Bernardini et al. (Infection and Immunity 69(2): 1072-1083, 2001) describe mutants of *Shigella flexneri* 5, which are an *aroC* mutant and a double *purE aroC* mutant.

Levine et al. (Behring Institute Mitteilungen 98: 120-123, 1997) described an attenuated *S. flexneri* 2a strain CVD 1203 which harbors mutations in chromosomal gene *aroA* and plasmid gene *virG*, as well as an *S. flexneri 2a* vaccine candidate CVD 1205 that harbors a deletion mutation in *guaB-A* rendering it defective in nucleic acid synthesis, and a deletion mutation in *virG*.

It is the objective of the present invention to provide improved Shigella vaccines, in particular for the prevention of diarrheal diseases that are highly relevant for travelers to endemic countries and young children living in developing countries. Such vaccines can be based on a live attenuated *Shigella flexneri* vaccine strain able to heterogeneously express antigens derived from different pathogens and induce broad protection against bacterial pathogens, and particularly shigellosis.

SUMMARY ~~OF THE INVENTION~~

The object is solved by the subject matter as claimed.

According to the disclosure~~invention~~ *there is provided a live attenuated Shigella* vaccine, which is based on a rough Shigella strain lacking LPS O antigen.

In one aspect, there is provided a live attenuated Shigella vaccine that is cross-protective against different serotypes and species of Shigella comprising a Shigella strain which is rough and non-invasive by a deletion of the *rfbF* gene, and a deletion of the *ipaB* and *ipaC* genes on the invasion plasmid.

Specifically, the Shigella strain is non-invasive by mutagenesis, in particular a mutation of the invasion plasmid.

Specifically the vaccine according to the disclosure~~invention~~ is attenuated by mutagenesis of one or more genes involved in the LPS synthesis, transport and expression, preferably selected from the group consisting of genes in the cluster of the *rfb* operon, e.g. located on the chromosome between Gnd (6-phosphogluconate dehydrogenase, 2089155-2090561) and GalF (UTP-glucose-1-phosphate uridyltransferase, 2101928-2102821) genes of the *Shigella flexneri* 2a 2457T strain (Wei et al. Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect Immun. 2003 May;71(5):2775-86.) or located on the virulence plasmid (*Shigella sonnei).* Specifically preferred is the mutagenesis of one or more genes within the *rfb/wbb* gene cluster encoding O-antigen synthesis, *waaL* encoding the O-antigen ligase, *wzx* encoding O-antigen flippase involved in O-antigen transport, *wzy/rfc* involved in O-antigen polymerization, genes within the *rfa/waa* gene cluster encoding

LPS-core synthesis, regulatory genes affecting O-antigen expression, such as *rfaH*, or loss of function(s) of which results in at least 90% reduction in the expression of O-antigens.

According to a specific embodiment, said mutagenesis is by a deletion of one or more of the *rfb* F, D, C, E, J and/or I genes, or a deletion of a part thereof, or corresponding genes in various Shigella serotypes. Alternatively, mutagenesis by inactivation, e.g. transient, conditional or constitutive inactivation, may be employed.

Said Shigella strain is preferably selected from the genus Shigella, e.g. from any Shigella serotypes or species, in particular *S. flexneri, S. sonnei, S. dysentheriae and S. boydii.*

Specifically, said Shigella strain expresses outer membrane proteins, which can induce cross-reactive antibodies, in particular conserved proteins, including OmpC, OmpA and OmpX, or those encoded on the invasion plasmid.

The vaccine according to the disclosure~~invention~~ is particularly cross-protective against different serotypes and species of Shigella, in particular against any of *S. flexneri 2a*, *S. flexneri 6* and *S. sonnei*, or enteroinvasive *Escherichia coli.*

According to a specific ~~aspect~~embodiment, said Shigella is non-invasive by further mutagenesis of the invasion plasmid, in particular a mutation of the invasion plasmid, which comprises a deletion of the *ipaB* and/or *ipaC* and/or other *ipa* genes.

Specifically, said Shigella comprises a recombinant endogenous invasion plasmid incorporating at least one gene encoding a heterologous antigen to secrete said antigen or to express said antigen on the bacterial cell surface.

Preferred embodiments refer to such vaccine, wherein said antigen is a protective antigen derived from a pathogen, e.g. selected from the group consisting of

- a bacterial antigen preferably a toxin or colonization factor,

-a viral antigen, preferably from a pathogen causing enteral or mucosal infections,

-a fungal antigen, preferably from a pathogen causing enteral or mucosal infections, and

- a parasitic antigen, preferably from a pathogen causing enteral infections.

Specifically, the bacterial antigen is originating from enteropathogenic bacteria, preferably selected from the group consisting of

a. *E. coli* antigens, in particular an enterotoxin selected from the group consisting of LTB, mutated LTA and ST of ETEC, subunits, or fusions thereof, antigens from enteroaggregative *E. coli* (EAEC), or Shiga-like toxin 1 or 2
b. *Campylobacter jejuni* antigens,
c. *Clostridium difficile* antigens, specifically toxins A and B
d. *Vibrio cholera* antigens, specifically the CT-B antigen, and
e. mutants or fusion proteins of a), b) c) or d).

Specifically, the bacterial antigen is an enterotoxin (ETEC) comprising the B subunit of heat labile toxin (LTB), the heat stable toxin (ST) or subunits or fusions thereof, preferably LTB/STm comprising an STm with an amino acid sequence as shown in SEQ ID 1, which optionally excludes the wild-type sequence of human ST.

In particular, said ETEC antigen is a fusion protein of the B subunit of LT and mutant ST, preferably a fusion protein LTB/STm with an amino acid sequence as derived from Figure 7 SEQ ID 11-18 (LTA-promoter-LTB-ST-LTB terminator nucleotide and LTB-ST amino acid sequences for 4 constructs), for example with ST mutations at position 13 and/or 12, such as P13F or P13G, and/or N12R or N12K).

Specifically, the viral antigen is originating from diarrheal viruses, preferably selected from the group consisting of rotaviruses and Norwalk virus (caliciviruses).

Specifically, the parasite antigen is originating from diarrhea-causing protozoa, preferably selected from the group consisting of *Giardia lamblia*, Cryptosporidium species and *Entameba histolytica*.

Specifically, the fungal antigen is originating from diarrhea-causing fungi, preferably selected from the group consisting of *Blastomyces dermatiditis* and Histoplasma spp.

According to a specific ~~aspect~~ embodiment of the disclosure~~invention~~, the Shigella vaccine strain further comprises a deletion of an essential chromosomal gene and an insertion of said gene into the invasion plasmid, in particular the *ppa* gene, or any of *accD*, *acpS*, *dapE*, *era*, *frr*, *ftsI*, *ftsL*, *ftsN*, *ftsZ*, *infA*, *lgt*, *lpxC*, *msbA*, *murA*, *murI*, *nadE*, *parC*, *proS*, *pyrB*, *rpsB*, *trmA*, *rho* and *rhoL*.

Yet, according to a specific embodiment of the disclosure~~invention~~, the vaccine is provided for use in the prophylaxis or immunoprophylaxis of a subject to prevent infectious diseases, in particular enteral disease, such as diarrheal disease. In one aspect, there is provided a vaccine as described herein for use in the prophylaxis of a subject to prevent infectious diseases. Specifically, the disease is selected from the group consisting of Shigellosis, dysentery and diarrhea.

According to the disclosure~~invention~~, there is further provided a method of preventing infectious disease in a subject, in particular enteral disease, specifically by vaccination and immunizing said subject, respectively.

Specifically, said enteral disease is caused by any Shigella serotype or species.

Preferably, said (immune) prophylaxis comprises administration of the vaccine in a mucosal or oral formulation.

Specifically, the vaccine is administered orally or intranasally.

A specific embodiment refers to a vaccine for use according to the disclosure~~invention~~, wherein

- a polyvalent vaccine is used expressing protective antigens of Shigella and at least one other one of a species other than Shigella by the incorporation of a protective antigen of said pathogen into the endogenous modified recombinant invasion plasmid, and wherein

- said infectious disease is caused by any Shigella serotype or species and/ or said pathogen.

According to a further ~~aspect~~ embodiment of the disclosure~~invention~~, there is provided a Shigella strain, which is a *S. flexneri* 2a strain, such as *S. flexneri* 2a 2457T, with a deletion of the *rfbF* and at least one of the *ipaB* and/or *ipaC* genes, or a deletion of essential parts thereof.

In one aspect, there is provided a Shigella strain, which is a *S. flexneri* 2a strain comprising a deletion of the *rfbF* gene, and the *ipaB* and *ipaC* genes on the invasion plasmid.

Specifically, said Shigella strain may further comprise a deletion of an essential chromosomal gene and an insertion of said gene into the invasion plasmid.

Preferably, said Shigella strain comprises a recombinant invasion plasmid incorporating at least one gene encoding a heterologous antigen to express and/or secrete said antigen.

Yet, according to a further embodimentaspect of the disclosureinvention, there is provided a recombinant plasmid vector based on a mutated Shigella invasion plasmid comprising a nucleotide sequence encoding at least one heterologous antigen, wherein the plasmid is mutated in at least one of the *ipaB* and/or *ipaC* genes. This specifically refers to a mutation for the deletion and/ or inactivation of a non-coding or coding region, such as regulatory sequences operably linked to a gene and a gene, respectively, preferably a deletion of the genes that renders the bacterial host cell non-invasive, in particular a deletion of the *ipaB* and/ or *ipaC* genes, or a deletion of a (substantial) part thereof.

A further specific embodimentaspect of the disclosureinvention relates to a bacterial host cell comprising the vector according to the disclosureinvention, wherein said host cell is specifically selected from the genera Shigella, Escherichia, Salmonella, Campylobacter or Yersinia.

Said host cell specifically comprises a mutation in the endogenous invasion plasmid. Specifically, the vector according to the disclosureinvention is an endogenous invasion plasmid, i.e. a plasmid endogenous or homologous to the host cell.

FIGURES

Figure 1. **Cross-protective capacity of live attenuated non-invasive strains of *S. flexneri* and *S. sonnei* lacking LPS O-antigen synthesis.**

Groups of 8 week-old BALB/c mice were immunized intranasally with CRP (10^6 CFU) and CRN (10^8 CFU) variants of *S. flexneri* 2a (a and b) mutants or alternatively phase I ($10^{5.5}$ CFU) and phase II ($10^{7.5}$ CFU) variants of *S. sonnei* (c) twice with two week-intervals. Control groups were mock vaccinated with saline. Subsequently, mice were challenged with either 10^6 CFU of wild-type *S. flexneri* 6 (a and c) or $10^{6.5}$ CFU of wild-type *S. sonnei* (b) via the same route. Survival was monitored subsequently for 14 days. Figures show combined data of three (b) or two (a and c) independent experiments with 5 mice in each group and repeat. Statistical analysis of the survival curves was performed by the Log-rank (Mantel-Cox) test. In case survival was significantly different from that of mock-vaccinated mice, the *p* value is shown on the graph.

CLAIMS

1. A live attenuated Shigella vaccine, which is based on a rough Shigella strain lacking LPS O-antigen, which is non-invasive by a mutation of the invasion plasmid.

2. Vaccine according to claim 1, which is attenuated by mutagenesis of one or more genes involved in the LPS synthesis, transport and expression, preferably selected from the group consisting of genes in the cluster of the *rfb* operon, or one or more genes within the *rfb/wbb* gene cluster encoding O-antigen synthesis, *waaL* encoding the O-antigen ligase, *wzx* encoding O-antigen flippase involved in O-antigen transport, *wzy/rfc* involved in O-antigen polymerization, genes within the *rfa/waa* gene cluster encoding LPS-core synthesis, regulatory genes affecting O-antigen expression, such as *rfaH*, or loss of function(s) of which results in at least 90% reduction in the expression of O-antigens.

3. Vaccine according to claims 1 or 2, wherein said Shigella strain is selected from the genus Shigella, in particular *S. flexneri, S. sonnei, S. dysentheriae and S. boydii.*

4. Vaccine according to any of claims 1 to 3, which is cross-protective against different serotypes and species of Shigella, in particular against any of *S. flexneri 2a*, *S. flexneri 6* and *S. sonnei*, or enterinvasive *Escherichia coli*.

5. Vaccine according to any of claims 1 to 4, wherein said mutation of the invasion plasmid comprisesa deletion of the *ipaB* and/or *ipaC* and/or other *ipa* genes.

6. Vaccine according to any of claims 1 to 5, wherein said Shigella comprises a recombinant endogenous invasion plasmid incorporating at least one gene encoding a heterologous antigen to secrete said antigen or to express said antigen on the bacterial cell surface.

7. Vaccine according to claim 6, wherein said antigen is selected from the group consisting of

- a bacterial antigen preferably a toxin or colonization factor,
- a viral antigen, preferably from a pathogen causing enteral or mucosal infections,
- a fungal antigen, preferably from a pathogen causing enteral or mucosal infections, and
- a parasitic antigen, preferably from a pathogen causing enteral infections.

8. Vaccine according to claim 7, wherein the bacterial antigen is an enterotoxin (ETEC) comprising the B subunit of heat labile toxin (LTB), the heat stable toxin (ST) or subunits or fusions thereof, preferably LTB/STm comprising a STm with an amino acid sequence as shown in SEQ ID 1.

9. Vaccine according to any of claims 1 to 8, wherein said Shigella further comprises a deletion of an essential chromosomal gene and an insertion of said gene into the invasion plasmid.

10. Vaccine according to any of claims 1 to 9, for use in the prophylaxis of a subject to prevent infectious diseases, preferably enteral disease.

11. Vaccine for use according to claim 10, wherein the vaccine is administered orally or intranasally.

12. Vaccine for use according to claim 10 or 11, wherein

- a polyvalent vaccine is used expressing protective antigens of Shigella and at least one pathogen of a species other than Shigella by the incorporation of a protective antigen of said pathogen into the endogenous invasion plasmid, and wherein
- said infectious disease is caused by any Shigella serotype or species and/ or said pathogen.

13. Shigella strain, which is a *S. flexneri* 2a strain with a deletion of the *rfbF* and at least one of the*ipaB* and/or *ipaC* genes, or a deletion of essential parts thereof.

14. Shigella strain according to claim 13, which comprises a recombinant invasion plasmid incorporating at least one gene encoding a heterologous antigen to express and/or secrete said antigen.

15. A recombinant plasmid vector based on a mutated Shigella invasion plasmid comprising a nucleotide sequence encoding at least one heterologous antigen, wherein the plasmid is mutated in at least one of the *ipaB* and/or *ipaC* genes.

CLAIMS

1. A live attenuated Shigella vaccine that is cross-protective against different serotypes and species of Shigella comprising a Shigella strain which is rough and non-invasive by a deletion of the *rfbF* gene, and a deletion of the *ipaB* and *ipaC* genes on the invasion plasmid.

2. Vaccine according to claim 1, wherein said Shigella strain is selected from the genus Shigella, in particular *S. flexneri, S. sonnei, S. dysenteriae and S. boydii.*

3. Vaccine according to claim 1 or 2, which is cross-protective against any of *S. flexneri 2a*, *S. flexneri 6* and *S. sonnei*, or enteroinvasive *Escherichia coli*.

4. Vaccine according to any of claims 1 to 3, wherein said invasion plasmid is a recombinant endogenous invasion plasmid incorporating at least one gene encoding a heterologous antigen to secrete said antigen or to express said antigen on the bacterial cell surface.

5. Vaccine according to claim 4, wherein said antigen is selected from the group consisting of

- a bacterial antigen, preferably a toxin or colonization factor,
- a viral antigen, preferably from a pathogen causing enteral or mucosal infections,
- a fungal antigen, preferably from a pathogen causing enteral or mucosal infections, and
- a parasitic antigen, preferably from a pathogen causing enteral infections.

6. Vaccine according to claim 5, wherein the bacterial antigen is an enterotoxin (ETEC) comprising the B subunit of heat labile toxin (LTB), the heat stable toxin (ST) or subunits or fusions thereof, preferably LTB/STm comprising a STm with an amino acid sequence as shown in SEQ ID 1.

7. Vaccine according to any of claims 1 to 6, wherein said Shigella further comprises a deletion of an essential chromosomal gene and an insertion of said gene into the invasion plasmid.

8. Vaccine according to any of claims 1 to 7, for use in the prophylaxis of a subject to prevent infectious diseases.

9. Vaccine for use according to claim 8, to prevent enteral disease.

10. Vaccine for use according to claim 8 or 9, wherein the vaccine is administered orally or intranasally.

11. Vaccine for use according to any of claims 8 to 10, wherein

- a polyvalent vaccine is used expressing protective antigens of Shigella and at least one pathogen of a species other than Shigella by the incorporation of a protective antigen of said pathogen into the endogenous invasion plasmid, and wherein

- said infectious disease is caused by any Shigella serotype or species and/ or said pathogen.

12. Shigella strain, which is a *S. flexneri* 2a strain comprising a deletion of the *rfbF* gene, and the *ipaB* and *ipaC* genes on the invasion plasmid.

13. Shigella strain according to claim 12, wheren said invasion plasmid incorporates at least one gene encoding a heterologous antigen to express and/or secrete said antigen.

14. Shigella strain according to claim 13, wherein the heterologous antigen is a bacterial enterotoxin (ETEC) comprising the B subunit of heat labile toxin (LTB), the heat stable toxin (ST) or subunits or fusions thereof, preferably LTB/STm comprising a STm with an amino acid sequence as shown in SEQ ID 1.