

Vaccines against human diarrheal pathogens

Current status and perspectives

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Worldwide, nearly 1.7 billion people per year contract diarrheal infectious diseases (DID) and almost 760 000 of infections are fatal. DID are a major problem in developing countries where poor sanitation prevails and food and water may become contaminated by fecal shedding. Diarrhea is caused by pathogens such as bacteria, protozoans and viruses. Important diarrheal pathogens are *Vibrio cholerae*, *Shigella* spp. and rotavirus, which can be prevented with vaccines for several years. The focus of this review is on currently available vaccines against these three pathogens, and on development of new vaccines. Currently, various types of vaccines based on traditional (killed, live attenuated, toxoid or conjugate vaccines) and reverse vaccinology (DNA/mRNA, vector, recombinant subunit, plant vaccines) are in development or already available. Development of new vaccines demands high levels of knowledge, experience, budget, and time, yet promising new vaccines often fail in preclinical and clinical studies. Efficacy of vaccination also depends on the route of delivery, and mucosal immunization in particular is of special interest for preventing DID. Furthermore, adjuvants, delivery systems and other vaccine components are essential for an adequate immune response. These aspects will be discussed in relation to the improvement of existing and development of new vaccines against DID.

Introduction

Infections of the gastrointestinal tract triggered by bacterial, parasitic and viral pathogens rank among the most frequent infectious diseases. Every year, 2 million people die of diarrheal diseases, 760 000 of these are children under 5 y of age.^{1,2} Diarrhea is defined as the passage of loose or watery stools occurring three or more times in a 24-h period. Three types of diarrhea are differentiated: (1) acute diarrhea, including Cholera, (2) acute bloody diarrhea, also called dysentery characterized by damage to the intestinal mucosa, and (3) the persistent diarrhea with disease duration of 14 or more days.² Most of the pathogens causing diarrhea share a similar way of transmission, i.e., the

fecal-oral transmission. However, the dose of the infectious agent and the specific process causing diarrheal symptoms varies widely between various pathogens.³

Water and sodium are necessary for life and if diarrhea leads to insufficient absorption of water and nutrients, patients will dehydrate and death occurs after losing 10% of body fluids.⁴ One of the main reasons for the prevalence of diarrheal infections is a shortage of potable water and sewage facilities. Therefore, infectious diarrhea is the leading causes of morbidity and mortality in less developed countries. Adding to the burden of morbidity and mortality, there are under-recognized long-term effects of frequent early childhood diarrheal, such as impaired absorption of nutrients, especially during the first two years of life in a phase of brain and synapse development. Along with the deficits in nutrients, significant developmental defects were observed in patients with childhood diarrhea. These include a permanent reduction in growth (up to 8 cm by the age of 7), a loss of 10 IQ points and lack of a year of school education by the age of nine.^{5,6} Despite of improving life conditions due to control of potable water and better sanitary facilities in developed areas, there are also problems with bacterial, parasitic and viral pathogens causing diarrhea. This disease mainly occurs in connection with hospitalization and the administering of antibiotics. The risk of infections can be reduced by prevention policies. However, only vaccines provide protection against disease outbreak and can guarantee long-term immunization.

The first vaccine against cowpox developed by Edward Jenner at the end of the 18th century together with new developed methods, that allow the attenuation and inactivation of microorganisms,^{7,8} laid the foundation for the scientific era of vaccinology.⁹ Today vaccines are the most cost efficient strategy to lower the burden of infectious diseases all over the world. To generate new vaccine candidates, it is required to know the virulence strategies of the bacterial, parasitic and viral pathogens and the specific immune mechanisms of the host. We will briefly describe important diarrheal pathogens with clinical presentation, main virulence factors and therapeutic approaches.

Important Pathogens Causing Diarrhea

There are numerous pathogens from different life forms such as bacteria, parasites and viruses that cause diarrheal infectious diseases (DID) in humans. The main focus of this article is on

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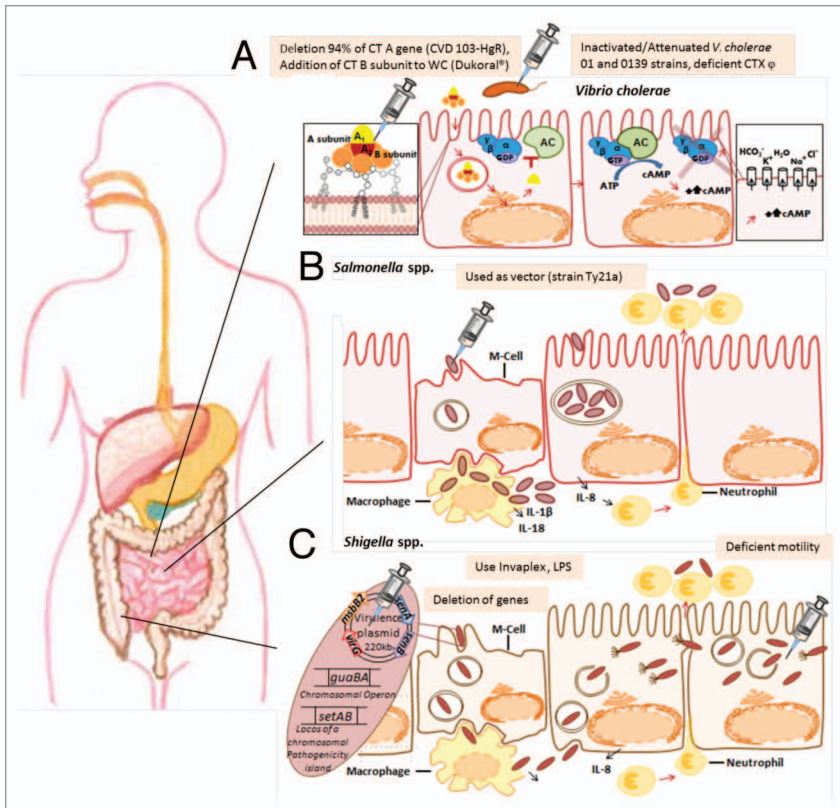


Figure 1. The gastrointestinal tract, interactions between pathogens and host and approaches to vaccine design. **(A)** *Vibrio cholerae* secretes an enterotoxin, which is an AB Toxin and comprises a single catalytic A subunit and a pentameric B subunit for specific binding to host cells. The receptor for CT is the glycolipid ganglioside GM1. Following internalization by receptor-mediated endocytosis, transport to the Golgi and the ER, the A1 subunit is finally transferred to the cytoplasm. The A1 fragment is a NAD-dependent ADP ribosyltransferase and activates the G protein G_{α} (GTP-bound), thereby continually stimulating adenylate cyclase (AC) produce cAMP. The high cAMP level enables the protein kinase A which induces a dramatic electrolyte transport, which is typically for diarrhea. Possible vaccine approaches (indicated by syringes) are the cholera toxin (CT), different virulence genes and use of inactivated/attenuated strains. **(B)** *Salmonella* spp. translocates effector proteins by a T3SS, encoded by *Salmonella* Pathogenicity Island 1, inducing pro-inflammatory responses and uptake of the pathogen via macropinocytosis. Uptake also occurs via M-cells or phagocytes. After phagocytosis by macrophages apoptosis is triggered, thereby triggering inflammation reactions with recruitment of neutrophils. Internalized *Salmonella* survive and replicate within the 'Salmonella-containing vacuole'. *Salmonella enterica* Serovar Typhi Ty21 represents a promising live vector for presentation of foreign antigens from unrelated bacterial, viral and parasitic pathogens. **(C)** *Shigella* spp. infects the epithelium from the intestinal lumen of the terminal small intestine and colon through M-cells. After phagocytosis, the bacteria are able to escape from the macrophage by triggering apoptosis. By remodeling the host cell actin cytoskeleton and forming large membrane protrusions, invasion occurs similar to *S. enterica*. Within host cells, *Shigella* is motile in the cytoplasm by a mechanism involving the formation of actin tails, also leading to infection into neighboring host cells. Spreading from cell to cell within intestinal tissue is accompanied by emission of bloody mucopurulent stools. Strains deficient in intracellular motility, in enterotoxin and further virulence genes present good vaccine candidates. Furthermore, cross-linked O-antigen polysaccharides of the relevant *Shigella* serotypes to a carrier protein is also a vaccine design approach.

Vibrio cholerae, *Shigella* spp. and rotavirus for which vaccines are available or in development (see further below). A comprehensive description of pathogens associated with DID can be found in the Supplemental Material and Table S1.

Vibrio cholerae

The Gram-negative curved rod *Vibrio cholerae* is responsible for 100,000 – 120,000 deaths annually (World Health Organization, WHO July 2012). The main virulence factor is Cholera toxin (CT), a secreted AB-toxin that triggers hyper-secretion of electrolytes by manipulation in second messenger levels in enterocytes (Fig. 1). The serotypes responsible for the disease defined clinically and epidemiologically as Cholera are *V. cholerae* O1 and O139.^{10,11} The currently prevailing 7th pandemic is caused by *V. cholerae* serotype O1. Despite of efforts to fight the disease by oral rehydration and in certain cases also antibiotic therapy, Cholera forms a major public health problem, especially in regions with low hygienic standards, such as developing countries and conflict areas.¹²

Shigella spp.

Further members of the Enterobacteriaceae family and major cause of diarrhea and dysentery are *Shigella* spp., which are closely related to *Escherichia coli*. *Shigella* spp. is transmitted through person-to-person contact and ingested by contaminated water or food. Only a small infective dose (10–100 bacteria)¹³ of one of the four *Shigella* species is enough to trigger epidemic mucosal ulceration and bloody diarrhea (see Fig. 1C). Despite of the self-limiting infection, the dysentery especially causes death of children in developing countries. Two virulence mechanisms are crucial for the pathogenesis, which are cell invasion and formation of the Shiga toxin. For invasion, bacteria attach to M cells of the terminal ileum and of the colon. Via transcytosis, they reach macrophages in the subserosa and are taken up by phagocytosis. After inducing apoptosis in the phagocytes, released *Shigella* may invade epithelial cells via their basolateral membranes. Within enterocytes, *Shigella* escapes into the cytosol and triggers F-actin formation at one cell pole, resulting in actin-mediated intracellular motility and intercellular spread.¹⁴

Rotavirus

The most common cause of diarrhea in young children is an infection with rotavirus.¹⁵ It is a non-enveloped virus and stable against environmental influences.¹⁶ Rotavirus infects intestinal epithelial cells, within it produces an enterotoxin called NSP4, which interacts with calcium-activated chloride channels.¹⁷ Furthermore, it reduces the effectiveness of digestive enzymes, leading to nutritional deficiencies.¹⁸ The disease is self-limiting in otherwise healthy

Table 1. Licensed oral vaccines against *V. cholerae* O1 (modified according to Ref^{6,102,103})* Produced by VABIOTECH, Vietnam and only available in Vietnam

Vaccine	No. of doses	Booster dose	Active component(s)	Licensed product name (manufacturer)	Duration of protection	Age range for vaccination
Inactivated whole <i>Vibrio</i> combination + CTX B subunit	2, given 7–42 d apart (3 for children aged 2–5 y)	Every 2 y (every 6 mo for children aged 2–5 y)	Mix of inactivated <i>V. cholerae</i> O1 of classical and El Tor biotypes and Inaba and Ogawa serotypes plus CT B subunit	Dukoral® (SBL)	2 y (6 mo for children aged 2–5 y)	≥ 2 y
Inactivated whole <i>Vibrio</i> combination	2, given 14 d apart	Every 2 y (likely to be extended to every 3 y)	Killed whole cells only (O1 classical and El Tor biotypes plus O139)	Shanchol® / mOrcVAX®**	3 y	Shanchol® ≥ 1 y; mORCVAX® ≥ 2 y
CVD 103-HgR recombinant live vaccine	1	Unknown	Recombinant classical, Inaba strain with deletion of 94% of the gene encoding the CT A, + Hg ²⁺ resistance gene (<i>mer</i>) introduced into the hemolysin A locus on the chromosome	Orochol®, Mutachol® (Berna Biotech)**	6 mo (established only in North American volunteers)	≥ 2 y

Note: *Produced by VABIOTECH, Vietnam and only available in Vietnam; ** Production discontinued; re-initiation requires modification of manufacturing facility

humans and therapy is based on rehydration.¹⁶ Worldwide, the number of deaths of young children associated with rotavirus was determined as 453 000 in 2008.¹⁹ To prevent and reduce rotavirus infections, in 2009 the WHO proposed to include rotavirus vaccination into the immunization plan.²⁰

Benefits and Limitations of Currently Available Vaccines Against Diarrheal Infections

Cholera immunization

Cholera is caused by two of more than 200 *V. cholerae* serogroups, i.e., O1 and O139. Furthermore, *V. cholerae* O1 can be classified into the classical or the predominant El-Tor biotypes. Although, prevention measures like the procurement of drinking water and improved sanitary facilities are important in areas with epidemic diseases, also vaccines against the pathogens are necessary. It could be demonstrated that only hygiene measures are not enough to prevent Cholera in Africa.²¹ In over 100 y of Cholera vaccine development, mainly the oral type of vaccine prevailed (see below).

Parenteral Cholera vaccines

Parenteral vaccines available during the 1990s consisted of phenol-killed whole cells of *V. cholerae* O1, which were about 50% protective for a short duration. Because of the short period of protection and painful local inflammatory reactions, the injectable vaccine has never been recommended for use.²² One parenteral Cholera vaccine, currently available in the United States, shows similar results and has no positive effect on controlling epidemic Cholera outbreaks.²³ Because of the low protective efficacy, the high reactogenicity of this vaccine type and the knowledge that oral administration of antigens results in mucosal immune response in the 1980s began the developing of oral Cholera vaccines.

Oral Cholera vaccines (OCV)

Two types of oral Cholera vaccines are currently available (Table 1): (1) Dukoral® (WC-rBS) a monovalent vaccine based on formalin and heat-killed whole-cells (WC) of *V. cholerae* O1 plus recombinant Cholera toxin B subunit (rBS), (2) Shanchol® and mORCVAX®, both identical bivalent vaccines based on serogroups O1 and O139, but formulated by different manufacturers. These oral Cholera vaccines received good results in controlling epidemic diseases²⁴ and trace all back to the early oral Cholera vaccine research of the 1980s.²⁵

Dukoral® has the advantage that the B subunit governs protection against ETEC. Shanchol® and mORCVAX® also have the advantages over Dukoral® that (1) no buffer (mostly clean water) is needed, (2) less storage space is required and (3) doses are less expensive.²⁶ The third cholera vaccine CVD 103-HgR, an oral live-attenuated vaccine (Table 1), ceased production in 2004 due to failure in a large field trial performed in Indonesia.²⁷ Further attenuated oral vaccines are still in different development phases and not yet licensed (Table 2). Many of these were created by removal of the CTX ϕ prophage, an inactivation of hemagglutinin/protease activity by sequence insertion of the *Clostridium thermocellum* endoglucanase A and modification of the *recA* region.

Shigella vaccine development

The immunity observed after infection with *Shigella* spp. is species-specific and each species divides into various serotypes and subtypes.²⁸ After infection, an IgM immune response is triggered against LPS O-antigen of the serotype.^{29,30} The serotype specificity and wide range of *Shigella* serotypes are reasons for the need of multivalent vaccines.

All vaccines against shigellosis developed so far are unlicensed, but some are currently undergoing clinical testing phases (see Table 2). Target populations for *Shigella* vaccine are infants

Table 2. New generation and unlicensed vaccines against *V. cholerae* and *Shigella* spp. (modified according Ref. 103)

Vaccine	Immunization route	No. of doses	Status	Cause of attenuation	Relevant immune response(s)	Developer, references
<i>V. cholerae</i> O1 attenuated vaccines						
Peru 15 or CholeraGarde® recombinant live vaccine	Oral	1 (10 ⁷ -10 ⁹ CFU)	Phase IIb	Core deletion of CT Modification of <i>recA</i>	Intestinal secretory IgA and serum IgG specific for LPS and other surface antigens: serum vibriocidal antibodies	Avant Immuno-therapeutics ¹⁰⁴
<i>V. cholerae</i> 638	Oral	1 (10 ⁹ CFU)	Phase I/II	Deletion of CTX ϕ prophage from VC strain C7258 El Tor Ogawa Inactivation of hemagglutinin/protease activity by sequence insertion of the <i>Clostridium thermocellum</i> endoglucanase A	Intestinal secretory IgA and serum IgG specific for LPS and other surface antigens: serum vibriocidal antibodies	Finlay Institute, Cuba ¹⁰⁵
VA1.3 and VA1.4	Oral	1 (5*10 ⁹ CFU) (VA1.3)	Phase I/II	Non-toxicogenic <i>V. cholerae</i> + insertion of the <i>ctxB</i> , VA1.3 + AmpR	Anti-CT-antibodies serum vibriocidal antibodies	Three different laboratories
IEM 108	Oral	1 (10 ⁹ CFU) in rabbits	Pre-clinical	deficient in CTX ϕ (IEM101) +introduced <i>ctxB</i> gene, + <i>rstR</i> gene	Anti-CT IgG and vibriocidal antibodies	China CDC ¹⁰⁶
<i>V. cholerae</i> O139 attenuated vaccines						
TLP01		1 (10 ⁹ CFU) in rabbits	Pre-clinical	CRC266 0139 strain with CTX ϕ , <i>mshA</i> deletion, inactivation of hemagglutinin/protease activity by sequence insertion of the <i>Clostridium thermocellum</i> endoglucanase A	Intestinal secretory IgA and serum IgG specific for LPS, IgM, vibriocidal antibodies	Finlay Institute, Cuba ¹⁰⁷
VCUSM2	Oral	1 (10 ⁹ CFU) in rabbits	Pre-clinical	VC strain C7258 El Tor Ogawa with <i>hema</i> deletion causes an ALA auxotrophy	Intestinal secretory IgAs and serum IgGs specific for LPS and anti-CT, vibriocidal antibodies	Universiti Sains Malaysia ¹⁰⁸
CVD 112		10 ⁷ CFU	Phase I	VC 0139 with <i>ctxA</i> , <i>zot</i> , <i>ace</i> , <i>cep</i> deletion	vibriocidal antibodies, anti-CT IgG	University of Maryland, US ¹⁰⁹
Attenuated <i>Shigella</i> spp. vaccines						
<i>S. sonnei</i> strain WRSS1	Oral	2 (10 ⁴ CFU)	Phase II	Deletion of the plasmid-encoded <i>virG</i> (<i>icsA</i>) protein → loss of actin-mediated motility ¹¹⁰	Intestinal secretory IgA and serum IgG specific for O-antigen and virulence plasmid proteins	Walter Reed Army Institute of Research ¹¹¹
<i>S. sonnei</i> strain WRSs2, WRSs3	Oral	2?	Pre-clinical	Deletion of the plasmid-encoded <i>virG</i> (<i>icsA</i>), <i>senA</i> , <i>senB</i> , <i>msbB2</i>	Unknown	112-114
<i>S. flexneri</i> 2a strain CVD 1208S	Oral	2	Phase I	Deletion of <i>guaBA</i> , <i>setAB</i> , and <i>senA</i> ¹¹⁰	Intestinal secretory IgA and serum IgG specific for O-antigen and virulence plasmid proteins	Center for Vaccine Development ¹¹⁵
<i>S. flexneri</i> 2a strain SC602	Oral	1-2 (10 ⁴ CFU)	Phase IIb	Deletion of the plasmid-encoded <i>virG</i> (<i>icsA</i>) protein → πρωτεϊν οφ τηρ πλασμιδ-ενχόδεδ υ ¹¹⁰	Intestinal secretory IgA and serum IgG specific for O antigen and virulence plasmid proteins	Pasteur Institute ¹¹⁶
<i>S. dysenteriae</i> 1 strain SC599	Oral	2	Phase II	Loss of invasion (<i>icsA</i> , iron chelation (<i>ent</i> , <i>fep</i>) and Shiga toxin A subunit (<i>stxA</i>)	Intestinal secretory IgA and serum IgG specific for O-antigen and virulence plasmid proteins	Pasteur Institute ³²
Inactivated and other <i>Shigella</i> spp. vaccines						
Parenteral <i>Shigella</i> glycoconjugates	Intra-muscular	2	Phase III	O polysaccharide covalently linked to carrier protein	Serum IgG specific O-antigen	NICHD ³³
<i>Shigella</i> invasion complex (Invaplex)	Nasal	3	Phase I	Invaplex is composed of the invasion plasmid antigen (<i>lpa</i>), proteins <i>lpaB</i> and <i>lpaC</i> in a native complex with <i>Shigella</i> LPS	Intestinal secretory IgA and serum IgG specific for O-antigen and virulence plasmid proteins	Walter Reed Army Institute of Research ¹¹⁷

Table 2. New generation and unlicensed vaccines against *V. cholerae* and *Shigella* spp. (modified according Ref. 103) (continued)

Vaccine	Immunization route	No. of doses	Status	Cause of attenuation	Relevant immune response(s)	Developer, references
Inactivated and other <i>Shigella</i> spp. vaccines (continued)						
Proteosomes to which <i>S. sonnei</i> or <i>S. flexneri</i> 2a LPS is adsorbed	Nasal	2	Phase II	outer membrane protein vesicles of Group B meningitides	Intestinal secretory IgA and serum IgG specific for O-antigen	ID BiomedicalA ¹¹⁸
Inactivated <i>S. sonnei</i>	Oral	3–5	Phase I	By formalin inactivation	Intestinal secretory IgA and serum IgG specific for O-antigen	Emergent Biosolutions ¹¹⁹
<i>Salmonella</i> vaccine vector Ty21a	Oral	3	Pre-clinical	live <i>Salmonella</i> Typhi vaccine vector expressing <i>S. sonnei</i> or <i>S. dysenteriae</i> antigens	Intestinal secretory IgA and serum IgG specific for O-antigen	Aridis ¹²⁰

and young children in developing countries and travelers from industrialized to developing countries.³¹

The unlicensed vaccines for shigellosis follow different approaches and are in various phases of trials. Two of the vaccine strategies demonstrated their efficacy by positive result in controlled large scale field trials.³² First vaccine strategy is the creation of parenteral conjugate vaccines in which O-antigen polysaccharides of the relevant *Shigella* serotypes are covalently linked to carrier proteins. The National Institute of Child Health and Human Development (NICHD) is investigating this approach,³³ because it has been suggested by several studies that immunity against shigellosis is conferred by anti O-antigen serum IgG³⁴ (see Table 2). This studies comprise several field studies, which yield following results: (1) A single dose of *S. sonnei* conjugate conferred 74% protection efficacy against *S. sonnei* gastroenteritis,³³ and (2) *S. sonnei* and *S. flexneri* 2a conjugate vaccine had no protection efficacy in younger than 3 y of age but *S. sonnei* showed a 71% protective efficacy in 3–4 y-old children.³⁵

The second strategy is creation of live oral vaccines based on attenuated strains of *Shigella*. The greatest benefits of this strategy are the needle-free delivery route, an easier way to manufacture than for other potential vaccines and good tolerability. Due to the fact that this approach uses living *Shigella*, the right degree of attenuation is a critical parameter. Over-attenuation will lead to insufficient immunogenicity, whereas moderate attenuation causes excessive reactogenicity.³⁶ For this, the invasion plasmid-encoded protein antigens (Ipas) are most qualified for reducing virulence.³⁷ The three distinct enterotoxins produced by *Shigella* strains can also be used for attenuation.³⁸ The most currently developed vaccines are the new generation, live attenuated vaccines, as shown in Table 2. The new approaches are based on the use of proteosomes, orally administered inactivated *S. sonnei*, a *Shigella* invasion complex (Invaplex), or a core-linked LPS expression of *S. dysenteriae* serotype 1 O-antigen in a living *Salmonella* Typhi vaccine vector.⁶ So far, these *Shigella* vaccine strategies have no documented evidence of protection in humans.

Rotavirus immunization

The first rotavirus vaccine RotaShield was licensed in 1998, but was removed from market in 1999 after intussusceptions were detected in immunized young children.³⁹ Since 2006, two new licensed rotavirus vaccines Rotarix® and RotaTeq® are available and drastically reduced worldwide infection rates.^{40,41} Rotarix®

is based on a monovalent, live, attenuated human G1P[8] (glycoprotein VP7 defines the G serotypes, protease-sensitive protein VP4 defines P serotypes) rotavirus strain (G1 serotype, P[8] genotype), the most common worldwide, but also prevents infections by serotypes G3, G4 and G9.^{42,43} This attenuated strain was found in a child at Cincinnati Children's Hospital.⁴⁴ After isolation, Vero cells (derived from monkey kidneys) were used for vaccine production.⁴⁵ The immunization is about 100% for the proposed serotypes, but decreases when infected with other serotypes.⁴³ However, the mechanism of protection is still unclear.⁴³

In contrast, RotaTeq® consist of genotypes G1, G2, G3, G4 and P1A(8) from human and bovine isolates.⁴⁶ Four of the five genotypes used are from human and each synthesizes one of the proteins of the outer capsid (G1, G2, G3 or G4) and a bovine adhesion protein (serotype P7). The fifth genotype P1A(8), express a bovine outer capsid protein (G6) and the human adhesion protein P1A (genotype P[8], serotype P1A[8]).⁴⁶ They are also multiplied in Vero cells and after vaccination, immunization is nearly 100%.⁴⁶ Similar to Rotarix®, the mechanism of immunization is not completely understood, but the serum anti-rotavirus IgA titers were 3-fold higher in volunteers with RotaTeq® treatment than those with placebo.⁴⁶

The vaccine is administered orally to infants.⁴⁷ First dose is given between the 6 and 12 wk after birth (1 mL Rotarix, 2 mL RotaTeq) and the latest vaccination before 24 wk (min. four weeks between both doses, Rotarix) or 32 wk (min. four weeks between all three doses, RotaTeq).^{43,46,47} In the first weeks after vaccination there is a small risk of an invagination (1–2 of 100 000 vaccinated infants), but negligible comparable to a rotavirus infection.^{42,47} Other side effects can be bloody stool, severe stomach pain or bilious emesis.⁴⁷

Since 2009, rotavirus vaccination is part of the immunization program.²⁰ However, both named vaccines are very expensive for developing countries, therefore non-profit organizations like the Global Alliance for Vaccines and Immunization (GAVI) offer them for a much lower price to reduce the infection rate in problematic regions.⁴⁸ In the meantime, Indian scientists developed a new rotavirus vaccine called Rotavac that is much cheaper (1 \$ per dose).⁴⁹ It is an oral, live attenuated vaccine applied in 3 doses (6, 10, 14 wk after birth), which passed clinical phase III.⁴⁹ The vaccine is based on a G9P[11] strain called 116E, originally isolated from a child in Delhi, with a VP4 similar to various bovine

Table 3. Types of vaccines

Name	Features	Advantage	Disadvantage	Example, Reference
Live, attenuated vaccine	Use of live attenuated bacteria and viruses, often isolated from patients or cell cultures	<ul style="list-style-type: none"> • booster effect (antibody production) • easy to create virus vaccines • mucosal immunization (IgA) 	<ul style="list-style-type: none"> • reverse mutation • high side effects • must be cultivable • not suitable for immunocompromised and pregnant people • difficult to create bacteria vaccines (high level of knowledge) • refrigeration 	Mumps, measles, rubella virus ^{74,121,122} diarrhea caused by rotavirus (see rotavirus immunization) tests with <i>S. Typhimurium ssaV</i> mutants in mice ¹²³ ETEC mutants tested in phase II ^{124,125}
Inactive/ killed pathogen vaccine	Consist of killed microorganisms such as bacteria and viruses inactivated with chemicals (formaldehyde), radiation, antibiotics or heat	<ul style="list-style-type: none"> • suitable for immunocompromised and pregnant people • no back mutation 	<ul style="list-style-type: none"> • low booster effect (immunization up to 2 y) • repeated dosing • must be cultivable 	Dukoral and Shanchol against Cholera (oral, see Cholera immunization) ^{26,63,74}
Subunit vaccine/ recombinant subunit vaccine	Use of an antigen or epitope, produced and isolated from microorganism	<ul style="list-style-type: none"> • use mixture of antigens • low side effects 	<ul style="list-style-type: none"> • genetic information required • low booster effect 	Hemagglutinin and neuraminidase of the influenza virus ^{74,126,127}
Toxoid vaccine	Use of inactivated toxins from pathogenic bacteria, can be combined with killed pathogens	<ul style="list-style-type: none"> • booster effect (serum antibody IgA, IgG) • combination of many toxins 	<ul style="list-style-type: none"> • only toxin producing bacteria • the toxin has to be known 	Cholera, diphtheria, tetanus, tests with <i>Clostridium difficile</i> ^{74,128,129}
Conjugate vaccine	Combination of the capsule lipopolysaccharide (LPS) and a toxin or an antigen subunit, because LPS often causes a weak immune response	<ul style="list-style-type: none"> • booster effect (T-cell dependent immunization) 	<ul style="list-style-type: none"> • only capsulated bacteria 	Diarrhea caused by <i>Campylobacter jejuni</i> , <i>Hemophilus influenzae</i> "type b" (Hib) ^{74,130,131} Pneumococcal conjugate vaccine (PCV) (Prenar, Synflorix and Prevnar 13) <i>N. meningitidis</i> Group C polysaccharide-tetanus toxoid (NeisVac-C)
DNA vaccine	The DNA contains the information of the antigen and is injected with a needle, some cells take it up and integrate it, after a time they synthesize the antigen and present it on the surface	<ul style="list-style-type: none"> • stimulate innate and adaptive immune response (in mice) • general side effects of the pathogen will be avoided • cheap and easy to create • no cold chain • stable DNA 	<ul style="list-style-type: none"> • the antigen and its sequence has to be known • integration of DNA can be a risk • uptake effectiveness? • low booster effect (in human) • gold particles are expensive 	Cancer, tests in mice with CpP2-DNA of <i>Cryptosporidium parvum</i> ^{74,132,133}
mRNA vaccine	See "DNA vaccine"	<ul style="list-style-type: none"> • see "DNA vaccine" • no risk of genome integration 	<ul style="list-style-type: none"> • see "DNA vaccine" • instable mRNA 	Prostate cancer ^{58,74}
Recombinant vector vaccine	Similar to "DNA vaccine," they use an attenuated virus or bacteria as a vector with the antigen information	<ul style="list-style-type: none"> • similar to a real infection -> better immune response • humoral and cellular response (in mice) • cheap and easy to create 	<ul style="list-style-type: none"> • back mutation? • suitable for immunosuppressed people? • integration of DNA can be a risk? • low booster effect (in human)? 	Vectors for HIV proteins, ^{74,134} tests in mice with <i>Cryptosporidium parvum</i> Cp15, profilin, and apyrase expressed in <i>Salmonella</i> Typhi vector ¹³⁵
Plant vaccine	Use of genetically modified plants (with viral and bacterial vectors) to produce antigens. Isolation of the antigen from plant or consumption of the plant	<ul style="list-style-type: none"> • booster effect? • no cold chain • long shelf life • cheap production 	<ul style="list-style-type: none"> • correct translation of the antigen information? • enough amount of antigen? • stability/delivery system? 	Tests in mice with rice expressing Cholera and rotavirus antigens ^{79,136,137}

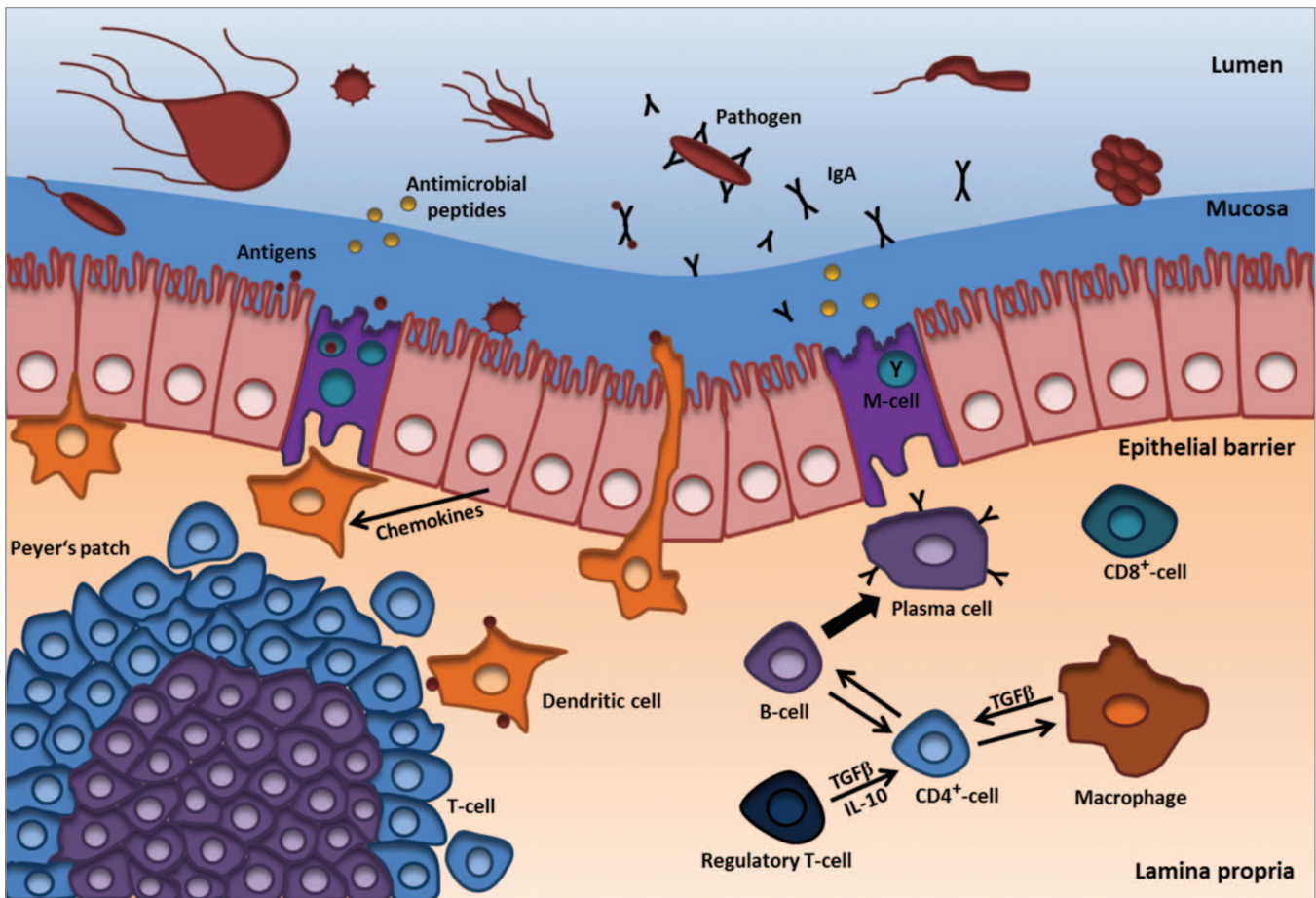


Figure 3. Mucosal immune responses. The gastrointestinal tract (GI) is covered by a mucus layer covering a monolayer of epithelial cells and M-cells that are connected by tight junctions (not shown). Inside the GI pathogens come in contact with the mucus, natural barrier not only against pathogens, but also against the gastric acid in the stomach. Mucus also protects against drying of the nasal mucosa and serves as an adhesion surface for the intestinal flora. Components of the mucus are mucin glycoprotein chains, antimicrobial peptides such as lysozyme, histatine and cystatine, defensins and specific secretory IgA. The mucus is produced from subjacent monolayer of enterocytes; ultimately they form the protective barrier. A further layer is the lamina propria with its gut-associated lymphoid tissues (GALT) such as Peyer's patches and isolated lymphoid follicles. It also contains a high amount of immune cells such as CD4⁺- and CD8⁺-T-cells, B-lymphocytes and plasma cells, dendritic cells and macrophages. For long-term immunization IgA producing plasma cells are very important. M-cells transport antigens via transcytosis to antigen presenting cells such as dendritic cells. These and macrophages interact with various types of T-cells (such as CD4⁺- and CD8⁺-cells) in the Peyer's patches, lamina propria and other lymphatic tissues through their receptors and various signal molecules. After T-cell activation, they also interact with B-cells, which then move to the target side and change into IgA producing plasma cells. IgA is secreted as mono- or dimer and binds to pathogens (e.g., viruses, bacteria and parasites) and antigens. Thus, the adaptive immune response does not proceed in an uncontrolled manner there are also regulatory T-cells. However, there are other defense mechanisms for instance antimicrobial peptides, defensins, digestive enzymes, the complement system and the mucin glycoprotein chains (not shown). Modified according to Macdonald and Monteleone et al. 2005.¹³⁸

RVs.⁵⁰⁻⁵³ There is also a second candidate based on live attenuated rotavirus strain called RV3, a G3P[6], which contains human RV-like VP7 and VP4 proteins and was isolated from neonates.⁵⁰

However, there is a newer approach reported from Wen et al. in 2012 based on truncated recombinant VP8* (Δ VP8*) proteins of human rotavirus strain Wa P[8], DS-1 P[4] or 1076 P[6], which was expressed in *E. coli* in high yield.⁵⁴ Three doses of 10–20 μ g of Δ VP8* vaccine (without adjuvants) were injected in guinea pigs and showed high amount of homotypic and heterotypic neutralizing antibodies.⁵⁴ Further, mice were treated with clinical relevant dosage of DS-1 P[4] Δ VP8* vaccine and produced increased level of VP8*-specific serum IgG antibodies.⁵⁴

This new development has the advantages of cost-effectiveness, lack of reversions, and based on the experience that rotavirus G types are often detected in parallel with P[8], P[4] and P[6] Δ VP8* proteins, excluded serotypes could be covered.⁵⁴

Problems and Limitations for Creating New Vaccines

In the previous parts, benefits and limitations of available vaccines against certain diarrheal infections were discussed. Next, problems and limitations during the research process of developing new vaccines will be described.

General obstacles in vaccination

Vaccinations against Cholera, shigellosis and other diarrheal diseases underlie limitations and barriers, such as general technical and logistical problems. Some vaccines require administration of further doses that are difficult for mobile populations. The use of oral vaccines, which sometimes required purified water for administration, the cost-effectiveness of the vaccines, strain variations from region to region, hygiene behavior and sanitation are additional wide ranged problems.

Moreover, logistical settings rely on stable infrastructure and human resources, both are scarce commodity in crisis areas, which are predestined for Cholera or other infection outbreaks. One further barrier is the transport of the bulky and large amount of vials, under respect of the cold chain principles. The public and political response in general and during times of crisis also plays a role in vaccination²¹

Barriers for vaccine development against parasites

Beside general problems discussed earlier in vaccine development, especially parasitic vaccine research and production is difficult. First, the parasitic (eukaryotic) genome size differs from prokaryotic bacteria and viruses which makes it hard to identify possible antigen targets. Further, suitable in vitro systems are missing and related expression vectors such as *E. coli* could be problematic due to a different codon usage which leads to a misfolded protein. If there is an existing organism for expression, the expression rate has to be high enough to guaranty sales turnover. In addition, protozoans e.g., *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* have complex lifecycles with different stages in which they form various virulence factors (see **Suppl. Material**). Often it is impossible to culture and analyze all stages of a lifecycle and virulence mechanisms. Accordingly, these parasites may cause chronic diseases. And last, investigations require long observation time and appropriate animal models are thus problematic (further animal model limitations are discussed below). However, potential targets and approaches exist in parasitic vaccine development (see **Table S1** and **Table 3**).

Antigen variation of gastrointestinal pathogens

Many pathogens causing diarrhea have developed strategies for avoiding recognition by the host immune system and thereby improve their long-term survival. One of these strategies is antigen variation. This method describes the change of antigenic molecules in infectious organisms such as bacterial, fungal and parasitic in response to the immune system of the host. This can be achieved with phase variation, epigenetic modifications, or DNA recombination. Phase variation is characterized by individual particular gene on/off switch. The switch can be made through slipped-strand mispairing at the level of transcription and translation or through only translational regulated mechanisms such as early dissociation of ribosomes and mRNA instability.⁵⁵ The majority of bacterial phase-variable molecules are surface structures involved in virulence, such as fimbriae (*S. Typhimurium*, *Proteus mirabilis*, *E. coli*), or flagella (*S. Typhimurium*, *Campylobacter coli*). Furthermore, multiples serotypes result from variation of LPS such as the O-antigen in *Shigella*⁵⁶ or the lipooligosaccharide (LOS) of *Campylobacter jejuni*.⁵⁷ The O-antigen modification

in *Shigella flexneri* is transmitted by serovar-specific temperate bacteriophages.⁵⁸

Antigen variation in pathogens may call the generation of multivalent vaccines, and recent examples are the pneumococcal polyvalent polysaccharide vaccines representing 23, or pneumococcal conjugate vaccines (PCV) representing 10 or 13 of the 83 capsule serotypes, respectively.⁵⁹ Production of polyvalent vaccines is cost intensive, since various antigens have to be produced and combined in amounts sufficiently high to trigger immunity against the important serotypes of the pathogen. The cost-effectiveness of new pneumococcal conjugate vaccines is under debate (example in ref. 60). Given a higher degree of serotype variation, as for example in non-typhoidal *Salmonella enterica*, generation of polyvalent vaccines may become technical difficult and expensive. Vaccination against a subset of serotypes also imposes selective pressure on pathogen. This may give rise to the infections by serotypes not represented by the polyvalent vaccine, and the need for reformulation of polyvalent vaccines.

Lack of suitable animal models

For *Shigella* vaccine development a major obstacle is the lack of an animal model that sufficiently resembles pathogenesis of human bacillary dysentery.³⁶ Various animals have been tested for developing disease models. For example, newborn mice were used, but fail in evaluation of protective immunity because of the short timeframe in which the vaccine has to be administered before the mice is infected.⁶¹ One further recently developed animal model is the guinea pig colitis model. After infection with wild-type *Shigella* strains, an acute inflammation of the colon was observed, mimicking human shigellosis.⁶² This limitation also applies to a number of other pathogens associated with DID.

Limitations of killed pathogen and subunit vaccines

A general problem of killed pathogen vaccines is the low duration of protection (< 2 y) and the need for repeated vaccine administration after this time.⁶³ This fact is problematic in regions where medical facilities are far away from the clients. The route of administration may partially compensate the lower duration of protection and mucosal immunization appears most efficient.⁶³

Killed pathogen vaccines require the propagation of bacteria, viruses or parasites. Subunit vaccines consist of certain antigenic constituents of pathogens, which can be toxins, capsules or cell envelope components such as LPS. These molecules have a better booster effect comparable to killed pathogen vaccines, for instance the Cholera toxoid vaccine showed a higher immunization than the killed pathogen alone.⁶⁴ Additionally, these fragments can be used as mono- or polyvalent vaccine or can be combined with inactivated vaccines. However, subunit vaccines are limited to toxin or capsule-producing pathogens. Furthermore, to identify the right toxin or antigen that causes a disease and effective in immunization is time-consuming and requires the proper expression vector and the right pathogen cultivation, a high concentration and an adequate purification. All of these vaccines are based on cultivatable microorganisms, but only a subset of disease-causing bacteria and viruses are so. A practical limitation of many subunits vaccines is the requirement for refrigerated transport chains.

Benefits and limitations of mucosal vaccination

Most microorganisms associated with DID enter the body orally via ingestion of food or water uptake, and the mucosal surface is one of the first body barriers against various pathogens (see Fig. 3). In order to overcome this barrier, or to persist in it, the pathogens need the necessary durability to survive. The ability to survive in this environment leads to initiation and transmission of diseases. To prevent DID at this stage, a local induced immune reaction through mucosal vaccination is of benefit. The mechanism of mucosal protection and reaction in humans against pathogens is described in the section "Mucosal immunization". For inducing an effective mucosal immune response, a vaccine should be directly delivered to the place of mucosal immune activation. Typical immunization routes for mucosal vaccination are nasal, oral, rectal and vaginal. Effective mucosal vaccines are available for rotavirus and *V. cholerae*. In particular, the nasal immunization route for mucosal vaccine generates the highest amount of systemic antibodies in human trials and mouse models.^{65,66} Several advantages of nasal and oral vaccines are known, especially compared with parenteral vaccines, because both mucosal and systematic immune reactions are activated. However, one advantage of the common intramuscularly/subcutaneously administration is the known quantity of vaccine that actually enters the body, plus the measurable amount of antibodies generated and lymphoid cells in the blood.⁶⁷ Nevertheless, the mucosal immunization enables rapid mass immunization and the needle- and syringe-free administration, which can preserve from infection by HIV, hepatitis B or C.

What are challenges in creating new mucosal vaccines? One uncertainty is the administration of the right amount of vaccine. Because of the mucosal host defense mechanisms, such as the attack through proteases, nucleases and the risk for dilution by bulk flow, a high dose is required.⁶⁸ Inducing tolerance by low intake of antigens and the host's own flora are further barriers for designing new vaccines. The lower immunogenicity of oral vaccines in developing nations compared with industrialized countries could be shown in some studies,⁶⁹⁻⁷² underlies probably unknown host factors⁷³ and is also to be considered for development. Application of multimeric and/or particulate vaccines mimicking mucosal adherent pathogens or adjuvants (see below) could increase and compensate the low immune response.⁶⁷

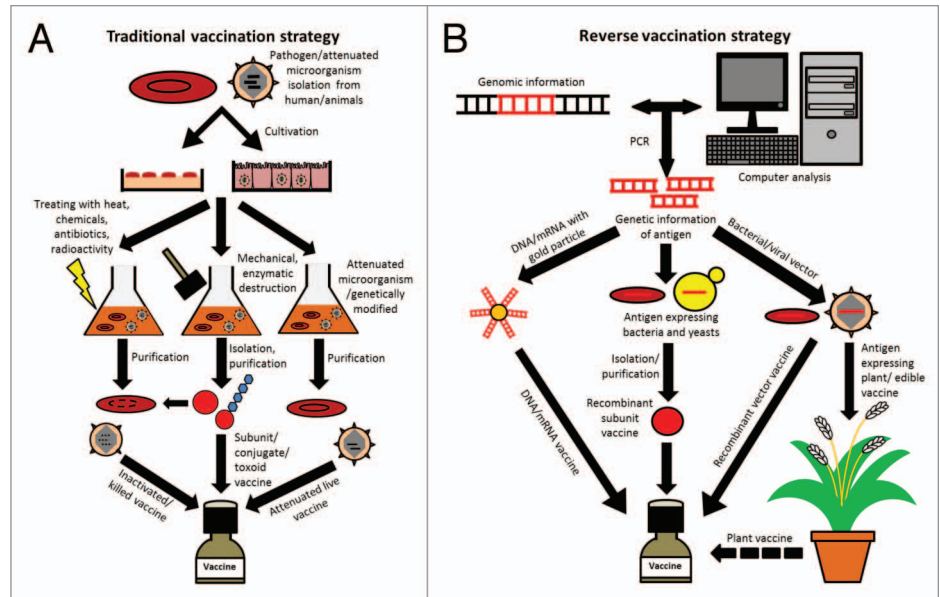


Figure 2. Traditional vaccine production and reverse genetics strategies. **(A)** Simplified representation of the individual steps of the traditional vaccine production by isolation of the pathogen over the cultivation, processing, up to the final vaccine. With this approach, different vaccines such as inactivated and attenuated live vaccines or subunit vaccines and special types like conjugate and toxoid vaccines can be produced and combined. Dashed line means destroyed DNA or RNA. **(B)** Simplified scheme of steps for reverse vaccine manufacturing. Different from traditional methods, reverse vaccinology starts with the analysis of the pathogen genome and epitope libraries searching for possible antigens used for immunization. After cloning/synthesis of the candidate sequence, it is transformed into vectors, antigen-expressing microorganisms or conjugated to gold particles. Vectors then can be used as vaccine or for plant modification. Antigen-expressing plants may be used as edible vaccines or are further processed to plant vaccines. Recombinant subunit vaccines are derived from antigen-expressing microorganisms. DNA- or mRNA-conjugated gold particles are also used as DNA or mRNA vaccines.

New Strategies for Vaccine Development

This section describes new strategies, the assets and drawbacks of vaccine candidates. A variety of new vaccines have been designed deploying distinct mechanisms to activate the innate and adaptive immune system.

The most known are killed/inactivated pathogen vaccines (*V. cholerae*), live attenuated vaccines and subunit vaccines (*V. cholerae*) such as conjugate and toxoid vaccines,⁷⁴ which all belong in this review to the traditional methods and are based on Pasteur's principles of isolation, inactivation, injection or additional purification of antigens and vaccination (see Fig. 2A). Further, bacterial subunit vaccines are commonly synthesized in the bacterium. In contrast, viral vaccines such as Rotarix® and Rotateq® against rotavirus are produced in Vero cells, other opportunities can be embryos or tissues, due to a lack of virus metabolism. However, the traditional way to produce new vaccines is lengthy, depends on the cultivation of pathogens and often failed the target, due to insufficient or absent or overreacting immune response.

A newer approach is the reverse vaccinology; with this strategy a vaccine against *N. meningitidis* has already been found. Based on the genome of an isolated pathogen, genes can be analyzed

and via databases and bioinformatics calculations, potential antigen candidates such as surface proteins are identified (see Fig. 2B). These target genes/proteins are synthesized recombinant in bacteria (e.g., *E. coli*) and injected in mice or other animal models.⁷⁵ Further, screening of immune sera of various tested antigen candidates can reveal their function as surface proteins and bactericidal effects.⁷⁵ With the last few candidates further tests and development are performed, which lead to a potential vaccine. By this method, mono- and polyvalent vaccines, DNA (e.g., first attempts with *C. parvum*), mRNA, recombinant vectors (e.g., first attempts with *S. Typhimurium*) and genetically modified plants (e.g., first attempts with rice expressing Cholera toxin or rotavirus antigen) can be generated (see Fig. 2B). All of these strategies have advantages and disadvantages as specified in Table 3.

Use of attenuated live vaccines or recombinant vaccines

Unlike inactivated vaccines, live attenuated vaccines have a high potential of long-term immunization, as exemplified by comparing rotavirus vaccines (live attenuated) with Cholera vaccines (recombinant, toxoid). However, live vaccines harbor risks such as back mutations and adverse side effects. Cultivation is required and application to immuno-compromized individuals may be critical. For live attenuated vaccine generation, bacteria and viruses can be isolated from patients without symptoms of disease and are cultivated. Another method is the use of genetically modified microorganisms. The deletion of virulent genes leads to attenuation, but requires a detailed knowledge about the bacteria or virus. In contrast, the transformation of antigen encoding genes into non-pathogenic bacteria or yeast cells results in recombinant proteins, which can be isolated and processed to recombinant vaccines. Important requirements for the production of such vaccines are a safe and rapidly growing microorganism with low nutrition claims and correct post-translational modifications. Recombinant vaccines are significantly more secure than live attenuated, have lower side effects, are accessible to all and can be used as mono- or polyvalent vaccines. An example of a recombinant vaccine is the inactivated Cholera vaccine with its recombinant toxin B subunit,^{26,76} also approaches with *Shigella* are based on recombinant protein synthesis (see Fig. 1C and Table 2).

Improving mucosal immunization

Another approach to improve vaccines is mucosal immunization, with various advantages such as easy administration even by untrained personnel, less waste by the avoidance of syringes and needles, less pain in application and no risk of infection by contaminated needles. Mucosal vaccine delivery also induces mucosal immunity by stimulation of IgA production (see Fig. 3) and, in some cases, a systemic immunity via IgG production.⁷⁷ Nonetheless, this type of immunization has also some disadvantages such as a complex composition of adjuvants and delivery systems, which are required to protect the antigen against gastric acid and digestive enzymes. These ingredients and the vaccine itself can cause allergies and other side effects, such as RotaShield against rotavirus.

Administration in form of aerosols is a possible type of mucosal immunization. An example for nasal vaccination is FluMist

for influenza vaccination, but there are also orally administered vaccines available for Cholera and rotavirus infections.^{47,76,78} Other approaches are based on genetically manipulated, edible plants, which expresses the antigen for immunization.⁷⁹

Improved adjuvants and delivery systems

Additional factors to significantly improve effects of vaccines are adjuvants and delivery systems. Most of the available vaccines are combined with adjuvants, substances that boost the immune response and promote long-term immunity.⁸⁰ In addition to the booster function, positive effects of adjuvants are reduction of the amount of antigen and increase in absorption.⁸¹ Only few adjuvants for human vaccines are licensed, one of the most commonly used is aluminum salt-based adjuvants (alum). Alum stimulate the immune and complement system, but little knowledge about the mechanism is available.^{82,83} Alum is part of many formulations such as tetanus, diphtheria or hepatitis B vaccines.^{84,85} Allergic reactions to alum-containing vaccines were observed.⁸⁶ A further adjuvant is squalene-oil-in-water suspension, as used in Freund's adjuvants.⁸⁷ First use was in an influenza vaccine (LUAD, Chiron) and also triggers an immune response.⁸⁸ Further adjuvants which are used in combating diarrhea are based on non-toxic Cholera toxin B subunit (CTB) from *V. cholerae* and heat-labile enterotoxin B subunit (LTB) from *E. coli*, which acts as pentamer and are essential for cell binding.⁸⁹ CTB has already been used for Cholera vaccine Dukoral®. Both CTB and LTB are well characterized toxin subunits which showed increased mucosal immune response in combination with coupled antigens and are therefore well suitable adjuvants.⁹⁰ Both bind together with their antigens to the GM1 ganglioside receptor on host cells such as antigen presenting cells and promote immune reaction.⁹⁰

Beside adjuvants, delivery systems such as liposomes and virosomes are also important for immunization. They protect the vaccine against degradation through gastric acid and proteases and deliver it to the target side. In case of influenza virus, it consists of the influenza virus envelope with its host cell binding and merging features, but without the genetic material.⁹¹ Vaccine formulations may require preservatives such as thimerosal, a mercury-containing compound.⁹² Other components can be amino acids and polysorbate for stabilization, formaldehyde and antibiotics for preventing bacterial growth and phenol and phenoxy-ethanol as preservatives. However, all these ingredients are often debated points.

How to reliably test vaccine performance?

Before a new vaccine enters the market, many obstacles and regulatory stages must be overcome. The whole process (research and development, preclinical and clinical studies, licensure) takes several years or decades and cost many millions of dollars. Often new vaccine candidates fail in the preclinical phase, because they could not guarantee safety and efficacy. After researchers found and developed a candidate vaccine, preclinical studies with cell cultures, tissues and animals begin. For example, the live, attenuated rotavirus vaccines were cultured and tested in monkey kidney cells MA-104.^{93,94} However, the main focus is still on animal experiments. Depending on the vaccine and route of administration, different animal models are used. Frequently, experiments are performed in rats, mice, dogs and primates. Parameters that

determine the attempt are the species, gender, age and group size.⁹⁵ Also important elements are the weight and food intake, as it might indicate side effects.

In order to verify vaccine efficacy, more parameters have to be monitored, i.e., (1) antibody titer (ELISA), (2) the dose that leads to an immunization, (3) types of immune cells responding, and (4) adverse effects (toxicity studies). In addition, possible influences of the vaccine on central and immune organs need to be investigated.⁹⁵ If the vaccine contains a new adjuvants, genotoxicity, carcinogenicity and pharmacokinetic studies have to be performed, in order to determine potential effects on the organism and to verify the effectiveness of the vaccine.⁹⁵ Interestingly, Cholera vaccines are available on the market, although there was no suitable animal model for analysis of immunization.⁹⁶

Future Perspectives

Despite various new approaches in developing vaccines, important in preventing DID are also improving hygiene measures and strengthening the immune system of the population at risk. This includes hand washing and disinfection, awareness campaign and, most important, non-contaminated water. Various approaches for water disinfection were implemented, such as filtration through Sari tissues (48% reduction of Cholera infections),⁹⁷ the Lifestraw family of filters with a pore-size as small as 20 nm,⁹⁸ and solar disinfection (SODIS) in PET bottles.⁹⁹ Another possibility to reduce the infection rate is household water chlorination, thus fecal-based contamination could be reduced.¹⁰⁰ Furthermore, hygiene education plays the major role. In addition to the hygiene, enhancing the immune system via breast milk feeding (contains maternal antibodies and nutrients) and zinc supplementation (important for enzyme synthesis)¹⁰¹ is also of interest. Although these methods reduce the frequency of infections, but do not necessarily provide protection by immunization.

There is still a lack of vaccines against diarrheal pathogens, especially against parasites. Here, economic constraints are an issue, since parasites are problematic in developing countries, but

not in industrialized nations. Vaccines may be too expensive for endemic regions and not profitable for business investment. As a consequence, research will be stopped.

Research and development lasts for decades until the vaccine is on the market. During this time, the antigen composition of the target pathogen may change, and adjustment of formulations may be required. Furthermore, often trained personnel for vaccination and correct storage places are missing. Lack the long-term immunity after vaccine may explain the need for repeated administration, again rising costs for vaccination campaigns.

Therefore, faster, more effective and more cost-effective vaccine development processes need to be developed. One area which deals with the development of new vaccines is reverse vaccinology. Based on genomic informations and computer analyses (epitope library), possible antigens can be found, modified for most effective immunization and produced recombinant (see Fig. 2B). Promising approaches are DNA-, mRNA-, plant-based and recombinant vector vaccines (see Table 3). Also, in order to enhance the immune response and reduce the administration rate, adjuvants and delivery systems (virosomes, liposomes, etc.) are used. However, in this area is a lot of potential for further vaccine improvement.

Attempts for an easier and safer delivery of vaccines are aerosols/nasal sprays, oral vaccines, special skin patches, powders and liquid jets, thus would reduce needles and trained personnel. Finally, for reducing costs, such as for packaging and refrigeration, plant-based vaccines could be an option, for example in form of rice, which is long lasting and does not need to be cooled and would act simultaneously immunogenic.

These are possible approaches with high potential for the optimization of vaccines and vaccine availability. However, this still requires further research and development.

Disclosure of Potential Conflicts of Interest

The Author states he has no conflict of interest

Supplemental Materials

Supplemental materials may be found here:

www.landesbioscience.com/journals/vaccines/article/29241

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