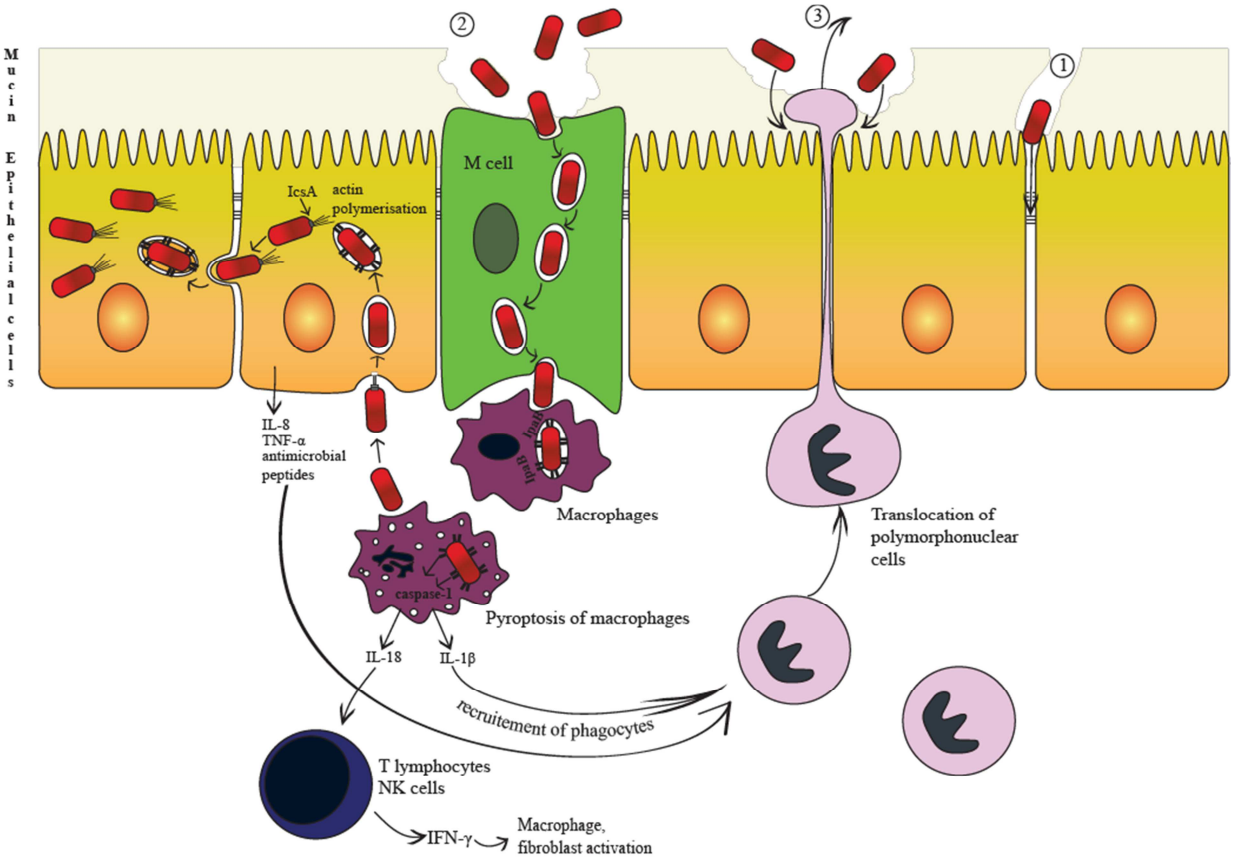


Figure 3 Schematic steps of the pathogenesis. Numbers encircled show the routes via *Shigella* reaches the subepithelium hence the basal membrane of the epithelial cells. Detailed description of the steps of pathogenesis can be found in the text.

M cells are able to transport antigens (as well as intact *Shigella* bacteria) from the intestinal lumen into the intraepithelial pocket of the follicular associated epithelium (FAE) filled with macrophages and lymphocytes. After being released the bacteria induce uptake by macrophages. *Shigella* lyses the phagosome of the macrophages due to the insertion of the invasion plasmid antigen B (IpaB) into the membrane vacuole (30). In the cytoplasm the bacteria release several bacterial antigens called pathogen-associated molecular patterns (PAMPs) and type 3 secretion system effectors, leading to the caspase-1 dependent pyroptosis of the macrophages and the production of IL-1β and IL-18 (31-33). IL-1 recruits polymorphonuclear cells (PMN-s) to the sub-mucosa, from where they migrate into the lumen in order to eliminate the bacteria. During the transmigration they disrupt the integrity of the epithelium, opening a new route for the luminal bacteria to reach the basolateral membrane of the epithelial cells (34). From the sub-mucosa, the bacteria adhere to the epithelial cells probably through a number of receptors. The α5β1 integrin can interact with invasion plasmid antigens (Ipa-s) (35), while the IpaB protein can bind to the CD44 receptor (36). The contact between the epithelial cell and the bacterium triggers the type three secretion apparatus (T3SS) encoded on the large virulence plasmid of *Shigella* (37). The secreted hydrophobic IpaC and IpaB proteins insert as a pore into the membrane of the host cell (38), and provide the entry of effector molecules into the cytoplasm. These effector molecules and the integrated IpaC protein interact with the cytoskeleton and lead to the internalisation of the bacterium in a macropinocytic vacuole (39-41). Similarly to the lysis of the phagosome this vacuole is lysed by IpaB and the bacterium is released into the cytosol, where it can replicate and spread to the neighbouring epithelial cells. The movement of this non-motile bacterium and the intercellular spread is achieved by the selective polymerisation of actin at the old pole of the bacterium (42). This step requires the expression and correct localisation of the outer membrane protein IcsA (VirG) (43;44) the latter being dependent on the structure of the LPS (45). By reaching the lateral membrane of the neighbouring cell, the bacterium is endocytosed. The surrounding membranes are rapidly lysed by the IpaB and IpaC proteins (46). This strategy involving several sophisticated virulence traits allows the bacterium to avoid being exposed to the extracellular environment.

1.3.1. Virulence factors

1.3.1.1. The endotoxin

1.3.1.1.1. Structure of the lipopolysaccharide

The lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. The molecule consist of the hydrophobic lipid component (lipid A) embedded in the outer leaflet of the outer membrane, while the polysaccharide part as a hydrophilic component is located on the surface of the bacteria. Based on structural diversity and genetic background the polysaccharide part can be divided into two structural domains; the core region, which is covalently linked to the lipid A, and the so called O-polysaccharide. The O-polysaccharide or O-antigen is a polymer of repeating subunits of 2-6 sugars and structural differences of the subunit provide the basic of the O-serotyping of the Gram-negative bacterial species. One single bacterium cell typically expresses on its surface lipid A-core molecules capped with no (rough form, R form), 1 (semi rough, SR form), 2 to up to 50 (smooth, S forms) O-antigen subunits (Fig. 4). Hence if purified LPS is separated electrophoretically, it typically forms a ladder like pattern, where the size difference of each ladder steps equals to the molecular weight of the O-antigen repeating unit.

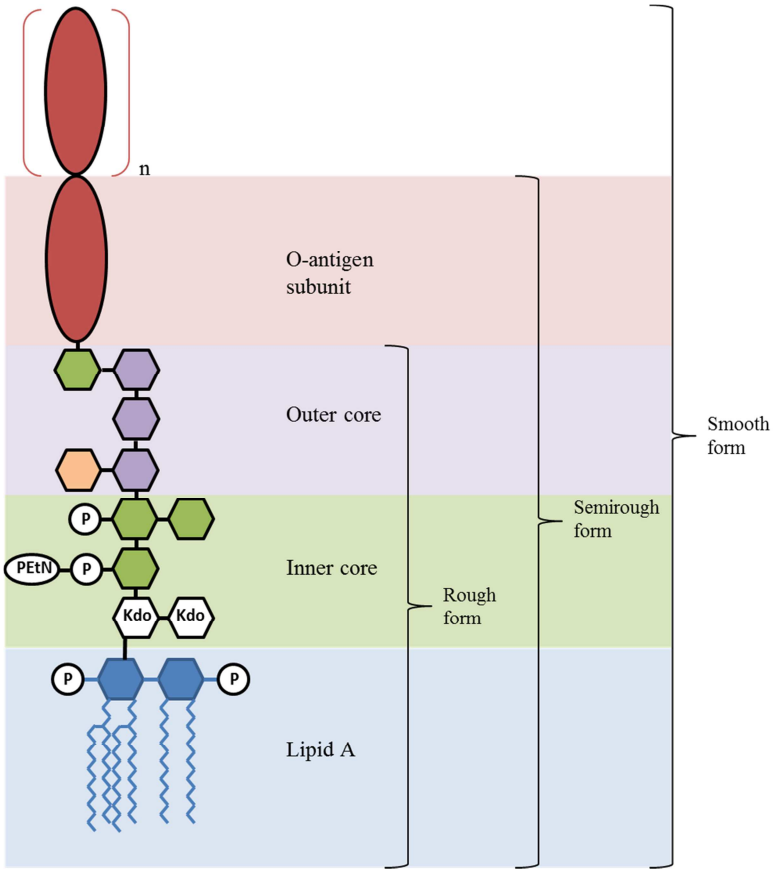


Figure 4 Schematic structure of the lipopolisaccharide

On a single Gram negative bacterium there are approximately 10^6 lipid A molecules (47). The chemical analysis of the lipid A part of *S. flexneri* 5a, X and *S. sonnei* revealed high similarity of the lipid moiety to that of *E. coli*. The sugar backbone of the lipid A consist β(1→ 6)-D-glucosamine dissacharide carrying two phosphate groups on the nonreducing glucosaminyl residues (48;49). The glucosamine disaccharide units are generally substituted by six fatty acids; lauric, myristic, 3-hydroxymyristic, Δ2-tetradecenoic and palmitic acids (49), two primary fatty acids (C14-OH) and two secondary ones (C14 and C12) in ester linkage and two primary C14-OH in amide linkage (50). The core oligosaccharide is linked to the O-6 position of the non-reducing glucosamine residue. However, recent studies revealed more heterogeneity within lipid A of *S. flexneri* variant X, whose diglucosamine backbone was shown to comprise non-stochiometric substitution by different number of acetyl and phosphoethanolamine groups. Moreover, variants with hepta-, penta- or tetra-acylated lipid A in *S. flexneri* strains were also described (51). Based on previous work of Kilár et al. at our institute, similar heterogenicity of the sugar backbone of lipid A was observed in *S. sonnei* (50).

The structure of the core region in several *Shigella* strains was found to be identical with the core types of *E. coli*, as described by Kontrohr et al. as well (52-54).
The fully conserved Lipid A- proximal portion (inner core) comprises of two 2-keto-3-deoxy-D-mannooctulosonic acid (Kdo) and three L-glycero-D-manno-heptose from which the first heptose is phosphorylated. In the different variants of the inner core (glycoforms) the phosphate group on the first heptose can be further decorated non-stochiometrically by 2-aminoethylphosphate (PEtN), the second heptose can be phosphorylated or the heptose in the third position can be glycosylated at the position 7 (Fig. 5) (55).

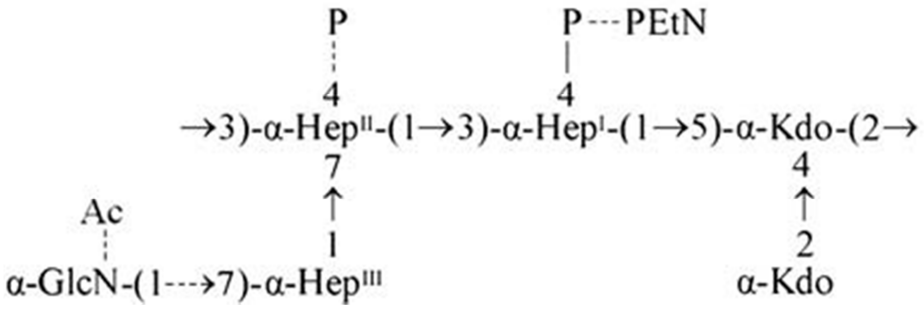


Figure 5 Structure of the inner core showing non-stochiometric modification sites (dashed lines)(figure adapted from Knirel et al. (55))

The outer core made up by the distal sugars shows moderate variability in *E. coli* resulting in 5 distinct outer core types; R1-R4 and K-12 (56). R1, R3 and R4 core types were also detected in *Shigella* and their distribution among the different *Shigella* serotypes is shown in Table 1.

Table 1 List of identified outer core types of *Shigella* serotypes

Strain	Core type	Reference
***S. boydii* 3**	R1	(55)
***S. boydii* 10**	R1	(55)
***S. boydii* 12**	R4	(55)
***S. boydii* 15**	R3	(55)
***S. boydii* 16**	R3b*	(55)
***S. boydii* 17**	R3	(55)
***S. dysenteriae* 1**	R4	(57)
***S. dysenteriae* 2**	R3	(55)
***S. dysenteriae* 3**	R1	(55)
***S. dysenteriae* 5**	R1	(55)
***S. dysenteriae* 6**	R1	(55)
***S. dysenteriae* 8**	R1	(55)
***S. dysenteriae* 9**	R1	(55)
***S. dysenteriae* 10**	R4	(55)
***S. dysenteriae* 13**	R1	(55)
S. flexneri 1	R3	(55)
***S. flexneri* 2a**	R3	(58)
S. flexneri 3	R3	(55)
S. flexneri 4	R3	(55)
S. flexneri 5b	R3	(59)
***S. flexneri* 6**	R1	(53)
S. flexneri X	R3	(55)
S. flexneri Y	R3	(55)
S. sonnei	R1	(60) (52)

NOTE * new core type identified in *S. boydii* 16

A fourth outer core was recently described in *S. boydii* 16 (55), which shares the backbone of the linear tetra-hexose of R3 (Fig. 6 a), but has a αddHep disaccharide extension on the same glucose group which provides the attachment site for the O-antigen polysaccharide (Fig. 6 b). Unlike the O-polysaccharide, the core structure has not been elucidated in all the different *Shigella* serotypes, therefore new core types could be described in the future.

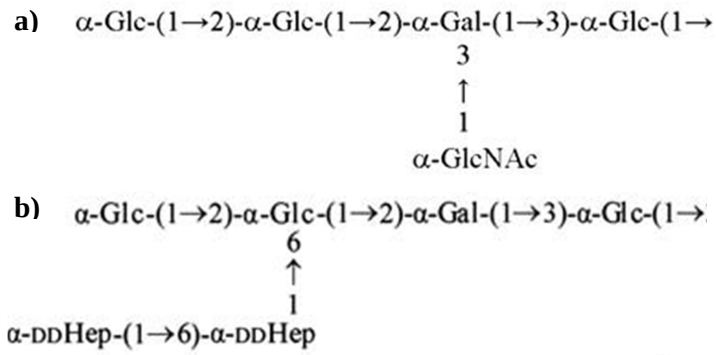


Figure 6 Structure of the outer core of a) R3 core of *S. flexneri* 2a, b) newly described R3b core of *S. boydii* 16 (structure adapted from Müller-Loennies et al. (61) and Knirel et al. (55))

The outermost portion of the LPS, the O-antigen is the most diverse surface moiety of Gram-negative bacteria. Currently there are close to 50 (sub)serotypes described in the *Shigella* genus; 16 for *S. flexneri*, 18 for *S. boydii*, one for *S. sonnei* and 13 serotypes for *S. dysenteriae* (62), but the number of the (sub)serotypes are constantly increasing.

With respect to *S. flexneri* O-antigens, all serotypes except type 6 share a common tetrasaccharide backbone made of one N-acetyl-d-glucosamine and three l-rhamnose residues. This basic subunit is recognized as type Y (Fig. 7). The immunological difference between the certain *S. flexneri* serotypes originates from decoration of this backbone. O-acetylation or α-D-glucopyranosyl attachment to the sugar backbone is encoded by bacteriophages (lysogenic conversion), while the newly described modification by phosphoethanolamin transferase that attaches PEtN group to one of the rhamnose residues in *S. flexneri* Xv, Yv and 4v (63) is plasmid encoded.

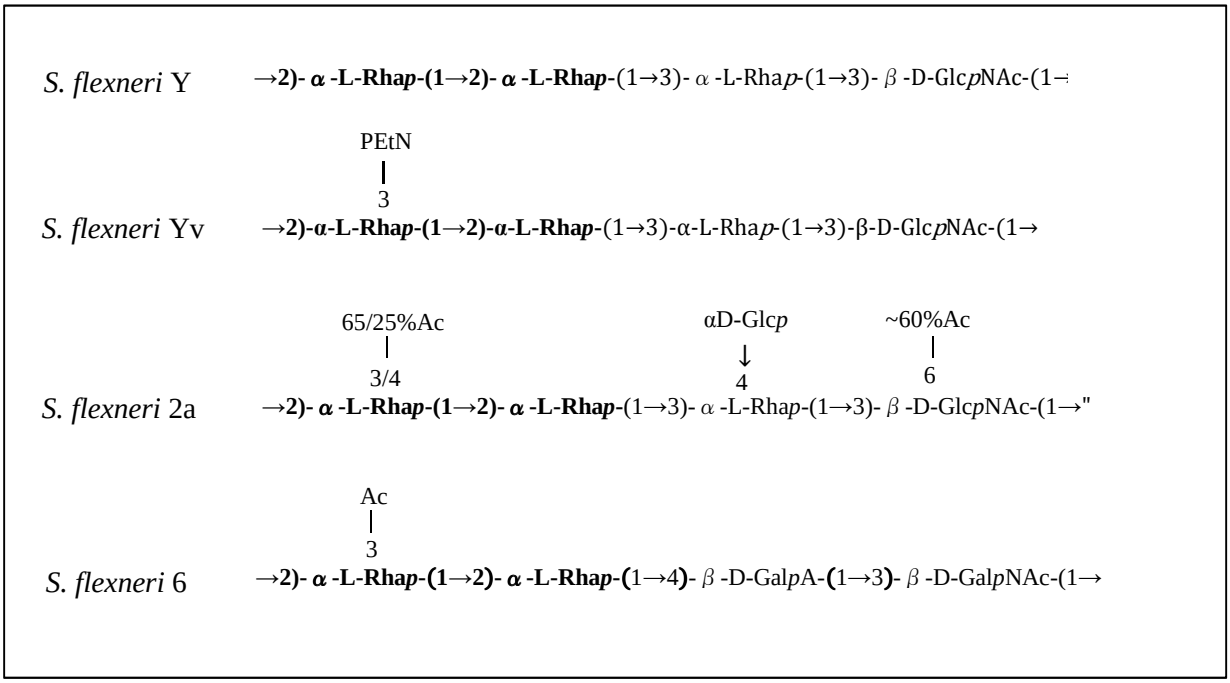


Figure 7 Structure of O-polysaccharide of *S. flexneri* Y, Yv, 2a and 6. Structural part responsible for serological cross-reaction is shown with boldface.

The repeating unit of *S. flexneri* serotype 6 is completely different with respect to the sugar composition. It is a tetrasaccharide of 2 rhamnoses, one galacturonic acid and one N-acetyl-galactosamine substituted with a single O-acetyl group on one rhamnose residue. The two rhamnose residues may provide the basis of the weak cross-reactivity of *S. flexneri* 6 with *S. flexneri* Y (Fig. 7).

S. boydii and *S. dysenteriae* are divided into 18 and 13 serotypes, respectively, and each of them has a distinct structure. Several serotypes show cross-reactivity with other shigellae (*S. boydii* 10 and 6 or *S. dysenteriae* 2 and *S. boydii* 15) or with *E. coli* (21 O-antigens are identical or highly similar to *E. coli* O-antigens resulting in serological cross-reaction). The structure of the O-antigens as well as the serological relationship is reviewed by Liu et al. (62).

The species *S. sonnei* bears a single serotype. The O-polysaccharide expressing smooth form is also designated as phase I or type I variant, whereas the O-antigen lacking rough form is the phase II or form II variant. The O-antigen of phase I is a unique structure, built by disaccharide of N-acetyl-L-altrosaminuronic acid and 4-amino-4-deoxy-N-acetyl-D-fucosamine residues, neither of the two sugars found in any of the described O-antigens of *E. coli*, but identical to the O-antigen structure of *Plesiomonas shigelloides* (Fig. 8).

$$\rightarrow 4) - \alpha - L - \text{Alt}p\text{NAcA} - (1 \rightarrow 3) - \beta - D - \text{Fuc}p\text{NAc4N} - (1 \rightarrow$$

Figure 8 Structure of O-subunit of *S. sonnei*

1.3.1.1.2. Synthesis of lipopolysaccharide

The lipid A molecules substituted with Kdo are required for the growth of *E. coli* and most of the Gram negative bacteria (47). The biosynthetic pathway of lipid A is best described in *E. coli* and as the enzymes are considered being highly conserved (64), does not essentially differs from the pathway in *Shigella*. The constitutive enzymes required for the synthesis of lipid A molecules are either in the cytoplasm as soluble (LpxA, LpxC and LpxD) or as peripheral membrane proteins (LpxH and LpxB) or on the inner membrane (LpxK, waaA, LpxL and LpxM) (64). The complete lipid A-Kdo molecule serves as the acceptor for the sequencial transfer of sugar molecules by the glycosyltransferases synthetizing the core oligosaccharide. These membrane associated glycosyltransferases and the modification enzymes are encoded in the *waa* (formerly known as *rfa*) cluster on the chromosome. The region consists of three operons between the genes *cysE* and *pyrE*. The first three genes encode enzymes responsible for the synthesis and transfer of L,D-heptose of the inner core. The central operon contains the genes responsible for the synthesis of the outer core as well as for the phosphorylation and for the attachment of the side-branch heptose of the inner core. The last gene in the cluster, *waaA* attaches Kdo to the lipid A molecule. The assembled core capped lipid A molecule is flipped to the periplasmic side by an ABC transporter protein MsbA (64) where the attachment of the O-polysaccharide and further extra-cytoplasmic modifications of the lipid A moiety occur. The exact lipid A synthesis pathway is reviewed by Raetz et al. (64), the assembly of the core region in E. coli is summarized by Muller-Loennies (56).

The genes involved in the synthesis of novel sugar nucleotides, encoding glycosyltransferases and enzymes required for the assembly and export of the O-antigen in *Shigella* (except in *S. sonnei*) are encoded in the *wbb* (also known as *rfb*) cluster between genes *galF* and *gnd*. The size of the cluster ranges from 9081 (*S. boydii* 14) to 17769 bp (*S. dysenteriae* 5) (62). In *Shigella* all the different rfb clusters have been sequenced and published by Liu et al. (62).

The GC content of the genes within the cluster – except for certain sugar synthesis genes – is lower than that of the genom; 30% compared to an average of 50% which suggests that these genes originate from another species and were acquired with lateral transfer during the evolution of *Shigella*. The transport and polymerization of the O-antigen follows the Wzx/Wzy dependent pathway (Fig. 9). The O-antigen subunits are synthesized on an undecaprenyl phosphate carrier (und-P) by specific glycosyltransferases at the cytoplasmic face of the inner membrane. In *Shigella* this is initiated by the transfer of GalNAc-1-phosphate or GlcNAc-1-phosphate onto und-P by the WecA protein forming GalNAc or GlcNAc und-PP (65). Subsequently individual sugars from their dinucleotide precursors are sequentially transferred to this carrier by specific sugar transferases. The assembled O-subunit und-PP is flipped to the periplasmic face by the Wzx/RfbX where it is polymerized by Wzy/Rfc (66;67). This means the transfer of the nascent O-subunit to the reducing end of another O-subunit-und-PP molecule resulting in a polymer of the O-antigen on und-PP. The length of the polymer is controlled by the chain length determinator protein Wzz resulting in the strain specific modal distribution of the O-antigen lengths. The polymer is ligated to the core-lipid A molecule by O-antigen ligase WaaL. Wzy is highly specific for the certain O-antigen serotype, whereas the presence of the specific WaaL protein – encoded within the *waa/rfa* operon - depends on the outer core structure (attachment site of the O-antigen).

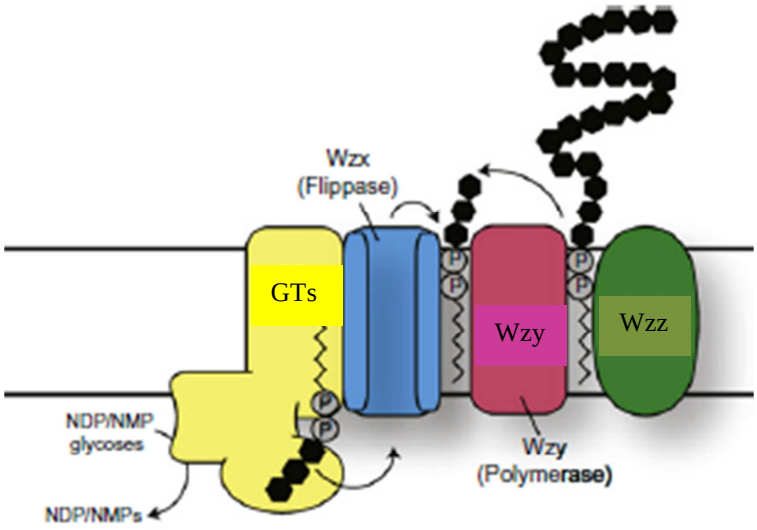


Figure 9 Wzy dependent synthesis of O-antigen. The individual O-antigen subunits are synthesized on an und-PP carrier on the cytoplasmic leaflet of the inner membrane by specific glycosyltransferases (GTs). These subunit-und-PP molecules are flipped to the periplasmid face by the Wzx flippase protein where polymerization occures. The Wzy polymerase transfers nascent O-subunits to the reducing end of another subunit bound to the und-PP carrier. The lenght of the polymer is regulated by the Wzz. Figure adapted from Greenfield et al. (68)

The synthesis of LPS results in core-capped lipid A (rough form), lipid A-core with one O-antigen subunit (semi-rough form) and smooth LPS with different length of O-polymers located at the periplasmic site of the inner membrane. The transfer of these molecules to the outer membrane has been elucidated recently and called as Lpt (LPS transport) pathway (69). The process involves at least seven Lpt proteins (LptA-G) and further four proteins with unknown functions (YftN, YfgH, YceK and YhjD) (70). These proteins are proposed to form a complex that reaches through the periplasm and can deliver LPS to the outer membrane (71) (Fig. 10).

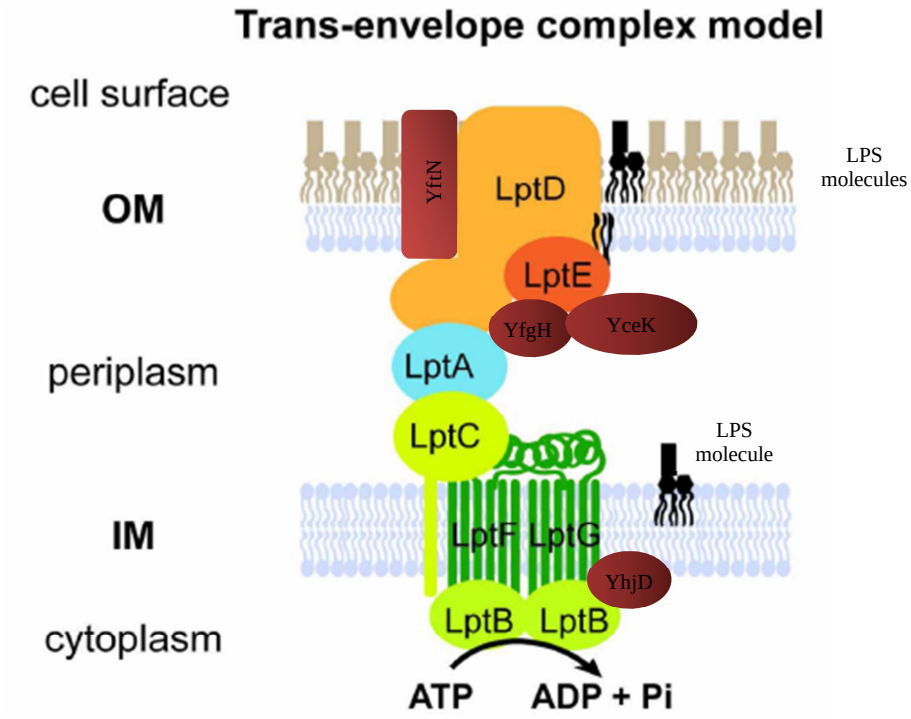


Figure 10 Transport of synthesized LPS molecules from the inner membrane to the outer membrane through the Lpt pathway. Figure shows localization of the seven Lpt proteins as well as the four unannotated proteins involved in the delivery of LPS molecules through the periplasm (figure adapted from Chng et al. (71))

In *S. flexneri* 1-5 the O-antigen comprises a common sugar backbone (see in 1.3.1.1.1). The serological difference between the serotypes is due to acetylation and glucosylation of the backbone at different positions (Fig. 11).

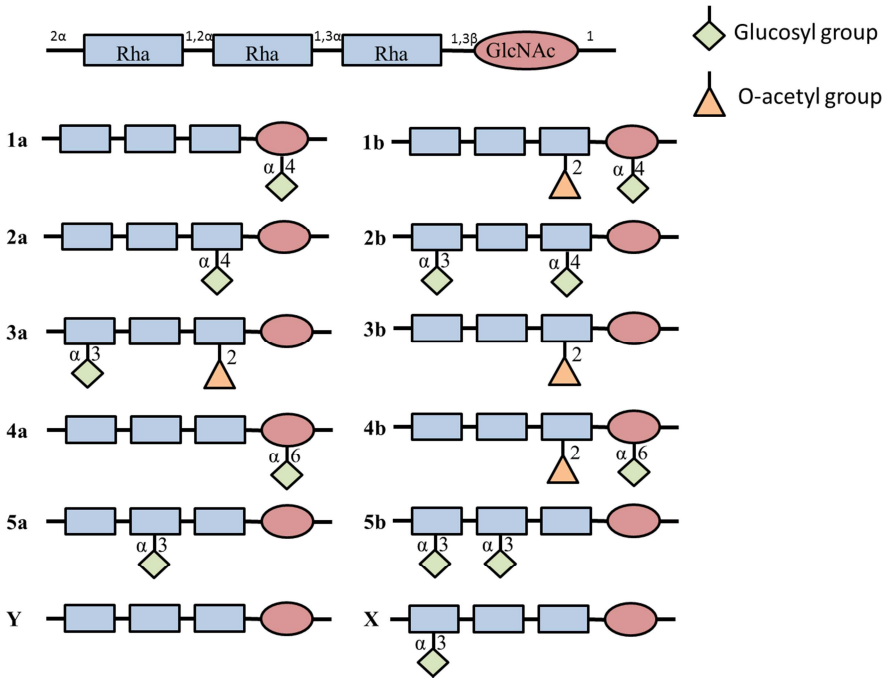


Figure 11 O-antigen structure of the different *S. flexneri* serotypes. The common O-antigen backbone is shown in the top row comprising rhamnose (Rha)-rhamnose (Rha)-rhamnose (Rha)-N-acetylglucosamine (GlcNAc) tetrasaccharide. The serotypes differ in the modification of the tetrasaccharide backbone by glucosylation or O-acetylation encoded by temperate bacteriophages.

These modifications are encoded by temperate bacteriophages (72). The phages are likely to integrate to the same site in the chromosome in the pro-lac region (73), where the modification genes are immediately downstream of the phage *attP* site. The glucosylation of the O-antigen is encoded by three genes, the product of *gtrA* and *gtrB* is highly conserved among the different serotypes (74-76), however the third gene is unique to each serotype and encodes the serotype specific glucosyl transferase. GtrB is proposed to catalyse the synthesis of und-P-glucose in the cytoplasm, which is then flipped to the periplasmic face by the GtrA protein. There the specific product of the third gene encodes the transfer of the glucose to the O-antigen subunit (72).

The second type of modification of the O-antigen results in the highly immunodominant O-acetyl group attachment to the tetrasaccharide unit. The O-acetyltransferase protein, Oac most likely acts at the cytoplasmic site of the inner membrane, still during the synthesis of the O-subunit (77).

S. sonnei differes from the other *Shigella* serotypes, as the *rfb* locus on the chromosome was deleted, and the genes involved in the O-antigen synthesis are encoded on the virulence plasmid (78) showing high level of similarity to the *rfb* genes of *Plesiomonas shigelloides.*

The length of the O polymer in *S. flexneri* 2a is regulated by two proteins; the Wzz_{SF} encoded on the chromosome is responsible for the short (11-17 repeating unit) LPS molecules (79), whereas the product of wzz_{SL} located on a small plasmid, pHS-2 results in "very long" (up to 90-100 subunits) LPS (80) unique for *S. flexneri* 2a.

1.3.1.1.3. Role of O-antigen in pathogenesis

The observation, that lack of the O-antigen results in an avirulent (in animal model) rough mutant of *Shigella* triggered extensive studies to elucidate the role of O-antigen in the pathogenesis of shigellosis (81). Although the truncation of the O-antigen did not abolish the invasiveness of *S. flexneri* (82), it was found to prevent intercellular spread, leading to the identification of O-antigen as a necessary factor for the correct localisation of the IcsA/VirG (45). Furthermore similarly to certain *E. coli* strains (83), LPS, particularly O-antigen has been shown to play a role in serum resistance; *S. flexneri* 2a strains expressing only shorter LPS forms (VS) showed higher sensitivity to complement mediated killing, which was further pronounced in rough mutants (82).

Recently, in vivo modification of the LPS molecules have been identified. To allow direct contact of the type III secretion system (T3SS) with the host cell, *S. flexneri* can glucosylate the O-antigen due to a phage conversion resulting in conformational changes of the O-polymer. Due to these changes, the LPS molecules will shorten by half revealing the tip of the T3SS (84). This modification improves invasion of the bacterium, however makes it more vulnerable for humoral factors, as complement. Furthermore it was shown, that low magnesium level in the growth medium leads to the overexpression of MsbB2 in *S. flexneri*, which acetylates lipid A, conferring increased resistance to antimicrobial peptides (85). Therefore, O-antigen length is considered as a "sword and shield", which has to be strictly regulated at various stages of the pathogenesis.

1.3.1.2. Exotoxins

Although the most typical clinical manifestation of *Shigella* infection is the bloody, mucous diarrhoea, called dysentery, most patients infected with *Shigella* present in the first phase of infection with watery diarrhoea, followed or not at all followed by dysentery. This observation led to the identification of two enterotoxins called enterotoxin-1 (ShET-1) and enterotoxin-2 (ShET-2)(86;87).

Enterotoxin-1 is a 55kD holotoxin, following the typical structure of the enterotoxins with a hypothesized configuration of A_1-B_5. Subunit A, and B have a size of 20kD and 7kD, respectively. The genes of the subunits (*set1A* and *set1B*) are located on the chromosome, separated by only 6bp (86). The toxin is expressed under iron-depleted conditions and leads to fluid accumulation in isolated rabbit ileal loops (86;88).

ShET-2 was first described in enteroinvasive *E. coli* (87). The 63,1kD protein was found in *Shigella* isolates as well, including members of all four species. It is encoded on the large virulence plasmid by the gene *sen*. The toxin is secreted by the type III secretion system (T3SS), which explain the lack of the signal peptide on the Sen protein (89). Beside its enterotoxic effect, ShET-2 plays a role in the formation of the inflammatory response against *Shigella* as a *sen* mutant provoked significantly less IL-8 production in *in vitro* assays compared to its wild-type parental strain (89).

The prevalence of the enterotoxins in clinical isolates of *Shigella* revealed that ShET-1 is only produced by *S. flexneri* 2a and 2b strains, whereas the *sen* gene is distributed equally among the four different species. Studies involving isolates from several geographical locations identified the *set* gene in 100% of *S. flexneri* 2a and 2b isolates (90-92). The elucidation of the real prevalence of *sen* is hindered by the rapid loss of the virulence plasmid during the storage and several subculturing of the clinical isolates. In studies involving older isolates, which probably underwent numerous culturing steps, the prevalence of *sen* ranges between 49.1-56% (90;92;93), whereas in the study of Yavzori et al., lacking prolonged storage of clinical isolates, all but some *S. flexneri* 1b strains carried the responsible sen gene. This study suggests that probably most if not all clinical isolates carry the *sen* gene encoding ShET-2, and the low detection rate is only due to the loss of the large virulence plasmid. Interestingly the vaccine strain T32 Istrati (see later) was shown to lack the *sen* gene (91).

A few years after the identification of *Shigella*, as a causative agent of bacterial dysentery, the presence of a “neurotoxin” in the autolysates from *S. dysenteriae* was demonstrated by Conradi et al.. Similar toxins were identified later in *E. coli* strains causing haemorrhagic colitis and haemolytic uremic syndrome and the toxins were referred to belong to the family Shiga toxins. In shigellae the Shiga toxin is produced nearly exclusively by *S. dysenteriae* 1 (94). The toxin has the characteristic structure of AB_5, where the A subunit is the enzymatically active moiety, and the B subunits provide the binding to the toxin receptor glycosphingolipid Gp3 (95). After binding to its receptor the toxin is endocytosed and

transported retrogradely to the endoplasmic reticulum, where the catalytically active cleaved fragment of the A subunit is translocated to the cytosol. The exact mechanism of the process is summarized in the review of Bergan et al (96). The active moiety of the toxin inhibits protein synthesis in the target cell by the inactivation of the 60S ribosomal subunit (97). Additionally the toxin induces apoptosis in several types of cells (98). The toxins belonging to the family typically are encoded by genes located on lambdoid bacteriophages, called Stx-phages. In *S. dysenteriae* 1 the phage is defective, due to loss of essential structural phage genes (99).

1.3.1.3. Type III secretion system

Beside LPS, the major virulence determinant of pathogenic *Shigella* bacteria is the ~200kb large virulence plasmid or invasion plasmid. The sequencing of the plasmid from different *Shigella* strains indicates the presence of roughly 100 genes, and proves that one-third of the plasmid is composed of IS elements (100-102). A 31-kb segment of the plasmid was shown to be necessary and sufficient for the invasion of the epithelial cells (103), hence was termed the "entry region". The 34 genes of the region are organised into 2 clusters (10 and 24 genes), transcribed in opposite directions (Fig. 12) (100).

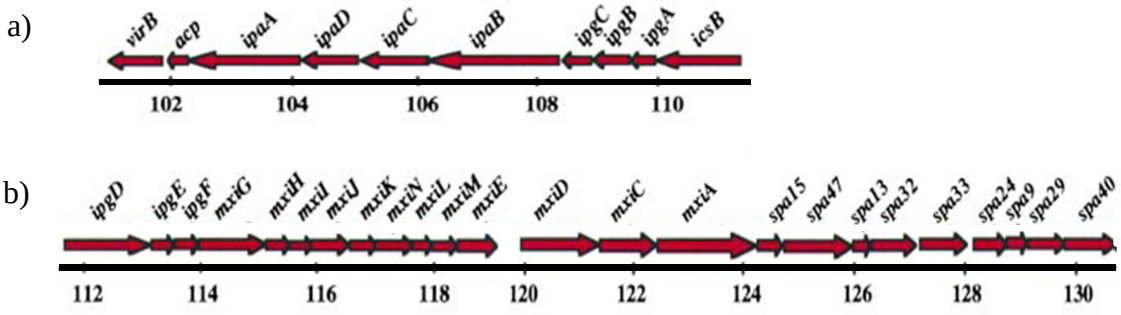


Figure 12 Genes of the „entry region" of pWR100 a) region between *icsB* and *virB*, b) cluster between *ipgD* and *spa40*. Numbers refer to the location of the genes regard to replicational origo. Figure adapted from Buchrieser et al. (100)

The operons containing the 24 genes known as the *mxi-spa* locus encode the type 3 secretion system (T3SS), responsible for the secretion of effector proteins into host cells. The second operon encodes effectors injected by the T3SS, translocator proteins regulating the secretion of other effectors and chaperons stabilizing proteins secreted by the T3SS in the cytoplasm of

the bacteria. There are several further effectors encoded outside of the entry region on the virulence plasmid or - in case of the up to 7 IpaH alleles - even on the chromosome (104). These effectors mainly modulate the immune response provoked by the bacterium (105). Most of the effector molecules are listed in Table 2 with their role in the pathogenesis.

Table 2 Role of T3SS effector proteins (106;107)

	Effector proteins	**Role**
Chaperons	IpgA	IcsB stabilization
	IpgE	IpgD chaperon
	Spa15	chaperon of IpaA, IpgB1, IpgB2, OspC3, OspC2, OspD1, OspC1
	OspD1	MxiE anti-activator
	IpgC	IpaC, IpaB binding in cytoplasm
	IpaD	N-terminal domain is an intramolecular chaperon
Entry locus	IpaC	pore formation, actin nucleation, ruffle formation
	IpaB	pore formation, CD44 interaction, invasion
	IpaA	actin disassembly in host cell
Secreted effectors	IcsB	prevention of autophagy through Atg5 binding
	IpgB1, IpgB2	manipulation of actin polymerisation in host cell
	IpgD	inhibition of chemokine induced T cell migration
	OspF, OspC1	modulation of MAPK pathway
	OspB	inhibition of inflammatory cytokine production
	VirA	microtubular reorganization in host cell
	OspE	epithelial detachment suppression
	OspG, IpaH	suppression of NFκB pathway through altering ubiquitination (innate immune response)
	OspZ	inhibition of NFκB activation (innate immune response)

1.3.1.3.1. The assembly and structure of the T3SS (the *mxi-spa* locus)

For targeting host cell signalling, bacteria have to transport effectors through 3 membranes; the inner and outer membranes of Gram negative bacteria and the membrane of the host cell. The type 3 secretion system of *Shigella* provides a one-step translocation system for these proteins by the needle-like structure of the complex. T3SS can be found in over 25 species, with a closely related structure to the flagellar T3SS (108). Among these pathogens the secretion system of *Shigella* shows high similarity to the T3SS of *Yersinia*, enteropathogenic *E. coli* and *Salmonella* (109).

The multiprotein system consist of the basal body extending through the inner-membrane, periplasm and outer membrane of the bacterium, and of a ~500Å long needle with a central 2-3nm diameter channel (Fig. 13) (110).

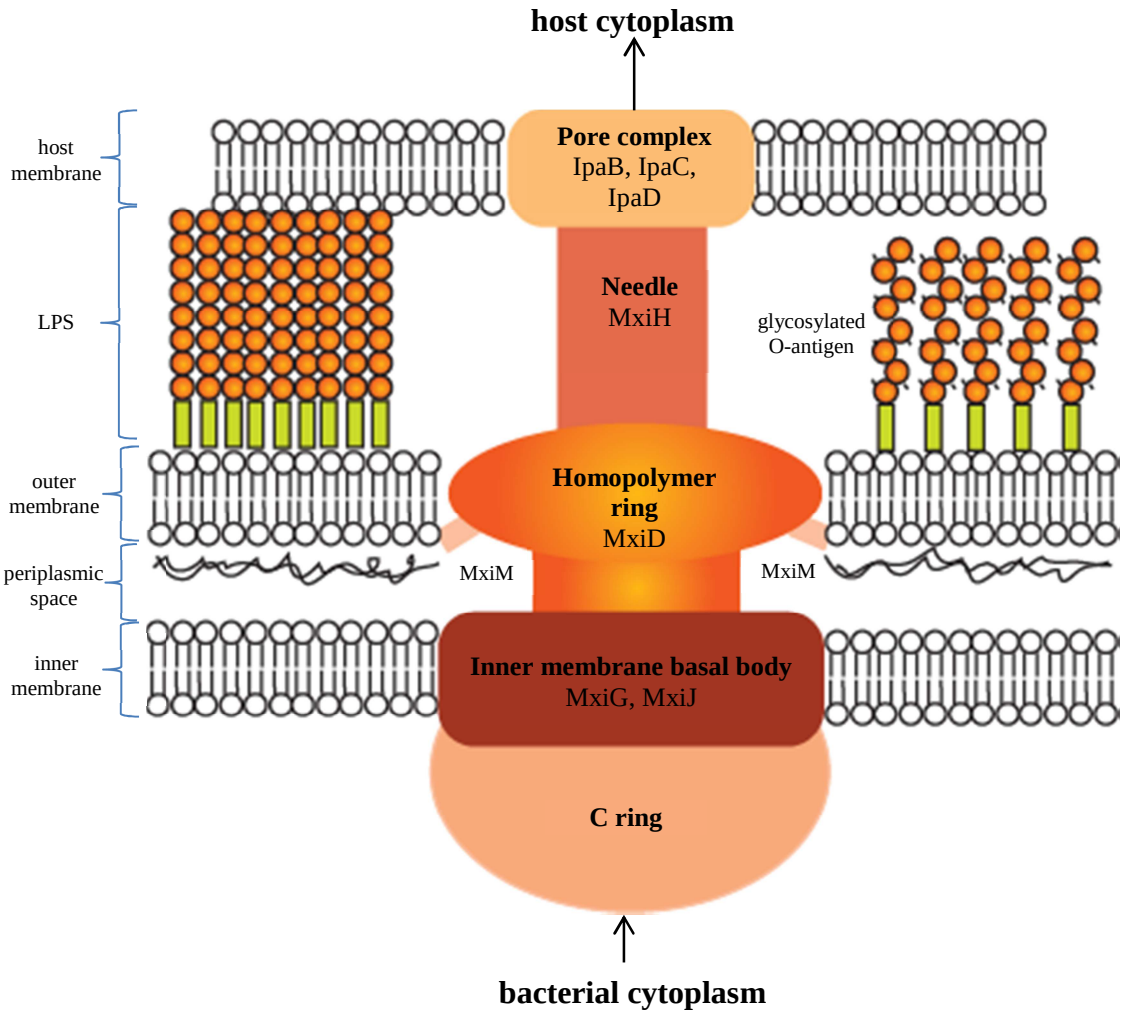


Figure 13 Schematic structure of the T3SS through three membranes. Arrows show the direction of effector transport. Detailed description of the system is found in the text.

The inner membrane and periplasmic part of the basal body comprises the MxiG and MxiJ proteins (111) connecting to the homopolymer ring of MxiD (112) protein which is anchored to the outer membrane by the lipoprotein MxiM (113). The protruding needle is assembled of polymerized MxiH subunits, while MxiI forms the inner rod in the basal body (114;115). The energy for the transport is provided by the ATPase Spa47 located in the cytoplasmic side of the basal body (116). Several further proteins are proposed to be involved in the assembly of the basal body (forming the so called C ring) (117), or required for the externalization of the needle subunits (118).

1.3.1.3.2. The function of the T3SS (role of effector molecules during the pathogenesis)

For a controlled secretion of the effector molecules first of all the transport of "translocator proteins" (IpaB, IpaC and IpaD) is necessary. These proteins are stored in the cytoplasm of the bacterium bound by chaperons preventing the premature association and degradation of the components (37). In the absence of contact with the host cell IpaD plugs the tip of the T3SS and retains IpaB inside the channel (111). During the activation of the T3SS due to conformational changes of IpaD the hydrophobic IpaB and IpaC can pass through the needle and insert into the host membrane, where it forms a pore (38) to allow translocation of approximately 25 effector proteins (Table 2) (100) which induce engulfment of the bacterium.

Invasion of epithelial cells requires the rearrangement of the cytoskeleton around the attachment site. Already the contact of bacterial surface proteins with their receptors primes the actin rearrangement. However the complete uptake requires the complex action of several effector proteins. These proteins induce the dissociation of the cytoskeleton from the cytosol membrane, massive actin polymerization with simultaneous depolymerisation, destabilization of the microtubule network and ultimately lead to the pinocytosis of the bacterium.
T3SS and the expression of the Ipa proteins are required for the escape of *Shigella* from the phagosome of macrophages, for the lysis of the pinocytotic vacuole and the double membrane formed during the intercellular spread.

1.3.1.4. Other virulence factors

Autotransporters belong to the T5SS family. In *Shigella* there are four main autotransporters described; IcsA or VirG is expressed during the invasion of the epithelial cells. The protein is involved in the intra- and intercellular motility of *Shigella* (see 1.3). Pic acts as a serine protease (25) during the disruption of the mucin layer covering the colon epithelium, as well

as it is an enterotoxin involved in the induction of diarrhoea. Its gene is located on the chromosome on the *she* pathogenicity island overlapping with *set* encoding ShET1 (119). The virulence plasmid encoded SepA shows sequence homology with IgA1 proteases (120) and shows enterotoxic activity in vitro (121), while SigA is involved in the cytoskeleton reorganization in tissue culture (122) and contributes to the fluid accumulation in the rabbit ileal loop model (123). Similarly to Pic, SigA is encoded on the *she* pathogenicity island of *Shigella*. The presence of the autotransporters in the epidemiologically most important serotypes justifies the proposed importance of these proteins in the pathogenesis of *Shigella*. Therefore several vaccine strategies rely on mutations of their genes.

1.4. Immune response against shigellosis

Shigellae evolved several mechanisms to evade the host's defence during an infection. Furthermore, these bacteria developed numerous methods to exploit the innate immune response in order to facilitate the spread of the bacteria and prolong their persistence in the mucosal epithelial cells. The inflammation in the early stage of the infection promotes the entrance of bacteria into the submucosa (as discussed in the 1.3 section), however it is essential in the prevention of a systemic disease and ultimately leads to the resolution of the infection. The immune protection after an infection is restricted to the infective serotype (homologous protection): the humoral immune response against LPS is implied to be the major, if not the sole, component of the protective immunity (124). While there are several data about the role of humoral immunity during a repeated infection with *Shigella*, there is little known about the cellular immune response to *Shigella*.

1.4.1. Innate immunity

After translocation of *Shigella* into the subepithelial layer, the bacterium is immediately engulfed by macrophages. Although macrophages and infiltrating monocytes are unable to kill the bacterium and the effector molecules of the T3SS ultimately lead to apoptosis of these cells (see references in 1.3), the IL-18 and IL-1β cytokines released by the apoptotic macrophages play a significant role in the innate immune response against *Shigella* (Fig. 2). IL-18 induces IFN-γ production in NK cells and T lymphocytes (125), which activates macrophages and fibroblast cells to eradicate bacteria. Without the activation with IFN-γ both cells would support the intracellular replication and intercellular spread of *Shigella* (126), which suggest major contribution of IFN-γ to the clearance of the bacterium.

Epithelial cells invaded by *Shigella* recognize peptidoglycan released from the bacterium (106) and LPS (127) and through the activation of NF-κB and MAPK signalling pathways secrete IL-8 and TNF-α as well as antimicrobial peptides. The same signal pathways are activated in the neighbouring non-infected epithelial cells, due to the transmission of the alarm signal through the intercellular connexin gap junctions (128). The released cytokines together with IL-1β from the apoptotic macrophages result in massive recruitment of PMNs. Although the neutrophil granulocytes migrating to the lumen disrupt the epithelial barrier thereby forming an entry portal to the basolateral surface for the luminal bacteria, they play major role in the restriction of the infection to the colon mucosa and in the ultimate resolution of the infection. On the one hand *Shigella* in unable to escape from the phagosome of neutrophils and is efficiently killed (129). Moreover, additionally recruited neutrophils release neutrophil extracellular traps (NETs), consisting of antimicrobial peptides and neutrophil elastase (130). These NETs entrap *Shigella* and degrade virulence associated proteins, preferentially IpaB, IpaC and VirG (131).

Antimicrobial peptides (β-defensin and LL-37) are demonstrated to display significant anti-infective properties in the control of enteric infections, and their role in the restriction of shigellosis is suggested by the susceptibility of four to five-day-old mice to intestinal *Shigella* infection, due to the lack of antimicrobial peptides before the age of six days (132).

Shigella is armed with a number of T3SS effector molecules to circumvent the innate immune system. These effectors modulate the pro-inflammatory signal pathways in the epithelial cells by dephosphorylation (OspF (105) or ubiquitination (OspG (133), OspZ (134) and IpaH9.8 (135)) of certain host factors.

1.4.2. Cellular response

Mainly due to the lack of natural intestinal murine model, there is little data available on the effect of the cellular immunity on shigellosis. In the widely used mouse lung model (136), the critical role of T and NK cells was demonstrated (137). T cell activation in patients undergoing *Shigella* infection (138), as well as the increased susceptibility of AIDS patients to shigellosis (139) confirms that cellular immunity, especially T cells can be important in the protective immune response against *Shigella*. Recent data show the involvement of Th17 cells in the clearance of the bacterium (140).

1.4.3. Humoral response

Natural *Shigella* infection provides an average of ~75% protection against an illness caused by a homologous strain (expressing the same O-antigen) (141). Both serum (IgG, IgM) and mucosal antibodies (sIgA) specific to the O-antigen of the LPS seem to play major role in this protection (142). sIgA can coat the surface of luminal *Shigella*, inhibiting the interaction of the bacterium with the mucosa (143). Furthermore sIgA anchored to the mucus lining of the epithelial cells can shield the cells and results in immune exclusion of the bacteria (144). The role of LPS specific IgA in the prevention of a re-infection with the same serotype of *Shigella* has been demonstrated in several clinical studies (145;146). However the protection of IgA-deficient vaccinated mice against homologous challenge suggests that IgG and IgM antibodies can also provide protection in the mouse lung model (147). In humans although there are IgG and IgM antibodies specific to *Shigella* detected in patients with a history of shigellosis (124;148;149), failed vaccine trials demonstrate that the parenteral stimulation of serum Ig alone does not guarantee protection against shigellosis (23;150).

Although there is a marked antibody response against the highly conserved invasion plasmid antigens Ipa-s (151), the lack of natural cross-protection implies that these antibodies are not protective.

1.5. Preventive and therapeutic options

Similarly to other infectious diseases, the most effective method to control shigellosis is prevention. Due to the lack of a licenced vaccine, to date this relies on hygienic measures (simple hand washing with soap, safe drinking water, proper disposal of waste, control of flies and avoiding contamination of food with faeces) and encouraging of breastfeeding (WHO, Guidelines for the control of shigellosis, including epidemics due to *S. dysenteriae* type 1).
In case of a diagnosed *Shigella* infection, based on current guidelines, beside proper rehydration, antibiotic treatment is urged to be initiated, as it reduces the duration of the symptoms, the period of *Shigella* excretion as well as highly decrease the risk of severe complications (152).

1.5.1. Antibiotic treatment and resistance

Over the past decades, increasing resistance of *Shigella* to several classes of antibiotics has been recorded. Due to worldwide high resistance to tetracycline, sulphonamides, ampicillin, TMP-SMX and nalidixic acid (13;153;154), current guidelines do not recommend the use of these antibiotics for the treatment of shigellosis. As first line antibiotic, ciprofloxacin is

recommended irrespectively of the age of the patient. Although fluoroquinolons can potentially cause cartilage damage, the effect was proved to be minimal in several studies (155). However increased use of this relatively safe and cheap drug resulted in increasing resistance, especially in Asian-African countries. The resistance of *Shigella* isolates to ciprofloxacin increased from 0.6% among isolates from 1998-2000 to 29.1% among strains from 2007-2009 (156). In comparison the ciprofloxacin resistance in European-American isolates remained very low (0.6% by 2007-2009). In China, the resistance reached 57.1% in 2008 among all *Shigella* isolates (153). The resistance to quinolones originates mainly from mutations in the quinolone resistance determining regions of *gyrA* (QRDR mutation) (157). The emergence of multi-drug resistant and fluoroquinolon resistant *Shigella* strains, especially in endemic countries leads to higher resistance among travel-associated shigellosis isolates as well (154;157).

If empirical treatment with ciprofloxacin does not improve the symptoms within 48 hours, switching to a second line antibiotic is recommended. These antibiotics can be pivmecillinam, ceftriaxone and - in adults - azithromycin, however the use of these antibiotics is limited due to higher cost, rapid emergence of resistance (pivmecillinam) or due to the necessity of parenteral injection (ceftriaxone). Furthermore, the spread of extended spectrum β-lactamases (ESBL) in Enterobacteriaceae affects *Shigella* as well; third-generation cephalosporin resistant strains were reported from Asia, especially from India with a frequency of 16.8% among *S. flexneri* isolates (158-160).
Importantly in all studies reviewed *S. flexneri* was reported to show higher resistance to most classes of antibiotics and more frequent among MDR isolates than *S. sonnei* (153;154;156;158).

1.5.2. Vaccine candidates

The huge medical need due to the high incidence as well as due to the emerging resistance of shigellae against the first as well as the 2^{nd}-3^{rd} choice antibiotics underlines the necessity of an effective vaccine against shigellosis. Since the beginning of the 20^{th} century numerous vaccine candidates have been developed against *Shigella*. Despite the number of vaccine studies and trials conferring protection experimentally, there is no licenced vaccine available against shigellosis mainly due to the lack of cross-protection between the several serotypes. The development of a successful vaccine is further hindered by the incomplete knowledge about the immune response required for the protection, as well as the unavailability of

intestinal rodent models. Even in non-human primates, the challenge dose required for the development of symptomatic dysentery exceeds that in humans by multiple logs, implying a (partly) different pathomechanism. Furthermore, the huge differences in basal immunity against shigellosis in developing vs. developed countries pose a great challenge for the clinical testing of vaccine candidates.

1.5.2.1. Killed whole cell vaccines

Killed vaccines provide a relatively safe form of vaccination. Nevertheless, in the 1960s, experiments showed that unlike a live attenuated vaccine strain, the oral administration of an acetone-killed *Shigella* inoculum was unable to prevent subsequent challenge in non-human primates (161). Therefore the main approach in vaccine development had been the live attenuated vaccine strategy with the constant problem of higher reactogenicity and side-effects. However, recently formalin-inactivated *Shigella* strains were found to be protective in a murine model (162). A formalin-killed *S. sonnei* strain (SsWC) was proven to be immunogenic and well-tolerated at a $2x10^{10}$ CFU dose in a phase I study (163). In spite of the initial success of this killed vaccine up to date there are no data available about the protective efficacy of this candidate in humans.

1.5.2.2. Live attenuated *Shigella* vaccines

1.5.2.2.1. Non-invasive live vaccines

Non-invasive and hence avirulent mutants of *Shigella* provide a possible solution against the high rate of side effects with live vaccines. Early trials with spontaneous non-invasive mutants, however, faced with the problem of reversion of the vaccine strain to its wild-type virulent form and with the marginal protection provided (164).

The most successful vaccine trials with non-invasive mutants were carried out between 1976 and 1980 using the spontaneous avirulent *S. flexneri* 2a strain Istrati T_{32} (165). The strain was isolated by Istrati in 1961 and proven to be avirulent, stable and effective in the guinea pig keratoconjunctivitis Serény test (166). The “Vadizen” vaccine was administered in Rumania to 32044 children and 4734 adults orally at $50\text{-}100x10^9$ CFU dose 5 times at 3-day intervals. It provided 78.91-87.9% protection against both homologous and heterologous (i.e. against different serotypes of *Shigella* species) infection with no major side effects recorded (low number of nausea, soft stool). The post-vaccination protection lasted 6 months and prolonged protection required re-vaccination twice a year. The vaccination was tested with similar

results in over 5000 individuals in China (167). Genetic analysis revealed that the vaccine strain carried a deleted form of the large virulence plasmid and lost at least three loci of the plasmid resulting in the non-invasive avirulent phenotype (168). These loci cover the genes *ipaBCD*, *invA* and *icsA*/*virG*. Although these early studies did not fulfil the present requirements of a double-blind placebo controlled clinical trial and the heterologous protection observed was controversial with any previous and later results, the efficacy elicited is exceptional among the anti-dysentery vaccine trials.

1.5.2.2.2. Invasive live vaccines

To mimic the pathogenesis of a natural *Shigella* infection, increasing number of invasive attenuated strains have been developed. These strains either harbour deletions in virulence genes involved in the intra- or intercellular spread of *Shigella* or in metabolic genes preventing its replication in the host. The major challenge for these vaccine candidates has been to find an optimal balance between immunogenicity and safety. There are two main trends; one part of the invasive vaccines is based on the auxotrophic mutant *S. flexneri* 2a Δ*guaBA* (169), the others carry a mutation in the *icsA/virG* gene required for the intracellular spread of *Shigella* (170). These basic mutants are further attenuated with the elimination of other virulence genes (Table 3) (171-175).

Table 3 Minimum phase I stage invasive live attenuated vaccine candidates

Vaccine candidate	Parental strain	Mutation	Route	Clinical phase	Reference
CVD 1204	*S. flexneri* 2a	Δ*guaBA*	oral	Phase I	(169)
CVD 1208S	*S. flexneri* 2a	Δ*guaBA;* Δ*set*; Δ*sen*	oral	Phase II	(171)
WRSs1	*S. sonnei*	Δ*icsA/virG*	oral	Phase I	(170)
SC602	*S. flexneri* 2a	Δ*icsA/virG;* Δ*iuc*	oral	Phase I-II	(173)
WRSd1	*S. dysenteriae* 1	Δ*icsA/virG;* Δ*stxAB*	oral	Phase I	(175)

Some of these candidates provided protection in clinical trials and propose a possible future vaccine against shigellosis; however, these strains alone could not elicit broad protection against multiple serotypes.

The obtain protection against most of the predominant *Shigella* serotypes, multiple-serotype vaccines have been developed. Based on antigenic characteristic of the LPS O-antigen, with the combination of an attenuated *S. flexneri* 2a and 3a Noriega et al. could elicit cross protection in the guinea pig keratoconjunctivitis model against *S. flexneri* 1b, 2b, 5b and Y (176). Alternatively different O-antigens can be expressed by one single strain; with the insertion of the serotype-conversion gene cluster of bacteriophages responsible for the different *S. flexneri* O-types, serotype-specific immune response could be provoked against two distinct *S. flexneri* serotypes in the mouse lung model (150). Subcutaneous administration of a hybrid T32 vaccine strain carrying a plasmid encoding *S. sonnei* O-antigen was reported to elicit 100% protection against intraperitoneal challenge of both *S. flexneri* 2a and *S. sonnei* (177).

1.5.2.3. Subunit vaccines

Similarly to the killed-whole cell vaccines, subunit vaccines provide a safe alternative of live attenuated mutants with low reactivity even in the increasing population of immunocompromised patients. However the high costs of the manufacturing and the discrepancy between the immune response elicited by the natural mucosal infection and the intramuscular administration of the subunit vaccines impose major challenges for these vaccines.

1.5.2.3.1. Serotype-targeted subunit vaccines

The most advanced vaccine trial is performed in Israel with a conjugate vaccine candidate. The O-polysaccharide of *S. flexneri* 2a or *S. sonnei* is covalently conjugated to the recombinant exoprotein A of *Pseudomonas* (rEPA). In a phase 3 study involving 2799 1-4 year children the conjugate was given intramuscularly twice in 6 weeks interval (178). The vaccine had minimal adverse effects (5% local pain, 4% fever) and elicited age-related rise against O-antigens. In correlation to the antibody response there was an age-related efficacy of the *S. sonnei* vaccine with 3.8% efficacy in the 1-2 years old group and 71.1% in the 3-4 years old group. Unfortunately, there were not enough cases for the statistical analysis of the *S. flexneri* 2a immunized group. These results may suggest that the conjugate effectively boosted the children old enough to have been primed with a possible previous infection, but it was unable to elicit protection in naïve population of the 1-2 year old children.

The conjugation of O-polysaccharide to tetanus toxoid provides an alternative method of the serotype targeting subunit vaccines. *S. flexneri* 2a O-antigen linked to tetanus toxoid induced

antibody response against *S. flexneri* 2a, and these antibodies when introduced simultaneously with the wild-type parent strain protected naïve mice (179).

1.5.2.3.2. Conserved antigen vaccines

An alternative of a multivalent vaccine combining several serotypes, conserved antigens can be used for immunization. In theory, epitopes shared by all serotypes could confer full cross-protection against *Shigella*. This highly attractive strategy encouraged several vaccine developments.

The most advanced among these candidates rely on two different strategies. Invaplex, the candidate of the Walter Reed Army Institute contains bacterial extract containing invasion plasmid antigens (Ipa-s) and LPS. It was expected to provide broad protection (based on the immune response against the Ipa-s), however due to the LPS contamination protection restricted to the same serotype was experienced in animals (180). Nevertheless, the vaccine administered intranasal was proven to be safe and immunogenic in human adults (181).

The second strategy involves genetically engineered outer membrane particles (GMMA Generalized Modules of Membrane Antigens) with outer membrane proteins of *Shigella* without the contamination of LPS (182). In the mouse model, this immunogen provided significant protection and seems to be a promising candidate against shigellosis.

2. Aims

Based on the unique cross-protection observed with the live-attenuated vaccine Istrati T_{32} we hypothesized that loss of a major surface antigen complex (i.e. the Ipa-s) and/or the consequent non-invasive character of the strain could have enhanced immune reactivity against conserved surface antigens. As the immunodominant antigens show high diversity, the protection elicited by a natural infection is restricted to the same serotype, however we hypothesized that the immunologically more silent structures could have remained conserved within the genus (or even within other members of the family) proposing the possibility of cross-protection. We speculated that these "minor" antigens cannot play a significant role in the formation of the immune response in the presence of other "major" immunodominant surface structures (Ipa-s, LPS O-antigens), however with the removal of these major antigens from the surface of the bacterium, the immune response will be driven by the naturally more silent minor antigens.

To provide a less expensive broad protective vaccination strategy, than the conserved antigen based subunit vaccines, we generated and tested live-attenuated vaccine candidate strains. The safety and cross-protective capacity of mutants of *S. flexneri* 2a and *S. sonnei* lacking major surface antigens were tested in the mouse lung model. We proved that highly attenuated non-invasive mutants, especially those lacking the serotype determining O-antigen are capable of triggering a protective immune response against lethal infections with heterologous serotypes in animal model. The identification of potential cross-protective proteins could help to improve our understanding about the efficacy of other subunit vaccine strategies as well.

3. Materials and Methods

3.1. Bacterial strains and culture conditions

Prototype sequenced *S. flexneri* 2a strain 2457T (183) was used as a parental strain to create isogenic attenuated *Shigella* mutants. Inactivation of the *aroC* results in an auxotrophic mutant unable to synthesize aromatic compound needed for intracellular multiplication (184). Deletion of *rfbF* leads to a rough LPS phenotype, as the enzyme RfbF is involved in the synthesis of the O-antigen (82). Serotype 2a (*S. flexneri* 544) and 6 (*S. flexneri* 542) and *S. sonnei* 598 challenge strains were clinical isolates from Kuwait (kindly provided by Prof Tibor Pál).

Bacteria were routinely grown at 37ºC in Luria Bertani (LB) broth or tryptic soy agar (TSA) plates supplemented with 0.01% Congo Red dye (Sigma-Aldrich) used for the detection of an intact invasion plasmid expressing the Ipa proteins (185). Where appropriate, media were supplemented with ampicillin (100 μg/ml), kanamycin (100 μg/ml) or chloramphenicol (25 μg/ml).

3.2. Plasmids and primers

Plasmids used for the generation of the mutants are listed in Table 4.

Table 4 List of plasmids used for mutagenesis with main characteristics and reference

Plasmid	Characteristics	Reference
pKD46	Helper plasmid encoding arabinose inducible λ Red recombinase, T_s ori, Ap^R	(186)
pKD3	template plasmid for the amplification of chloramphenicol cassette (*cat*)	(186)
pKD4	template plasmid for the amplification of kanamycin cassette (*kan*)	(186)

The sequence of oligonucleotides used for the generation and genotypic confirmation of the mutations are provided in Table 5.

Table 5 List of primers used for mutagenesis and genotypic confirmation, underlined sequence part shows the region aligning with the antibiotic cassette

Oligonucleotide	Function	Oligonucleotide sequence
aroCpKD-F	Amplification of PCR product used for the allelic exchange of *aroC*	5’ CGC ACG GGC TGG CGC TCG GCT GCT GCA TCG TCG ATG GTG TTC C*GT GTA GGC TGG AGC TGC TTC* 3’
aroCpKD-R	Amplification of PCR product used for the allelic exchange of *aroC*	5’ TAT CAG TCT TCA CAT CGG CAT TTT GCG CCC GCT GCC GTA A*CA TAT GAA TAT CCT CCT TAG TTC CTA TTA A* 3’
rfbFpKD1	Amplification of PCR product used for the allelic exchange of *rfbF*	5’ GAA TAG TAA TAT TTA CGC TGT CAT TGT GAC ATA TAA TCC C*GG TGT AGG CTG GAG CTG CTT C* 3’
rfbFpKD2	Amplification of PCR product used for the allelic exchange of *rfbF*	5’ GCA TTA TAA CGA CCG CCC CCA GTA ATT CCT CTT ATT CC*C ATA TGA ATA TCC TCC TTA GTT CCT AAT CC* 3’
aro-ko1	Confirmation of allelic exchange within *aroC*	5’ GAG CCG TGA TGG CTG GAA ACA C 3’
aro-ko2	Confirmation of allelic exchange within *aroC*	5’ AGC GCA ATC GCG GTT TTG TTC A 3’
rfbF-ko1	Confirmation of allelic exchange within *rfbF*	5’ GGG TTA CTG GGT GCC GCA ATA TCC 3’
rfbF-ko2	Confirmation of allelic exchange within *rfbF*	5’ CCT CAA TCC AGC ATT CGC CAT TAT ACG 3’
rep-1	Detection of *repA* on virulence plasmid	5’ GTG GCG TAG CAT GCT AGA TTA CTG 3’
rep-2	Detection of *repA* on virulence plasmid	5’ CAG TGC AGA TGT GAA CGT GAT ATC 3’

virF-V	Detection of *virF* on virulence plasmid	5’ GGG CTT GAT ATT CCG ATA AGT C 3’
virF-I	Detection of *virF* on virulence plasmid	5’ GCA AAT ACT TAG CTT GTT GTT GCA CAG AG 3’

3.3. Generation of isogenic *Shigella* vaccine candidates

Inactivation of the *aroC* and *rfbF* genes was performed by the Red recombinase technique (186). As a first step a helper plasmid (pKD46) encoding the inducible λ Red recombinase was transformed into *S. flexneri* 2a CRP. Overnight culture of bacteria were washed with decreasing volume of sterile 0.9% physiological saline twice, and then washed with 5 ml 10% sterile glycerol. The pellet was resuspended in ~100 µl 10% glycerol and transformed immediately with purified plasmid. All centrifugation steps were carried out with 4,400 rpm for 10 min at 4˚C, the culture was kept during the whole procedure strictly on ice.

For electroporation a Gene Pulser Xcell (Bio Rad) was used. 50 µl electrocompetent bacteria were mixed with pKD46, transferred into electroporation cuvette (2 mm gap, Bio Rad) and electroporated with 2.5 kV. After pulse was provided bacteria were resuspended in 10 ml LB, incubated at 30˚C for 1.5 h. Transformed bacteria were plated on ampicillin TSA plates and incubated overnight at 30˚C.

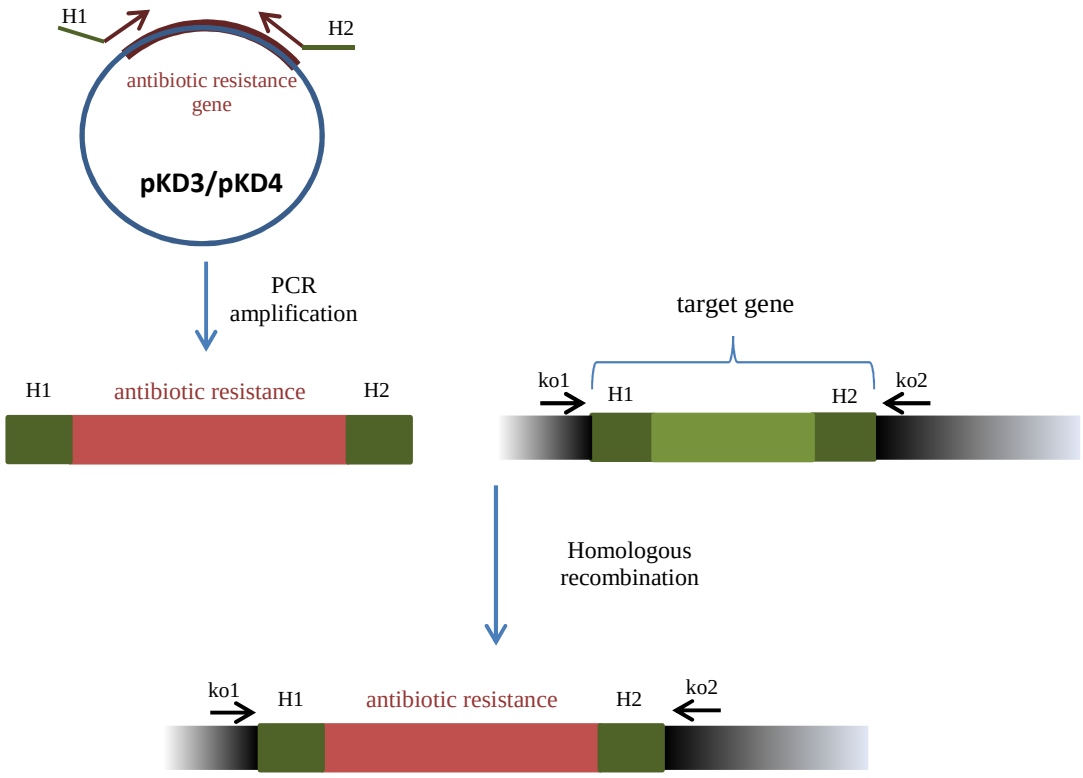


Figure 14 Schematic steps of the mutagenesis. Detailed description of the method is in the text.

An antibiotic cassette encoding resistance to kanamycin (kan) or chloramphenicol (cat) flanked by 30-50 bp region homologous to the *aroC* or to the *rfbF* genes (H1 and H2) was generated with proof reading KlenTaq® LA polymerase from a helper plasmid pKD3 (cat) or pKD4 (kan). The amplified product was extracted from 1.5% agarose gel with Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The eluted purified cassette was precipitated with 2.5-times volume of denatured ethanol and 0.1-times volume of 3 M sodium-acetate (pH 5.2), and kept at -20°C overnight. The precipitate was centrifuged with 13.000 rpm in a table top microcentrifuge (Eppendorf) for 20 minutes at 4°C. The pellet was washed twice with ice-cold 70% ethanol and centrifuged as previously. DNA was rehydrated in 5 µl nuclease-free distilled water (Fermentas). Different volumes of concentrated antibiotic cassette were electroporated into electrocompetent *S. flexneri* 2a 2457T CRP strain containing pKD46 helper plasmid grown in the presence of 40 mM/ml D-arabinose. In the presence of arabinose the λ Red recombinase is induced, and the targeted gene is replaced by the antibiotic cassette based on homologous recombination between H1 and H2.

Electrocompetent cells were generated according to the protocol described. Transformants were incubated at 37˚C for 2hours followed by 3 hours at room temperature. Pelleted bacteria were plated on TSA plates with the corresponding antibiotics and incubated overnight at 37˚C. Antibiotic resistant clones were subjected to genotypic confirmation with PCR. Amplification with control primers listed in Table 5 was performed with MasterMixx® (Fermentas) with the following program: initial denaturation at 95˚C for 2 min, denaturation at 95˚C for 30 sec, at 58˚C annealing for 30 sec and 72˚C elongation for 90 sec and with 30 cycles. The mutants were detected based on the different size of the product in wild-type strains compared to clones with cassette inserted.

2457T Δ*aroC::kan* and 2457T Δ*rfbF::cat* mutants were subsequently cultured on TSA plates supplemented with Congo red (CR TSA) to select Congo red positive (CRP) and Congo red negative (CRN) colonies. The loss of the invasion plasmid in CRN isolates was confirmed with the PCR based detection of plasmid encoded virulence determinants VirF and Ial (187) and of the RepA protein with the program used for the detection of mutants. PCR product was run on 1.5% agarose gel in 1% Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide.
Similarly phase I and phase II (i.e. non-invasive and rough) variants of *S. sonnei* were differentiated on CR TSA plates and tested for the presence of the virulence plasmid.

3.4. Adhesion-invasion assay

Int407 and HeLa cell lines from the collection of the Department of Medical Microbiology and Immunology, University of Pécs were used to test the adhesion and invasion as well as the *in vitro* survival/multiplication of *Shigella* strains. Confluent cells ($2x10^5$ cells in a 24-well plate) were incubated in 10% FCS RPMI solution without antibiotics at 37°C in the presence of 5% CO_2. Given CFU of bacteria were added to the cells to reach a MOI of 10. For the determination of the adhesion and invasion, after 1 hour incubation at 37°C in the presence of 5% CO_2 cells were washed with 1ml sterile PBS twice and lysed with 1% Triton-X-100 (cell associated bacterial count) or incubated for an additional 30 minutes with 1 ml of 40 µg/ml gentamycin (Sanofi-Aventis) in RPMI followed by washing and lysis of the cells (intracellular/invasive bacterial count). The bacterial count was determined with plating of duplicates of dilutions.

For the testing of the *in vitro* intracellular survival/multiplication, following 1 hour incubation with the bacteria, cells were washed with PBS and incubated for 24 hours with 40 µg/ml gentamycin (Sanofi-Aventis) in RPMI at 37°C 5% CO_2. On the following day cells were washed and lysed as described previously. The bacterial count determined equalled to the intracellular survived bacterial count.

The cells were controlled with microscope before each experiment as well as after 24 hours incubation with the intracellular bacteria. All *Shigella* strains tested were sensitive to 40 µg/ml gentamycin and resistant to 1% Triton-X-100. Experiments were run in duplicates.

3.5. Serum resistance of *Shigella* mutants

To test the survival of the vaccine strains in serum of healthy human donors, ~10^6 CFU bacteria were incubated at 37°C in 200 µl 50% human serum diluted with sterile PBS (active serum) for 30-60-120 minutes or 24 hours. As control, same amount of bacteria was incubated the same way in 50% heat inactivated serum (complement inactivated at 56°C for 30 minutes). Bacterial count (CFU) was determined based on plating different dilutions after the incubation and compared to the bacterial count at the beginning of the experiment. This starting cell count was determined for each experiment individually.

3.6. Animal experiments

In vivo studies were performed in the mouse lung model (136). All assays were conducted according to the principles set forth in the guide for the care and use of laboratory animals in a laboratory as authorized by Hungarian decree (no. XXVII, 1998) and by the subsequent regulation (government order no. 243/1998). Six to eight-week-old female specific pathogen free (SPF) BALB/c mice were anesthetised intraperitoneally with a mixture of 5 mg/ml ketamine (Calipsol, Richter Gedeon, Hungary) and 0.3 mg/ml xilazine (Primasine, Alfasan). Immunizations and challenges were performed intranasally with 50µl of inoculum (diluted in physiological saline) containing the required CFU of bacteria.

3.6.1. *In vivo* survival of *Shigella* vaccine candidates

Mice were infected with 10^6 CFU or 10^8 CFU bacteria intranasally. On day 1, 2, 3 and 4 post-infection 2 mice from each group were euthanized with cervical dislocation and their lungs were removed, homogenised with a T25basic (IKA Labortechnik) homogeniser in 10 ml 0.1% TritonX-100 in PBS. Serial dilutions of the suspension were plated on TSA plates with chloramphenicol (*S. flexneri* 2a 2457T Δ*rfbF* CRP and CRN) or kanamycin (*S. flexneri* 2a 2457T Δ*aroC* CRP and CRN). The bacterial count recovered was compared to the dose used for the infection.

3.6.2. Determination of the 50% lethal dose (LD_{50})

Fifty per cent lethal doses (LD_{50} values) were calculated from results of infections with doses of 0.5 log-serial dilutions ranging between 10^5 and 10^8 CFU/mouse. Groups of 5 mice were infected with the given doses, their survival was monitored for 14 days post-infection. The LD_{50} value was calculated with statistical methods.

3.6.3. Homologous challenge

Group of 5 anesthetised mice were immunized intranasally with sublethal dose of *S. flexneri* 2a 2457T Δ*aroC* CRP (10^6 CFU), CRN (10^8 CFU) and Δ*rfbF* CRP (10^6 CFU), CRN (10^8 CFU) twice with 2-week intervals. Control mice received sterile physiological saline. Two weeks after the booster immunization mice were challenged intranasally with lethal dose of clinical isolate *S. flexneri* 2a 544. Survival and weight was monitored daily for 14 days post-infection.

3.6.4. Heterologous challenge

Group of 5 mice were immunized with *S. flexneri* 2a 2457T Δ*aroC* CRP (10^6 CFU), CRN (10^8 CFU) or with Δ*rfbF* CRP (10^6 CFU) or CRN (10^8 CFU) twice with two-week intervals. Control group received sterile physiological saline. Two weeks following the booster immunization, mice were infected with lethal dose of heterologous clinical isolate of *S. flexneri* 6 strain 542 or *S. sonnei* strain 598. Survival and weight of the mice was monitored daily for 14 days.

Alternatively group of 5 mice were immunized with *S. sonnei* 598 phase I ($10^{5.5}$ CFU) or phase II ($10^{7.5}$ CFU) strains twice with two-weeks intervals. Two weeks after the booster immunization, mice were challenged with lethal dose of either *S. flexneri* 2a or *S. flexneri* 6. Survival and weight was measured every day for 14 days. Control group received physiological saline.

3.6.5. Immunization with heat-killed bacteria

Group of 15 mice were immunized intranasally with 10^8 CFU heat-killed *S. flexneri* 2a 2457T Δ*aroC* CRP, CRN and Δ*rfbF* CRP or CRN twice with 2-week interval. For the heat-inactivation washed bacteria were concentrated to the required CFU and incubated at 100 °C for 1 hour. One week after the booster blood was taken from the tail vein of each mice, let to clot at room temperature, and centrifuged with 4,000 g for 10 minutes to gain serum. The samples were stored at -20 °C till use

3.6.6. Collection of blood and bronchoalveolar lavage from immunized mice

One week after the booster immunization ~50 µl blood samples were taken from the tail vein of immunized mice. The blood was let to clot at room temperature, centrifuged at 4,000 g 10 minutes to gain serum. Alternatively, mice immunized were euthanized with cervical dislocation two weeks after the booster and their trachea was prepared, cannulised with a blunt end needle and 200 µl saline was injected into their bronchi. Bronchoalveolar lavage fluid was retracted subsequently.
Blood and BAL samples of mice belonging to the same immunization group were pooled and stored at -20 °C.

3.7. ELISA

The immune response of the vaccinated animals was measured in whole cell ELISA on parallel 96-well plates (Sigma 3D) sensitized with either the smooth, invasive strain or with its rough, non-invasive counterpart. Wells were coated overnight at 4°C with 90 µl bacterial suspension (5 x 10^8 CFU/ml in freshly prepared pH 9.5 carbonate buffer). On the following

day plates were washed three-times with 200 µl PBS containing 0.05% Tween 20 (washing buffer). Blocking of the wells was performed with 200 µl/well 2% BSA (Sigma-Aldrich) in PBS for one hour at room temperature. BAL and serum samples were serially diluted in 100µl PBS containing 0.1% BSA and 0.05% Tween 20 and incubated with the antigen-coated plates for 1h at 37°C. After 3 washes, plates were probed with 100 µl 1:2,000 diluted anti-mouse IgG (serum samples) or anti-mouse IgA (for BAL samples) immunoglobulin conjugated with horseradish-peroxidase (HRP) (Sigma-Aldrich). To determine the ratio of IgG2a and IgG1 isotypes, HRP conjugated anti-mouse IgG2a and IgG1 (Sigma-Aldrich) were used. Following 1 h incubation at 37°C with the secondary antibodies, plates were washed three times with washing solution. The ELISA substrate was o-phenylenediamine (Sigma-Aldrich) dissolved in citric acid buffer containing H_2O_2 (0.56% citric-acid-1-hidrate, 2.24% Na_2HPO_4 x $12H_2O$ in distilled water, 0.0334% orto-phenylenediamine, 0.016 V/V% 30% hydrogen peroxid). The reaction was stopped with 2 N sulphuric acid. The OD was measured at 492 nm with FLUOstar OPTIMA (BMG Labtech) ELISA reader. The immune response following immunization with the different vaccine strains was expressed in relation to the one elicited by the Δ*aroC* CRP (smooth, invasive strain) BAL or serum sample at the same dilution (1:10 for BAL samples, 1:400 for serum IgG). Means and SEM were calculated from at least 4 independent assay with samples from different immunization studies.

3.8. Protein identification by mass spectrometry

For the sonication overnight culture of bacteria were centrifuged and washed 3-times in physiological saline and resuspended in sonicating buffer (pH 7.4) in the presence of protease inhibitor (cOmplete Mini tablet, Roche, 1 tablet/10 ml). Suspension was sonicated with 30% amplitude 0.5 sec bursts for 2 minutes repeated twice on ice, then centrifuged with 6,000 rpm for 10 min at 4˚C. The supernatant (whole cell lysate) was stored at -20˚C. 10-15 µl of pools (sera of 5 mice) of hyperimmune sera collected from mice immunized twice either with 2457T Δ*rfbF* CRN (rough, non-invasive) or belonging to control group (received sterile physiological saline) were reacted with 100 µl aliquots of sonicated whole cell lysates of *S. flexneri* 2a 2457T Δ*rfbF* CRN o/n at 4˚C with constant rotation. Immune complexes were captured with 20 µl protein G beads (Sigma-Aldrich). Following 4 washes with 500 µl PBS, proteins were eluted with Laemmli buffer and run on 8% polyacrylamide gels with constant 120 V with a PROTEAN® II xi Cell system (Bio-Rad). Proteins were visualized with

SyproRubi (Invitrogen) on UV-transilluminator. Protein bands detectable by serum obtained with the double mutant but not with the control mouse sera were excised and stored in ultra-pure water at 4˚C. Identification of the proteins was done in collaboration with Éva Hunyadi-Gulyás in the Proteomics Research Group at the Biological Research Centre, Hungarian Academy of Sciences, Szeged. Following reduction (DTT) and alkylation (IAM) they were in-gel digested with trypsin (porcine Trypsin, Promega) and subjected to mass spectrometry analysis. RP-LC-MSMS experiments have been carried out on an ion-trap (LCQ-Fleet, Thermo) mass spectrometer on-line coupled with a nano-HPLC system (nano-Acquity, Waters). MSMS peak lists were generated by mascot Distiller (ver. 2.2.1.0) and submitted for database search on Mascot search engine against the NCBI100220 protein database. Two searches were run, the first without species specification (10448260 sequences) and the second restricted to ‘Bacteria’ (82322302 sequences).

3.9. Statistical analysis

The 50% lethality dose was calculated with the statistical method of Reed and Muench (188). The statistical analysis of the survival curves was performed with the LogRank (Mantel-Cox) test, ELISA titres of BAL and sera as well as serum sensitivity and adhesion-invasion capacity were compared with the Mann-Whitney non-parametric analysis using GraphPad Prism version 5.00 for Windows. The p value was considered significant if lower than 0.05. Graphs show mean with standard error of the mean or for the presentation of ratios geometrical means with the corresponding standard error.

4. Results

4.1. Construction of vaccine candidates and their characterization

Isogenic attenuated mutants of the prototype *S. flexneri* 2a strain 2457T were constructed (Table 6). Deletion of *aroC* resulted in a mutant auxotrophic for aromatic amino acids, PABA and dehydroxybenzoic acid (184). The inactivation of *rfbF* resulted in a rough LPS phenotype (82). With the selection of Congo red negative (CRN) variants of both mutants, double mutant clones devoid of invasive potential encoded on the large virulence plasmid were constructed. Phase II form of *S. sonnei* 598 designates a mutant lacking the large virulence plasmid. In *S. sonnei* this plasmid carries the *rfb* operon responsible for the synthesis of the O-antigen (see before), hence *S. sonnei* phase II variant is both non-invasive and expresses a rough LPS (189). The phenotype and genotype of the mutants were confirmed with multiple methods.

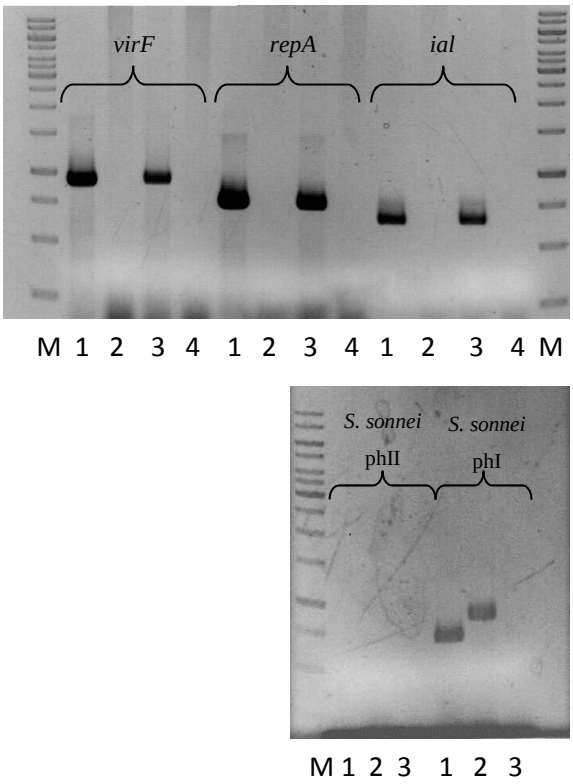
Table 6 Characteristics of vaccine strains tested

Strain	**Phenotype**	
	O-antigen	Ipa[a]
***S. flexneri* 2a 2457T (wild type)**	+	+
2457T Δ*aroC* CRP	+	+
2457T Δ*aroC* CRN	+	-
2457T Δ*rfbF* CRP	-	+
2457T Δ*rfbF* CRN	-	-
***S. sonnei* 598 phase I**	+	+
***S. sonnei* 598 phase II**	-	-

[a] **Invasion plasmid antigen**

4.1.1. Confirmation of the loss of the virulence plasmid

The lack of Congo red binding (CRN phenotype) does not necessary originate from the loss of the whole virulence plasmid, but could theoretically come from smaller deletions or mutations within the invasion genes. In order to confirm that the tested CRN mutants carry no remnants of the plasmid we tested the presence of three genes located on the plasmid by PCR. The amplification of *virF* (encoding a conserved regulator protein of IcsA/VirG (190) and *ial* (invasion associated loci) is used for the detection of *Shigella* species from clinical samples (187), whereas the lack of the replication protein determinant *repA* confirms the loss of the whole plasmid (Nagy et al. unpublished). For subsequent experiments CRP isolates carrying all, as well as CRN strains devoid of all three genes were selected (Fig. 16).



M: 1kb DNA ladder (Fermentas)
1: *S. flexneri* 2a 2457T Δ*aroC* CRP
2: *S. flexneri* 2a 2457T Δ*aroC* CRN
3: *S. flexneri* 2a 2457T Δ*rfbF* CRP
4: *S. flexneri* 2a 2457T Δ*rfbF* CRN

M: 1kb DNA ladder (Fermentas)
1: *repA*
2: *virF*
3: *ial*

Figure 15 Visualization of PCR product controlling virulence genes encoded on the virulence plasmid. Products were run on 1.5% agarose gel with 6x Loading Dye (Fermentas). Gels were stained with ethidium bromide.

The lack of amplicon with primers annealing to the *ial* gene in the tested *S. sonnei* 598 phase I clone (Fig. 16) suggested the partial deletion of the large virulence plasmid. As *ial* is among the most likely deleted genes of the plasmid,, this was not completely unexpected (187).

Subsequently, a phase I clone of *S. sonnei* that was positive for all three genes tested was selected (not shown), which was used for all experiments.

4.1.2. Testing of adhesion and invasion potentials

Based on the literature, the entry region of the large virulence plasmid is necessary and sufficient for the invasiveness of *Shigella*. In order to confirm the invasive phenotype of the CRP mutants of *S. flexneri* 2a 2457T invasion assay was performed on HeLa cells.

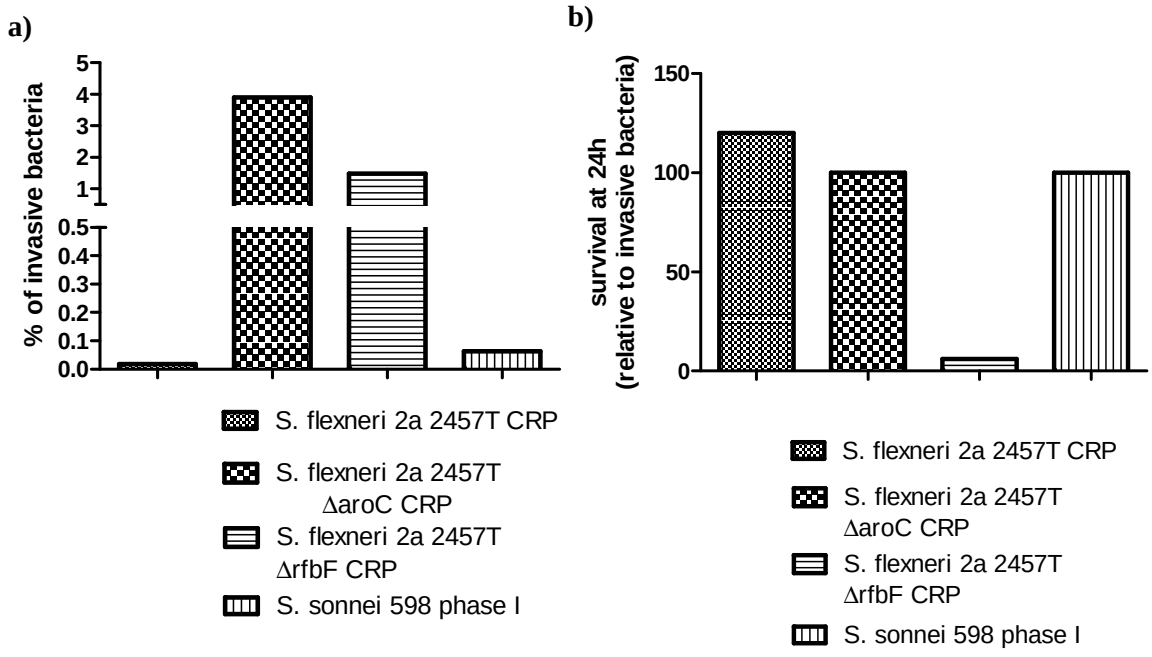


Figure 16 Invasiveness (a) and 24h intracellular survival (b) of CRP strains on HeLa cells in one representative experiment. Invasiveness shown as percentage of intracellular bacterial count compared to the number of cell associated bacteria. Intracellular survival shows percentage of intracellular bacteria after 24h incubation relative to the invasive bacterial count.

Figure 17 shows the intracellular bacterial cell count in relation to the number of adherent bacteria on HeLa cells after 30 minutes (Fig. 17. a) and 24 hours (Fig. 17. b). Invasiveness and intracellular survival (although at different degree) was shown for all tested *S. flexneri* 2a 2457T CRP mutants. In contrast, all CRN strains were repeatedly unable to invade eukaryotic cells (data not shown). Interestingly, although the rough strain (*S. flexneri* 2a 2457T Δ*rfbF* CRP) showed an increased invasiveness, its intracellular survival after 24 hours was found to be compromised. To confirm that unlike the higher invasiveness of the rough strain, there is no significant difference in the number of cell adherent bacteria in case of the Δ*rfbF* mutant, adhesion assay was performed.

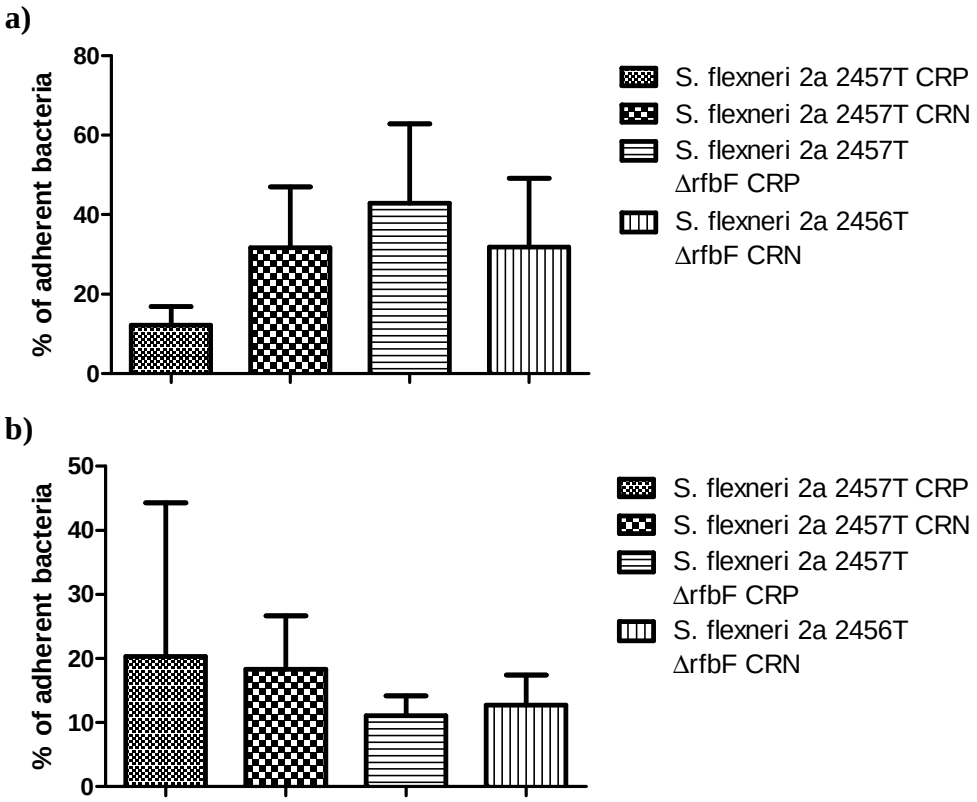


Figure 17 Ratio of adherent bacteria compared to the inoculum size in percentage on a) Int407 and b) HeLa cells. Graphs show mean of 3 and 4 experiments respectively with the SEM.

Adherence of CRP and CRN mutants of the *S. flexneri* 2a 2457T wild type and *S. flexneri* 2a 2457T Δ*rfbF* strains to Int407 and HeLa cells was tested. Figure 18 shows the percentage of adherent bacteria compared to the bacterial count of the inoculum given onto the cells. We detected that both the loss of the virulence plasmid as well as the rough LPS phenotype resulted in higher, however statistically not different ($p=0.25$ or $p=0.20$ respectively) adhesion of *S. flexneri* 2a 2457T to Int407 cells (Fig. 18. a). This difference was not observed in case of the HeLa cell line (Fig. 18. b). Therefore, the increased number of intracellular bacteria observed for the rough strain at early time points described above cannot be attributed to a higher number of bacteria associated with the cells.

4.1.3. Serum resistance of vaccine candidates

Survival and multiplication of pathogenic bacteria in serum (serum/complement-resistance) is an important characteristic of virulent strains. To test the effect of the lack of the virulence

plasmid and/or rough phenotype of the bacteria on serum resistance, the mutants were incubated in active and heat-inactivated (i.e. complement inactivated) human serum and the surviving proportion was determined at various time points (Fig. 19).

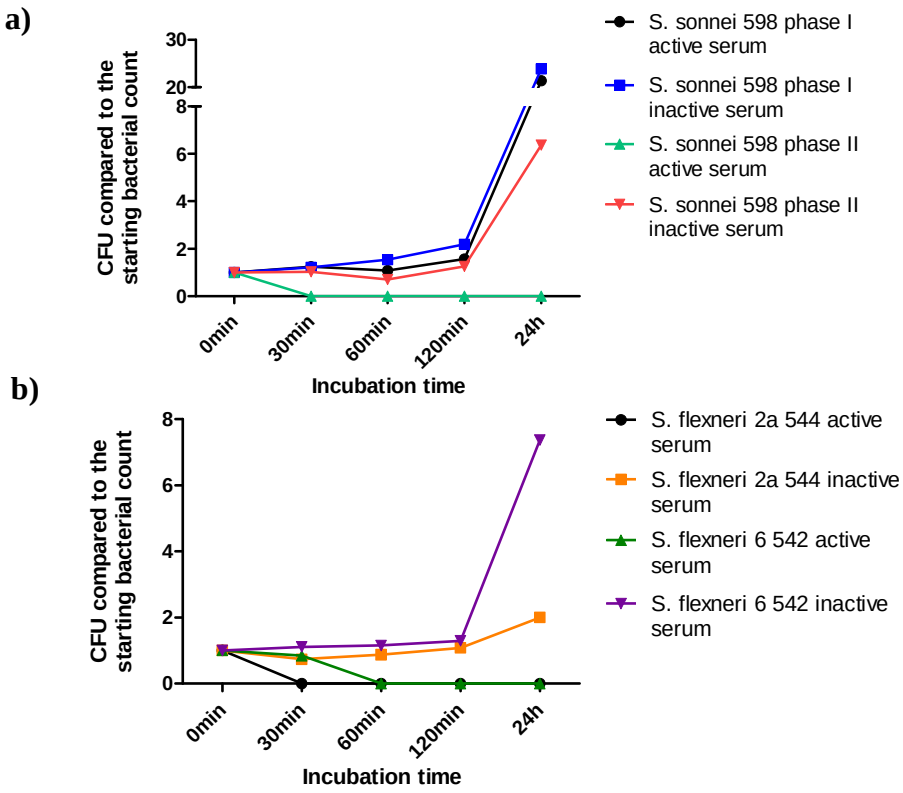


Figure 18 Survival of *Shigella* strains in 50% human serum. CFU count after 30, 60, 120 minutes and 24 hours incubation in active or heat inactivated serum of healthy donors relative to the bacterial count of the inoculum. Graphs show geometrical mean of 5 (a) and 3 (b) experiments. Statistical comparison at each time point was performed with Mann-Whitney test. Significant difference was found only between the CFU of *S. sonnei* phase I, *S. flexneri* 2a and *S. flexneri* 6 in active and in heat-inactivated serum ($p<0.05$) and between bacterial count recovered from active serum with strains *S. sonnei* phase I and phase II ($p=0.02$).

Unfortunately *S. flexneri* 2a 2457T CRP wild-type strain was found to be sensitive to 50% complement: no bacteria could be detected in active serum after 60 minutes, unlike in heat-inactivated serum). Since both the Δ*rfbF* and Δ*aroC* mutants showed the same characteristic, no difference could be detected, when incubated in the presence of 50% human serum. On the other hand, wild-type *S. sonnei* 598 phase I showed survival and multiplication in 5 independent experiment performed with fresh serum samples obtained from 5 individuals, whereas the phase II form of the same strain could not be recovered from active serum samples after 30 minutes incubation (statistically significant difference at each time-point examined) (Fig. 19. a). Although the bacterial count of phase II *S. sonnei* in heat-inactivated

serum after 24 hours was lower than that of phase I *S. sonnei*, the difference was found not to be statistically significant (p=0.2286). *S. flexneri* 2a 544 and *S. flexneri* 6 542 used for the challenge experiments were tested in the same setting with serum of 3 donors (Fig. 19. b). Like the other 2a serotype strain, *S. flexneri* 2a 544 was rapidly killed in active serum after 60 minutes. *S. flexneri* 6 542 could be detected in low cell count for 120 minutes, however after 24 h, no survival was found. The difference between the survival of *S. flexneri* 2a and *S. flexneri* 6 in active serum was not found to be statistically significant with the nonparametric statistical test.

4.1.4. *In vivo* survival of *Shigella* mutants in the mouse lung model

To exclude the possibility that during the immunization prolonged survival of the mutant bacteria enhances the immune response provoked, we tested the *in vivo* survival of sublethal amount of 2457T mutants.

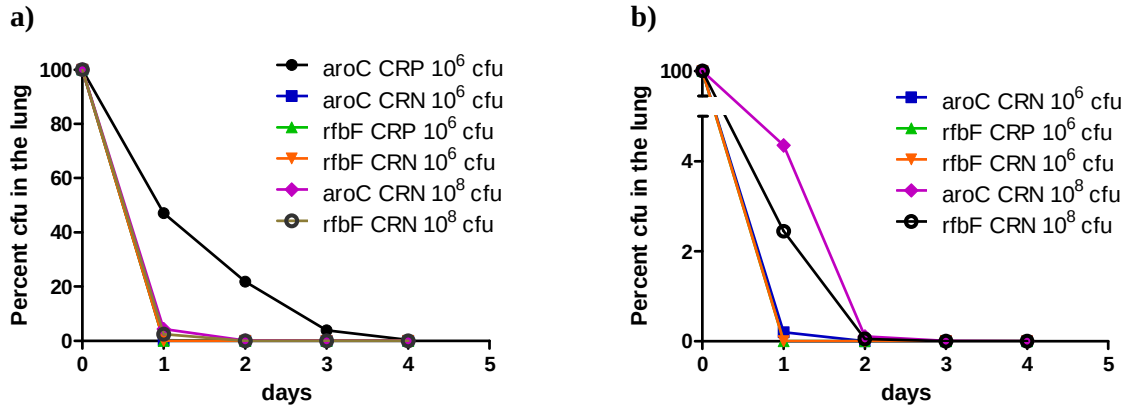


Figure 19 Bacterial count recovered from lung of infected mice after 1-4 days post-infection. Graph shows result as mean CFU from 4 mice per data point. Panel B shows results focused on Δ*aroC* CRN, Δ*rfbF* CRP and CRN in order to show subtle differences between the survival of these three strains.

Figure 20 shows the number of surviving bacteria in the lung in relation to the total CFU instilled. The invasive auxotrophic mutant (2457T Δ*aroC* CRP) showed the best survival. Although the number of live bacteria was rapidly decreasing, viable bacteria could be found even 4-days post-infection. The CRN form of the same mutant showed lower survival; in case the same bacterial cell number was used for the infection, no live bacteria were detected after 48 hours, however at a 100-fold higher infectious dose, live bacteria were found even after 4 days in the lung of one mouse. In comparison, at 10^6 CFU/mouse infectious dose both the invasive and the non-invasive rough mutants (*S. flexneri* 2a 2457T Δ*rfbF* CRP and CRN) were cleared from the lung already after 24 hours in repeated experiments. At higher dose, the

Δ*rfbF* CRN mutant was able to survive 48 hours, but was completely cleared by day 3. The low level of *in vivo* survival of the *S. flexneri* 2a 2457T Δ*rfbF* CRP confirms the results of the *in vitro* assay with HeLa cells, where the invasive rough strain showed impaired intracellular survival after 24 hours.

4.1.5. Virulence in the mouse lung model

To assess the level of attenuation as well as to find the optimal dose for immunization and challenge, 50% lethal doses (LD_{50}) were calculated from results of infections with different doses of bacteria in the mouse lung model (Table 7). The virulence of CRP auxotrophic and rough mutants of *S. flexneri* 2a 2457T was comparable; the LD_{50} of *S. flexneri* 2a 2457T Δ*aroC* and Δ*fbF* CRP was 8 and 7-times higher, respectively, than that of their parent strain. However, loss of the virulence plasmid decreased virulence to an undetectable level in case of both the smooth and rough backgrounds. Even the highest doses tested (10^8 CFU or $10^{7,5}$ CFU/mouse) elicited no lethality, therefore an exact LD_{50} value could not be established. This high level of attenuation of the CRN and phase II strains combined with the shorter persistence of the non-invasive bacteria *in vivo* allowed a 100-fold higher immunization dose.

Table 7 50% lethal dose (LD_{50}) of strains used in this study and degree of attenuation relative to the wild-type strain

		Attenuation
	LD_{50} (CFU)	relative to wild-type
***S. flexneri* 2a 2457T CRP (wild-type)**	2.19×10^6	-
***S. flexneri* 2a 2457T CRN**	$>10^8$	>45.66
2457T Δ*aroC* CRP	1.78×10^7	8.13
2457T Δ*aroC* CRN	$>10^8$	>45.66
2457T Δ*rfbF* CRP	1.54×10^7	7.03
2457T Δ*rfbF* CRN	$>10^8$	>45.66
***S. sonnei* 598 phase I**	1.69×10^6	-
***S. sonnei* 598 phase II**	$>10^{7,5}$	>18

4.2. Protective capacity of *S. flexneri* 2a Δ*rfbF* against homologous challenge in the mouse lung model

Series of attenuated *S. flexneri* 2a mutants lacking either O-antigens (Δ*rfbF* CRP), the invasion plasmid (Δ*aroC* CRN), both (Δ*rfbF* CRN) or none (Δ*aroC* CRP) of these antigens were used to immunize mice intranasally at sublethal doses. Subsequently groups of mice were challenged with lethal doses of a clinical isolate (*S. flexneri* 2a 544), belonging to the same serogroup (homologous challenge) and their survival was monitored (Fig. 21).

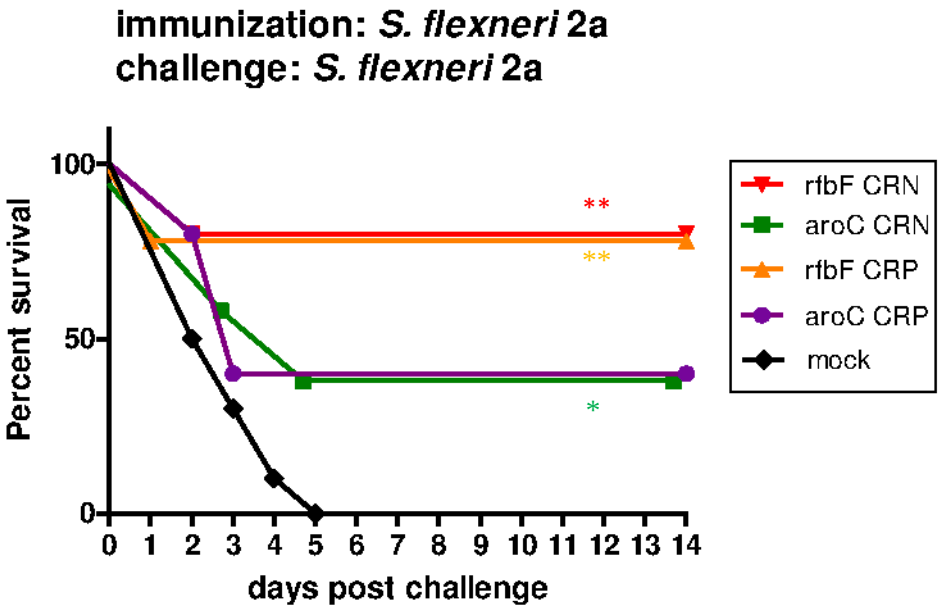


Figure 20 Protective capacity of *S. flexneri* 2a mutants. Groups of 6-8 weeks old BALB/c mice were immunized intranasally with CRP (10^6 CFU) or CRN (10^8 CFU) mutants twice with two-weeks interval. Control group received physiological saline. Challenge was performed two weeks following booster with 10^6 CFU of wild-type *S. flexneri* 2a 544 the same route. Survival was monitored for 14 days. Figure shows combined result of two experiments with 5 mice/group/experiment. Statistical analysis was performed with Log-rank (Mantel-Cox) test, significant p values are indicated with asterixes (*p<0.05, **p<0.01)

Naturally immunization with sublethal dose of the wild-type strain resulted in full protection against a homologous challenge (data not shown). Furthermore, as expected both the invasive and non-invasive vaccine strains expressing O-antigens (Δ*aroC* CRP and CRN) elicited partial, but statistically significant protection resulting in approximately 40% survival. However surprisingly the invasive rough mutant (Δ*rfbF* CRP) in spite of the lower in vivo survival and comparable level of attenuation indicated by the similar LD_{50} values provided even higher protection, than the smooth vaccine strain; 8 out of 10 mice survived challenge

with 100% lethal dose of homologous clinical isolate. Moreover the protection elicited by the rough strain was found to be irrespective of the invasiveness of the strain, immunization with Δ*rfbF* CRN (rough, non-invasive) mutant resulted in the same level of protection.

4.3. Cross protective capacity of *Shigella* mutants

4.3.1. Protection against heterologous challenge with *S. flexneri* 6 or *S. sonnei*

In order to assess the spectrum of protective capacity of rough, non-invasive *Shigella* mutants, challenge of immunized mice was performed with clinical isolates expressing heterologous O-antigens. In the first set of experiments mice were immunized with CRP and CRN mutants of smooth and rough *S. flexneri* 2a strains and subsequently infected with a lethal dose of *S. flexneri* 6 542 (Fig. 22. a).

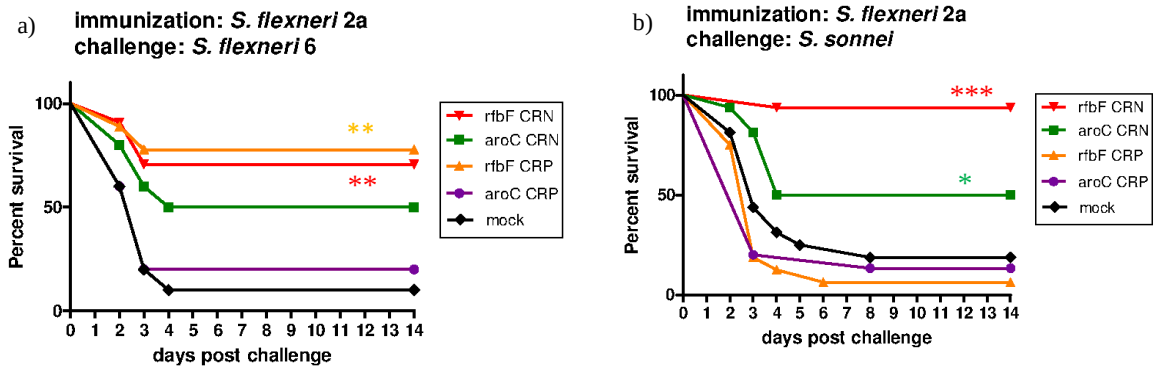


Figure 21 Cross-protection capacity of *S. flexneri* 2a mutants. Group of 5 6-8 weeks old BALB/c mice were immunized with CRP (10^6 CFU) or CRN (10^8 CFU) variants of *S. flexneri* 2a mutants twice in two-weeks interval. Two weeks after the booster, mice were challenged with a) 10^6 CFU of *S. flexneri* 6 542 or b) $10^{6,5}$ CFU of *S. sonnei*. Survival was monitored for 14 days. Figures show data from a) two or b) three experiments with 5-5 mice in each grou. Statistical analysis was performed with the Log-rank (Mantel-Cox) survival test. In case of significant difference compared to the mock-vaccinated mice, it is indicated as follows: *p<0.05, **p<0.01, ***p<0.001

Based on previous data from the literature, no or minimal cross-protection provided by the smooth strains was expected. When mice were vaccinated with a Δ*aroC* CRP (smooth invasive) mutant, no protection was registered against the heterologous challenge. The non-invasive counterpart of the strain (Δ*aroC* CRN) elicited partial, but statistically non-significant, 50% protection. However, similarly to the homologous challenge experiment, the rough vaccine strains provided a nearly complete protection against a *S. flexneri* 6 challenge (8/10 mouse survived), which was again independent of the presence of the invasion plasmid.

In the second set of immunizations, mice vaccinated by the same mutants were infected with a lethal dose of invasive *S. sonnei* 598 strain (Fig. 22. b). Immunization with the rough non-invasive mutant (Δ*rfbF* CRN) resulted in an almost 100% protection against a *S. sonnei* challenge, 14 out of 15 immunized mice survived the heterologous infection compared to 1/15 of the mock group. Interestingly the rough invasive strain (Δ*rfbF* CRP), highly effective against a heterologous *S. flexneri* 6 542 challenge, did not protect against *S. sonnei*, just as did not the smooth invasive mutant (Δ*aroC* CRP). The protection provided by the smooth non-invasive strain (Δ*aroC* CRN) was similar to that against *S. flexneri* 6, but in this set of experiments was found to be also statistically significant compared to the mock immunization. However importantly the survival of the Δ*aroC* CRN immunized mice remained significantly lower than the nearly complete survival of the rough non-invasive mutant immunized group (Δ*rfbF* CRN).

4.3.2. Cross protection provided by *S. sonnei* phase II immunization against *S. flexneri* 6 and *S. flexneri* 2a challenge

To corroborate the cross-protection elicited by the non-invasive rough mutant of *S. flexneri* 2a, a partially reversed experiment was performed. Mice were immunized with either the smooth, invasive phase I form of *S. sonnei* 598 or with the rough, non-invasive phase II variant. Subsequent challenge with a heterologous *S. flexneri* 6 isolate confirmed the previous results; vaccination with the wild-type strain did not elicit heterologous protection, while the rough, non-invasive strain (phase II form) lacking both the O-antigen and the virulence plasmid elicited high, in this case, full protection reproducibly (Fig. 23).

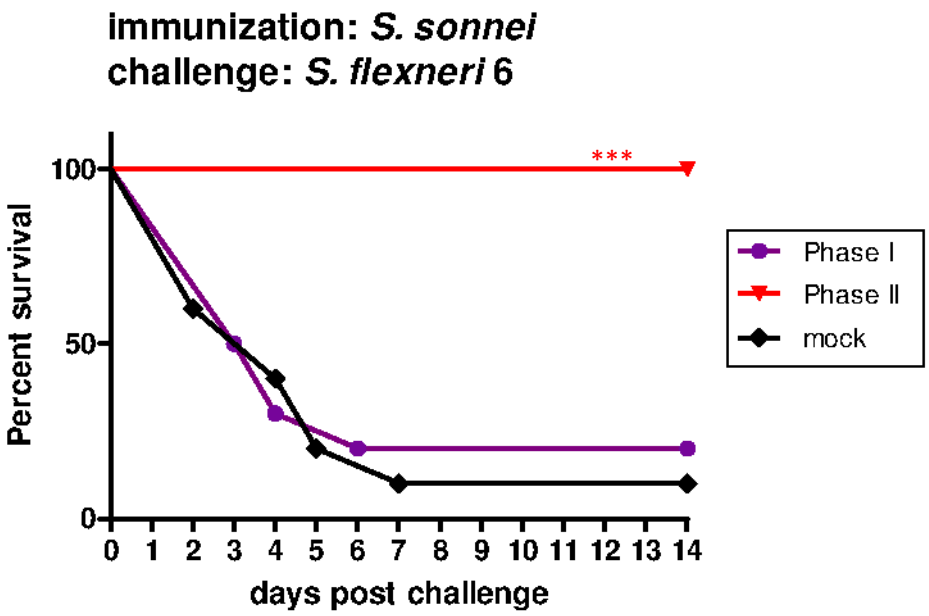


Figure 22 Protection provided by *S. sonnei* phase II variant against lethal dose of *S. flexneri* 6 542. Mice immunized twice intranasally with $10^{5.5}$ CFU of phase I or $10^{7,5}$ CFU of phase II varians of *S. sonnei* 598 were challenged with 10^{6} CFU of *S. flexneri* 6 542. Survival was monitored for 14 days. Survival curve shows result of two independent experiments with 5 mice/group/experiment. Statistical analysis was performed with Log-rank (Mantel-Cox) test. Significant difference between immunized and mock mice is indicated with asterix. ***p<0.001

In a parallel experiment, mice immunized with the *S. sonnei* vaccine strains were infected with a *S. flexneri* 2a clinical isolate. Although this challenge did not result in lethal infection, the infected mice showed signs of systemic infection. As an alternative read-out, weight was measured daily for 14 days post-infection (Fig.24)

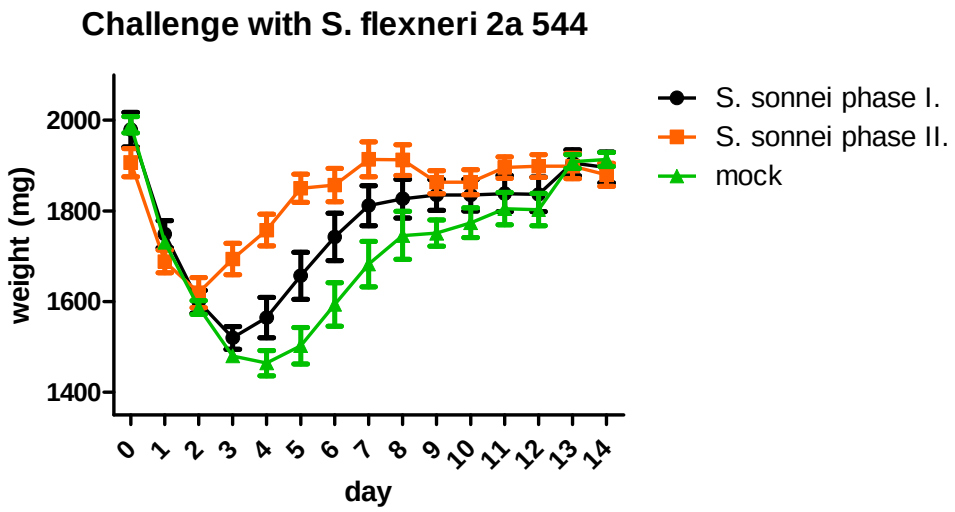


Figure 23 Cross-protection provided by phase I and phase II of *S. sonnei*. Groups of 5 mice were immunized intranasally twice with two weeks interval with sublethal doses (phase I $10^{5.5}$ CFU, phase II $10^{7.5}$CFU) of *S. sonnei* 598. Control group received saline. Two week following the last immunization mice were challenged with 10^6 CFU of wild-type *S. flexneri* 2a 544 via the same route. Weight of mice was monitored daily for 15 days at the same period of the day. Graph shows average of two independent experiments with 10 mice/group in total. Weights were compared with Mann-Whitney test each day. P values are the following: weights following *S. sonnei* phase II immunization compared to mock were significantly different on days 3-5 (p<0.0001), day 6 (p=0.0005), day 7 (p=0.0029), day 8 (p=0.0376), day 9 (p=0,0115); following *S. sonnei* phase I compared to mock immunization on day 4 (p=0.0279), day 5 (p=0.022), day 6 (p=0.0279); while those of *S. sonnei* phase II vs phase I on day 3 (p=0.0062), day 4 (p=0.0057) and day 5 (p=0.0041)

The weight loss following a heterologous challenge of mice immunized with phase II *S. sonnei* (lacking O-antigen and invasive potential) was statistically significantly less between days 3-9 compared to the weight loss of mock vaccinated mice. Similarly, weight loss of the two groups immunized either with the wild-type phase I *S. sonnei* or with sterile saline (mock) differed significantly from each other on days 4-6 post-infection suggesting some degree of protection elicited against the heterologous challenge. However, comparison of weights of phase I and phase II immunized mice revealed significant difference on days 3-5; the groups immunized with the rough non-invasive strain (phase II) showed weight loss only until day 2 p.i. and started gaining weight already on day 3, whereas mice immunized with sublethal dose of phase I *S. sonnei* lost more weight (average of 4.5 g on day 3), and the recovery started only on day 4 post-infection. The results suggest that in comparison to the smooth invasive counterpart, the phase II form of *S. sonnei* elicited higher protection against a heterologous *S. flexneri* 2a challenge.

4.4. Humoral immune response against the vaccine strains

The humoral immune response provoked by the vaccine strains was measured by detection of specific antibody levels by ELISA.

4.4.1. *Shigella*-specific antibodies raised upon immunizations

The serum IgG response as well as the specific mucosal IgA response from broncho-alveolar lavage fluid was determined by ELISA. The immunoreactivity was measured to the invasive smooth (2457T wild type or 2457T Δ*aroC* CRP) and to non-invasive rough (2457T Δ*rfbF* CRN) whole bacteria of serial dilutions of both samples recovered from mice vaccinated with various live attenuated *S. flexneri* 2a strains. In order to compare several ELISA experiments, as well as to compare results on different target bacteria exhibiting different coating characteristic, we calculated ratios of OD values gained with the different immune sera. The reactivity of sera of mice immunized with *S. flexneri* 2a ΔrfbF CRN was divided by the corresponding reactivity of sera from mice immunized with *S. flexneri* 2a Δ*aroC* CRP.

$$\frac{OD(\Delta rfbF\ CRN\ immune\ sera)}{OD(\Delta aroC\ CRPimmune\ sera)}$$

Therefore, values lower than 1 suggest higher reactivity of the Δ*aroC* CRP immune sera and vice versa.

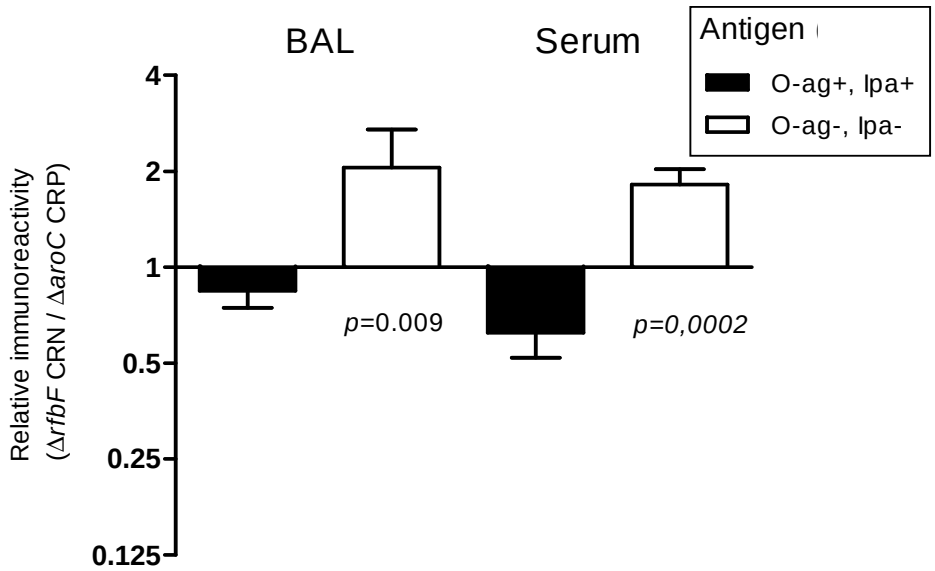


Figure 24 Reactivity to whole bacteria of mucosal IgA and serum IgG obtained from mice vaccinated with live attenuated *S. flexneri* 2a strains. BAL or serum samples were collected in pools following two immunizations with the smooth invasive (Δ*aroC* CRP) or with the double mutant (Δ*rfbF* CRN). Immune reactivity was determined on different target cells; expressing both O-antigen and Ipa-s or non of these major antigens. Reactivity was expressed as ratio (reactivity of the Δ*rfbF* CRN divided by the reactivity of the Δ*aroC* CRP sample) at the same dilution (BAL1:10, serum 1:400). Graphs show means with SEM of at least four experiments performed with sample obtained from independent vaccinations. Reactivity ratios were compared with non parametric Mann-Whitney test. P values are indicated on the graph.

As expected, both serum and BAL specimens obtained from mice vaccinated with the *S. flexneri* 2a 2457T Δ*aroC* CRP (expressing both major antigen complexes) were more reactive to the invasive smooth homologous target verifying that the O and Ipa antigens, indeed, dominate the immune response (Fig.25). On the other hand, loss of both immunodominant antigens on the vaccine strain (2457T Δ*rfbF* CRN) resulted in an improved reactivity to the homologous target strain devoid of these antigens. These results corroborate that there is a higher titre of antibodies against minor antigens following vaccination with strains expressing neither of the major antigenic complexes. Importantly this phenomenon was apparent in case of both serum IgG and mucosal IgA.

4.4.2. Detection of IgG isotypes from the serum of immunized mice

The relative amount of IgG2a and IgG1 antibodies specific to the corresponding vaccine strain were also measured in ELISA coated with whole cells.

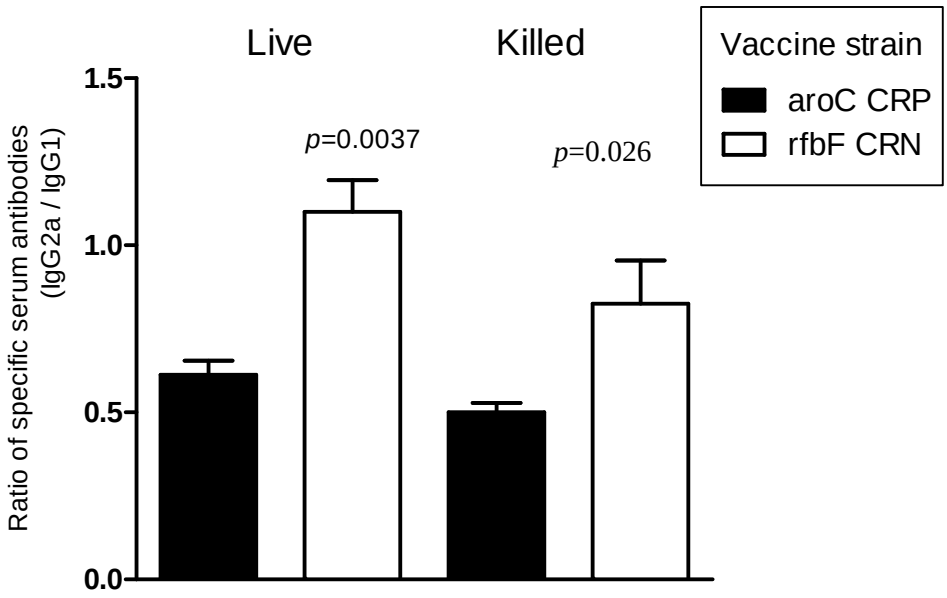


Figure 25 Ratio of IgG2a and IgG1 antibody levels detected in serum of mice immunized either with live *S. flexneri* 2a mutants or with the same amount (10^8CFU) of heat-killed lysate of these strains. Ratio of the reactivity of specific isotypes against the whole cell of corresponding vaccine strain in 1:400 dilution is shown on the graph. Statistical difference was calculated with the Mann-Whitney test. P values are indicated on the figure.

As shown on Figure 26 the IgG2a/IgG1 ratios differed significantly depending on the nature of the vaccine strains. In case of immunization with the non-invasive rough strain (*S. flexneri* 2a 2457T Δ*rfbF* CRN) an increased specific serum IgG2a/IgG1 ratio was observed (compared to that of the auxotrophic invasive mutant) implying a Th1 dominance of the immune response. In order to show that this difference originated from the different antigen repertoire on the bacterial surface rather than from differences in invasiveness, sera obtained from vaccination using both strains at the same CFU in heat-killed form were also investigated. The difference between the IgG2a/IgG1 ratios remained statistically significant upon heat-inactivation of the vaccine strains.

4.5. Identification of potential cross-protective antigens

In order to identify antigens responsible for the observed protection provided by the rough non-invasive strain, immune sera obtained upon vaccination with *S. flexneri* 2a 2457T Δ*rfbF* CRN was mixed with aliquots of bacterial lysate of the same strain and the resulting immune complexes were purified by protein G beads. Following eluation, proteins were separated electrophoretically and visualized. Proteins that were captured by antibodies against the double mutant but not (or significantly less) by sera of mock mice were excised and subjected to mass spectrometry based protein identification. MS experiments were done in collaboration with the Proteomics Research Group at the Biological Research Center of Hungarian Academy of Sciences in Szeged.

Table 8 List of identified proteins by mass spectrometry

NCBI accession number	Protein name	Species	MW	Exp #1		Exp #2		Exp #3	
				Peptide number	Sequence coverage	Peptide number	Sequence coverage	Peptide number	Sequence coverage
24111543	preprotein translocase subunit SecA	*Shigella flexneri* 2a str. 301	102217	3	5%				
24111558	pyruvate dehydrogenase subunit E1	*Shigella flexneri* 2a str. 301	99948	8	10%	16	22%		
24111559	dihydrolipoamide acetyltransferase	*Shigella flexneri* 2a str. 301	65878	15	33%	17	34%	4	10%
24112311	formate acetyltransferase 1	*Shigella flexneri* 2a str. 301	85554	3	5%				
24112636	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	*Shigella flexneri* 2a str. 301	96638	23	40%	7	12%	3	4%
24113309	putative outer membrane porin protein C	*Shigella flexneri* 2a str. 301	39628	3	11%				

	precursor								
24113600	outer membrane porin protein C	*Shigella flexneri* 2a str. 301	41377	13	47%	11	27%	6	21%
24113669	phosphate acetyltransferase	*Shigella flexneri* 2a str. 301	77466	3	6%				
24113841	outer membrane protein assembly complex subunit YfgL	*Shigella flexneri* 2a str. 301	41858	3	9%				
24114156	glycine dehydrogenase	*Shigella flexneri* 2a str. 301	105041	9	13%	9	14%	4	6%
24115265	elongation factor Tu	*Shigella flexneri* 2a str. 301	43427	2	7%			3	8%
38000008	outer membrane protein A precursor	*Shigella flexneri* 2a str. 301	35347					5	16%
56404015	IpaC, secreted by the Mxi-Spa secretion machinery	*Shigella flexneri* 2a str. 301	38838			21	69%		
56479896	glyceraldehyde-3-phosphate dehydrogenase	*Shigella flexneri* 2a str. 301	35681	2	16%	3	15%		
24114459	translation initiation factor IF-2	*Shigella flexneri* 2a str. 301	96520	8	16%				

The sole antigen identified in all three independent experiments with at least 10% protein coverage was outer membrane protein C (OmpC, NCBI accession number 24113600). Other proteins also identified in all 3 independent experiments were: bifunctional acetaldehyde-CaA/alcohol dehydrogenase (24112636), glycine dehydrogenase (24114156) and dihydrolipoamide acetyltransferase (24111559). A full list of identified proteins is provided in Table 8. Besides these hits, mouse immunoglobulin and protein G were also identified as contaminants.

5. Discussion

Current vaccine approaches (in general, as well as for *Shigella* in particular) usually rely on major antigens of the pathogens (191;192). In order to evade the immune response, however, evolutionary pressure has selected multiple immunologically distinct variants of these antigens, which form the basis of classifying pathogens into serotypes. Utilization of serotype-determining major antigens might therefore confer only partial protection against a pathogen, unless all serotypes can be included in the vaccine (e.g. in case of poliovirus vaccines). Combination of the most prevalent serotypes can give a relatively broad protection (176), however, this could be transient due to serotype replacement, i.e. less common serotypes emerge by filling the gap opened by the eradication of the vaccine serotypes. This necessitates vaccine optimization from time to time, for example by including additional serotypes in the multivalent vaccines (as experienced recently in case of pneumococcal vaccines) (193). Due to phenomena as antigenic competition and interference, as well as to financial considerations, however, the maximum number of serotypes possibly covered remains limited.

On the other hand, the various serotypes of a microbial pathogen share a considerable number of conserved antigens. The fact that they could have remained conserved implies that they are either not accessible for antibodies (i.e. are not protective antigens) and/or their function is so indispensable for pathogenesis that allows no modification in their antigenic structure. This is exemplified by the *Shigella* Ipa proteins, which are highly conserved (due to their sophisticated function in invasion) and very immunogenic. However, they still cannot elicit cross-protection, probably because they are only expressed upon contact to the target cell (194).

It has been shown earlier that the majority of antibodies generated upon an invasive *Shigella* infection are against the O-antigen and subsequently, (upon repeated infection) against invasion antigens (195;196). Given that Ipa-s are not considered protective and O-antigens are highly variable with as many as 50 different structural variants, the immunity elicited is usually restricted against the same O-serotype (homologous protection). Having evolved this high number of different O-types, *Shigella* pathogens can efficiently evade the immune response. Due to the role of O-antigen specific antibodies in the protective immune response,

current vaccine approaches in clinical phase (independent of the nature of the vaccine) rely on the presence of O-antigen and in order to elicit protection against the most important serotypes numerous serotypes would need to be combined (176).

We have proposed, that immunodominant antigens such as Ipa and O-antigens may hijack the immune response in a way that allows less antibodies to be raised against minor antigens, preventing the production of cross-protective antibodies during a natural infection (Fig. 27).

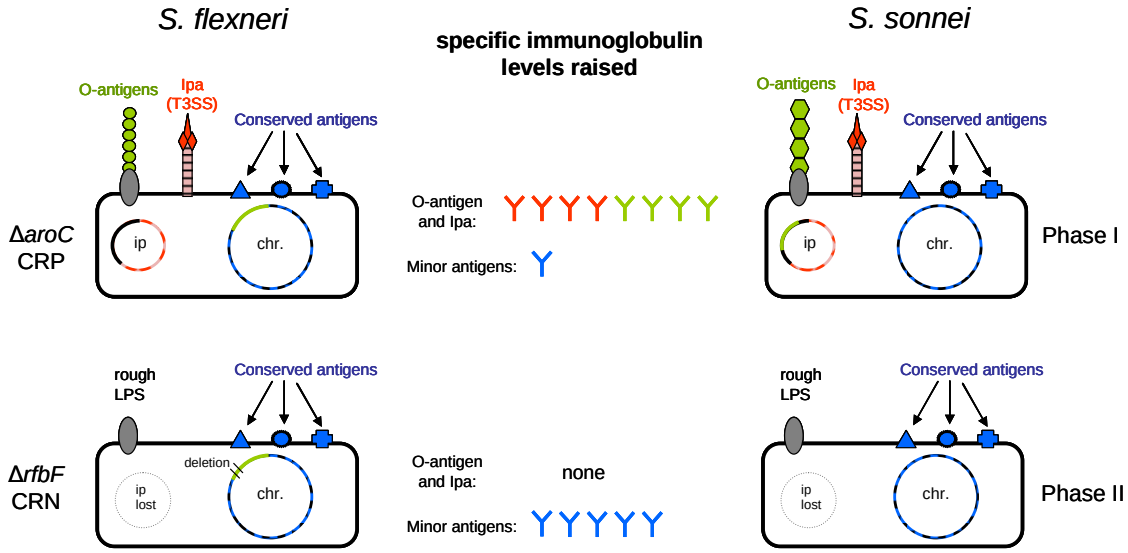


Figure 26 Schematic representation of antigens expressed by the various mutants used as well as proposed antibody response against these mutants. Green color represent O-antigen as well as antibodies specific to it. Analogously red shows Ipa-s with the corresponding antibody response. . Mutants expressing both major antigens (O-antigen and Ipa-s) trigger an antibody response mainly against these epitopes. „Minor" conserved antigens are indicated with blue. Strains lacking both O-antigen and Ipa-s induce higher antibody response against the minor shared epitopes. Ip invasion plasmid, chr.: chromosome, T3SS: type three secretion system

To corroborate our hypothesis, we generated a set of isogenic mutants of *S. flexneri* 2a strain 2457T lacking the invasion plasmid - hence Ipa-s - (Δ*aroC* CRN), the O-antigen (Δ*rfbF* CRP), both (Δ*rfbF* CRN) or none of these antigens (Δ*aroC* CRP). The rough as well as the non-invasive characteristic of the mutants was confirmed both genetically and phenotypically. All CRN mutants tested were unable to invade both HeLa and Int407 cell lines in contrast to their CRP counterparts. Furthermore, the *in vivo* survival of the tested strains in the mouse lung model revealed that the lack of O-antigen results in rapid clearance of the rough strains. When administered at 10^6 CFU dose, both invasive and non-invasive Δ*rfbF* mutants were cleared within 24 hours. Comparison of the virulence in the mouse lung model showed, that while the lack of the O-antigen (Δ*rfbF* CRP) results in a moderate, 7-fold higher LD_{50}, loss of the invasion plasmid (2457T CRN, Δ*aroC* CRN, Δ*rfbF* CRN) renders the bacteria essentially

avirulent, with no lethality and no visible signs of infection even at 10^8 CFU dose. We confirmed our findings with an isogenic *S. sonnei* phase I (smooth and invasive) and a rough non-invasive *S. sonnei* phase II strain pair. The lack of the invasion plasmid and the O-antigen rendered the phase II variant non-invasive on human epithelial cells, resulted in sensitivity to 50% human serum, as well as increased the lethal dose in the mouse model used at least 18-fold. Our results suggested that non-invasive mutants (especially those expressing no O-antigen) of *S. flexneri* 2a as well as *S. sonnei* are highly attenutated and may fulfill the requirements for vaccine candidates with respect to safety.

For the detection of the mutants' vaccine potential we used the mouse lung model. As Shigellae are human pathogens, there are only few animal-models considered predictive for the detection of virulence as well as protective capacity of vaccine candidates.

The gold standard for the detection of virulence is the guinea pig keratoconjunctivitis test (Serény test) (166). The Serény test can be useful for protection studies as well; avirulent vaccine candidates applied intragastrically, intranasally can prevent keratoconjunctivitis caused by a virulent strain (197). Another recent guinea pig model applies virulent strains intrarectally to elicit dysentery (198). All these models with guinea pigs however are not routinely used for vaccine candidate testing due to ethical considerations.

Ileal-ligated loop model in rabbits has been established as well for studying shigellosis, however the applicability of the model is very low and the model may be too artificial to assess protective efficacy of vaccine candidates.

Non-human primates (macaques, chimpanzees and baboons) are naturally susceptible to intestinal infection with *Shigella*, their symptoms, immunological reaction as well as histological changes due to the bacterium resemble human infections. Therefore non-human primate protection studies are considered as highly predictive for vaccine studies (199). However in contrast to the low inoculum necessary for clinical symptoms in human, 10^{10}-10^{11} CFU (applied intragastrically with bicarbonate buffer) is needed to elicit shigellosis in non-human primates, suggesting a partially different pathomechanism (200).

Mice are naturally resistant to intestinal *Shigella* infections, although intragastric instillation of high dose *Shigella* till the age of 4-5 days results in intestinal inflammation (201). However this model cannot be used for protection studies due to the short period of susceptibility.

A simplified but easily accessible model for studying vaccine capacity of *Shigella* strains is the mouse lung model. The mucosal similarities (epithelium, lymphoid follicles) between the lung and the intestine allowed numerous studies of the pathogenesis and immunology of

shigellosis (136). If given intranasally *Shigella* invades the bronchiolar and alveolar epitelium resulting in bronchitis and pneumonia. The infection remains localized to the lung (202). Although the lack of microbiota in the lung proposes the advantage, that no previous exposure to bacteria alters the immune response, however the “sterile” environment - besides the obviously different target organ - is one of the disadvantages of the mouse lung model. Despite these differences, up to date the mouse lung model has been the most widely used animal model for shigellosis (including early testing of vaccine candidates currently in clinical phase), therefore this model was used in our experiments as well.

We showed that deletion of major antigens (Ipa and O-antigens) not only rendered the mutants (2457T Δ*rfbF* CRN and *S. sonnei* 598 phase II) avirulent in the animal model, but immunization with the 2457T Δ*rfbF* CRN mutant resulted in significant protection against lethal challenge with the homologous, *S. flexneri* 2a strain. Furthermore the absence of the immunodominant antigens highly improved cross-protective potential of live vaccine strains. Significant protection was provided against a heterologous *S. flexneri* 6 and *S. sonnei* challenge by the non-invasive rough mutant 2457T Δ*rfbF* CRN. Similarly, immunization with *S. sonnei* 598 phase II form protected 10/10 mice against a 90% lethal dose of *S. flexneri* 6 and resulted significantly lower weight loss of immunized mice during a sublethal infection with *S. flexneri* 2a, than immunization with the wild-type *S. sonnei* phase I strain. Furthermore, it was also shown that relative immunogenicity of putative shared conserved antigens (both in serum and bronchoalveolar lavage) had been increased in the absence of the major antigens on the vaccine strain. Earlier, very similar conclusions has been reached for related bacteria, i.e. extraintestinal pathogenic *E. coli* (203) and *S. enterica* (204). In both bacterial species the loss of O-antigens in the vaccine strains was shown to trigger a higher antibody response to shared (i.e., serotype independent) minor antigens. This phenomenon (reviewed by Nagy et al (191)) is not restricted to enterobacterial pathogens as for instance immunization with capsule-less pneumococci was also shown to trigger a serotype independent protection (205).

Furthermore, we observed that immunization with the non-invasive rough mutant (vs. isogenic smooth, invasive strain) triggered a response with an isotype pattern of specific serum antibodies typical for a Th1-directed immune response (i.e, IgG2a dominance). This observation was especially surprising given that immunizations were performed in Balb/C mice, which is considered to be a Th2-prone mouse strain (206). Moreover, opposite direction

in Th1/Th2 balance was reported by others when live vs. dead *Salmonella typhimurium* strains were used for immunization in the same mouse strain (207;208). The authors of these studies concluded that the invasive phenotype was responsible for the Th1 dominance, which was inverted to Th2 response upon heat inactivation (i.e. loss of invasiveness). In our case, however, immunization with the noninvasive rough *Shigella* strain retained the capacity to generate higher IgG2a levels even when both vaccine strains were applied at the same CFU in heat-killed form. This observation argues that the antigenic composition of the vaccine strains might also be crucial factor in determining the IgG subclasses in this case. In fact mouse IgG1 is thought to correspond to human IgG4, which is a typical subclass of antibodies generated against carbohydrate antigens. On the other hand, mouse IgG2a is similar to human IgG1, characteristic for anti-protein antibodies. Therefore, the presence or lack of the bulk of O-antigens in the vaccine strains might be responsible for the dominant IgG isotypes in the immune sera, suggesting again the higher potential of raising specific antibodies against conserved protein antigens in the rough background. Since mouse IgG2a antibodies can activate complement, whereas IgG1 cannot, it is tempting to speculate that not only the qualitative, but also the quantitative antibody repertoire induced by the rough non-invasive mutant contributes to the superior protection by this vaccine strain. Certainly, the importance of isotype dominance induced by whole cell vaccines warrants further investigations in order to optimize specific protection against bacterial pathogens.

Attempts were made to identify the nature of the conserved antigens whose higher immunogenicity might be responsible for the improved cross-protection upon vaccination. Besides OmpC, we repeatedly identified three cytoplasmic enzymes: bifunctional acetaldehyde-CoA/alcohol dehydrogenase, glycine dehydrogenase and dihydrolipoamide acetyltransferase. Higher immune response to these enzymes (as to any other shared proteins irrespective of cellular localization) is not surprising based on the hypothesis depicted on Fig. 15. However, given the intracellular localization, it is not likely that antibodies against cytoplasmic enzymes would contribute to protection. Nevertheless, it has been shown by several studies that metabolic enzymes could not only be present on the surface of bacterial (209) pathogens, but in some instances were shown to serve as targets of protective antibodies (210). Based on these observations, protection mediated by these proteins cannot be ruled out and certainly warrants further investigation. The role of major porin OmpC as a potential target responsible for mediating serotype-independent cross-protection appears to be more straightforward. This antigen is embedded in the outer membrane and generally considered to

be masked by more superficial structures (i.e. smooth LPS) limiting its accessibility for antibodies. Some recent studies, however, showed protection in murine model against lethal infections by *S. flexneri* 2a following immunization with the highly related purified OmpA protein (211). Similarly, DNA vaccination of mice with DNA encoding *K. pneumoniae* OmpA elicited opsonophagocytic antibodies and protection against lethal i.p. challenge by wild-type *K. pneumoniae* (212). Moreover, an analogous study on the search for conserved antigens responsible for serotype-independent protection among heterologous *S. enterica* strains also identified both OmpA and OmpC as candidates for cross-protective antigens (204).

Although it is difficult to directly extrapolate data from an extraintestinal animal model to potential human applications, high attenuation in a human host of non-invasive *Shigella* was proven by previous clinical studies with the T_{32}-Istrati strain (165). While by this vaccine a heterologous protection was also repeatedly demonstrated one should emphasize that only limited similarities exist between the T_{32}-Istrati strain and any mutants used in this study. As far as it has been investigated in T_{32}-Istrati the attenuation was exclusively due to a deletion in the invasion plasmid resulting in non-invasive phenotype of the vaccine strain (168). Contrary to this, our most effective vaccine strain was not only non-invasive, but also rough (lacking both the O-antigen and the invasion plasmid). As fas as surface antigens are concerned, the closest to the T_{32}-Istrati strain was our smooth but non-invasive 2457T Δ*aroC* CRN mutant expressing smooth LPS but no Ipa-s. However our strain beyond being non-invasive also had a further attenuating characteristic, with the deletion of the *aroC*. Nevertheless it was noteworthy that this strain (2457T Δ*aroC* CRN), just as claimed for the T_{32}-Istrati, did evoke significant heterologous protection against a heterologous *S. sonnei* challenge, compared to the invasive counterpart. Still, the non-invasive and rough mutants elicited an even higher protection, which was statistically significant against all heterologous challenges tested. Comparing these figures corroborate our hypothesis, that the less major antigens (O-antigen, Ipa) the vaccine expressed, the better heterologous protection is induced.

Our study and the vaccine approach we propose have some limitations and obviously still have some questions to be addressed.

One of the major limitations is the animal model used. As discussed previously to assess the vaccine capacity of our mutants, a mouse lung model was used, in which the pathological process took place extraintestinally. Nevertheless several studies have shown before that

certain aspects of shigellosis can indeed be assessed in this model (136;137;145). It is particularly true, if these experiments are only meant to establish some basic principles, such as our findings showing that cross-immunity indeed can be provoked *in vivo* as well between unrelated *Shigella* serotypes. Further studies increasingly involving primates and eventually humans should answer how these promising observations can be translated into human use.

Another question remaining unanswered is whether with the high level of attenuation the non-invasive rough mutants has remained immunogenic enough to provoke strong immune response in humans. With any vaccines, but particularly with those envisioned to be given per os, persistence to provide a sufficient antigen load is a reason for concern. In case of the T_{32}-Istrati vaccine multiple extremely high doses were necessary to achieve protection (165). Unless experimentally assessed, one cannot predict with certainty how a rough, non-invasive strain would behave once given orally. However a recent report showed that rough strains adhered to intestinal human epithelial cells more efficiently than their isogenic smooth form (213). Although we found that rough 2457T Δ*rfbF* mutants showed higher adherence to Int407 but not to HeLa cells, the difference between the rough and the smooth strains remained statistically insignificant. Hence, in case of a higher adherence, our rough vaccine strains may persist longer, than the T_{32}-Istrati providing superior antigen load.

As we aimed to induce cross-protection via immune reaction against conserved antigens (probably conserved between *Shigella* and *E. coli* strains as well), the vaccine may also disturb the homeostasis of the gut microbiota. Mucosal immune response is – by a yet not fully elucidated mechanism – efficiently eliminated to antigens of commensal intestinal bacteria. Most healthy individuals have serum antibody titer against conserved *E. coli* antigens, e.g. outer membrane proteins (214) or LPS inner core (215) without any known detrimental effect on commensal *E. coli*. Nevertheless these antibodies might contribute to immunity against invasive *E. coli* infections. Whether presence of the normal microbiota will pose any problems to induce an effective response in the human gut (unlike in the sterile mouse lung) needs to be addressed as well. There are experimental data in non-human primates suggesting that the different gut microbiota of macaques results in different susceptibility and importantly in different immune response against oral immunization with attenuated *Shigella* vaccine candidate (216)

Despite these limitations – answering the raised questions was beyond the scope of our work - evidence is provided that both attenuating mutations, i.e. loss of O-antigens as well as the

non-invasive phenotype alone is responsible for a highly attenuated virulence. Given that both mutations are mediated by deletion of corresponding genes, the likelihood of reversion to a virulent phenotype of the proposed vaccine strain is negligible; hence such double mutants would fulfill safety regulations. On the other hand, live oral vaccines are relatively cheap to manufacture, and require no trained medical personnel for administration, which are important factors when considering the target population in endemic countries. Therefore, we feel that mutants with the above-described phenotype might serve the basis for broadly protective live *Shigella* vaccines in endemic regions.

List of publications related to the present work

Szijártó V, Hunyadi-Gulyás E, Emődy L, Pál T, Nagy G. Cross-protection provided by live *Shigella* mutants lacking major antigens. Int J Med Microbiol **2013 May**; 303(4):167-75. (IF: 4.173)

List of additional publications

Schneider G, Dobrindt U, Middendorf B, Hochhut B, **Szijártó V**, Emődy L, Hacker J. Mobilisation and remobilisation of a large archetypal pathogenicity island of uropathogenic *Escherichia coli in vitro* support the role of conjugation for horizontal transfer of genomic islands. BMC Microbiol **2011**; 11:210. (IF: 3.04)

Szijártó V, Pál T, Nagy G, Nagy E, Ghazawi A, al-Haj M, El Kurdi S, Sonnevend Á.. The rapidly emerging ESBL-producing *Escherichia coli* O25-ST131 clone carries LPS core synthesis genes of the K-12 type. FEMS Microbiol Lett **2012 Jul**; 332(2):131-6. (IF: 2.044)

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