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1. Diarrhoeal diseases

1.1. Diarrhoeal diseases

1.1.1. Overview

Diarrhoeal diseases represent a major health problem in developing countries and also a high risk to travellers who visit these countries. Conservative estimates place the global death toll from diarrhoeal diseases at about two million deaths per year (1.7 – 2.5 million deaths), ranking third among all causes of infectious disease deaths worldwide. Most of these deaths occur in children under five years of age [1] [2] [3]. An average morbidity attack rate of 3.2 episodes of diarrhoea per year per child has been reported, but in some settings in developing countries, this number can be as high as 12 episodes per year per child. Evidence has been accumulating for long-term consequences of such heavy disease burden in early childhood on physical and mental development of children that may eventually translate into costly impairment of human fitness and productivity at an adult age [4]. Moreover, outbreaks of cholera, shigellosis and typhoid fever most often occur in resource-poor countries, adding to the burden of disease among the most vulnerable such as refugees, internally displaced populations and groups living in shanty towns.

The wide diversity of bacterial and viral infections that may cause diarrhoea [5] complicates accurate surveillance and diagnosis, especially in developing countries with little or no access to modern laboratory procedures. The specific disease burden attributable to a particular infectious agent is particularly complex, given the multiplicity of these agents and their serotypes, and its accurate documentation depends largely on laboratory facilities. While, in the long term, access to clean water, better hygiene, adequate nutrition, and improvement of sanitary measures would certainly have the greatest impact on diarrhoeal diseases, immunization against specific diseases is the best hope for the short- and mid-term. The development of vaccines against enteric pathogens however represents a serious challenge because of the large number of pathogens and the requirement to induce mucosal immunity in the gut [6] [7].

Among the principal bacterial agents of diarrhoeal diseases are *Vibrio cholerae* (cholera), a variety of *Salmonella* spp, including *S typhi* (typhoid fever), and of *Shigella* spp, the agents of shigellosis (bacterial dysentery), *Campylobacter* spp (especially *C jejuni*) and a variety of enteropathogenic *Escherichia coli* strains, including the enterotoxigenic ETEC strains that are the main agents of travellers' diarrhoea.

Diarrhoeas can also be caused by a variety of bacterial pathogens such as *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium difficile* or *Klebsiella*, as well as by various protozoa including *Giardia*, *Cyclospora* and *Cryptosporidium* spp (e.g. *Giardia lamblia*, *Cryptosporidium parvum*) and *Entamoeba histolytica*.

Among the enteric viruses, rotaviruses remain the leading cause of diarrhoeal disease in young children in the world. Every year, before the advent of rotavirus vaccines, an

estimated 527 000 young children died from severe diarrhoea caused by rotavirus infection, most of them occurring in developing countries in South Asia and sub-Saharan Africa [8]. The progressive implementation of rotavirus vaccines in the field will hopefully soon change this bleak picture. Other viruses causing diarrhoeas include enteric adenoviruses, astroviruses and caliciviruses, the latter (noroviruses) being responsible for most gastroenteritis winter outbreaks in industrialized countries [9].

Diarrhoea also is the most common health problem among travellers from industrialized countries who visit developing areas, especially in the tropics [10]. Up to 80% of diarrhoeal episodes in travellers are bacterial in nature, caused principally by enterotoxigenic *Escherichia coli* (ETEC) strains, as well as, quite commonly, by *Shigella*, *Campylobacter* and *Salmonella spp.* In addition, mild cases of cholera, caused by *Vibrio cholerae*, are often indistinguishable from other causes of acute diarrhoeal disease. The increased frequency of antibacterial drug-resistance among these pathogens is a source of major concern [11] [12] [13].

The CDC's Foodborne Diseases Active Surveillance Network (FoodNet) recently estimated that the incidence of illnesses caused by foodborne pathogens in the USA had not decreased since 2004 as far as campylobacter, listeria, *E coli* O157, salmonella, shigella, vibrio and yersinia infections were concerned, whereas cryptosporidium infections increased by 44% [14]. Salmonella remained the most common cause of foodborne illness as of 2007 in the USA. Foodborne outbreaks attributable to leafy greens such as lettuce, cabbage and spinach increased substantially during the past 35 years and continue to cause public health problems throughout the USA, in part due to increased consumption of ready-to-eat products and in part due to the modified atmosphere packaging that is used to keep the produce looking fresh.

All these pathogens are transmitted by the faecal-oral route.

1.2. [Caliciviruses](#)

Viral gastroenteritis is one of the most common illnesses in humans worldwide and caliciviruses, especially noroviruses (NoV), are one of its major agents. The prototype strain of NoV is the Norwalk virus, which was originally discovered in 1968 in an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, USA [15].

1.2.1. *Disease burden*

The role of human caliciviruses, including noroviruses, as agents of gastroenteric diseases has long been unrecognized and under-appreciated because diagnostic tools were not commonly available as these viruses remain uncultivable by standard cell culture assays. The application of new molecular diagnosis tools such as RT-PCR has shown that they are significant contributors to diarrhoeal disease burden in both children and adults. They appear to be the most common cause of winter gastroenteritis outbreaks in humans, popularly known as "stomach flu" in the UK ("grippe intestinale" in France), and a common cause of sporadic cases and outbreaks of acute gastroenteritis with vomiting, abdominal cramps, diarrhoea, headache and fever which occur in various epidemic settings such as restaurants, schools, day care centers, hospitals, nursing homes and cruise ships [9] [16] [17] [18] [19] [20]. The implicated vehicles of infection are contaminated water, shellfish, and food contaminated either at its source or by food handlers. Transmission also readily occurs from person-to-person or through contact with contaminated objects. Asymptomatic virus shedding can persist for up to two weeks, and the virus can survive freezing and heating to

60°C, as well as chlorinated waters, permitting its spread in recreational and drinking water as well as in steamed shellfish.

From several studies, it appears that, in industrialized countries, NoV are the second most common agent of non bacterial acute gastroenteritis in children after rotavirus, with an incidence of about 12% in children less than five years of age with severe diarrhoea, and the most common cause of outbreaks of acute viral gastroenteritis in adults, including those that are foodborne [21] [22] [23] [24] [25]. In the USA alone, NoV may account every year for more than 235 000 clinic visits, 91 000 emergency visits and 23 000 hospitalizations among children less than 5 years of age [26]. The role of NoV in developing countries has been less firmly established. However, in many Asian and African countries, most children appear to acquire serum antibodies to NoV early in life, suggesting that the virus probably plays a pre-eminent role in pediatric diarrhoeal diseases [27] [28] [29]. In Beijing, China, infants had a seroprevalence rate of 41% at 7 months of age, 65% at 1 year, 85% at 3 years, and 100% at 8–9 years of age. A recent estimate puts at more than 1 million the number of hospitalizations and at more than 200 000 the number of deaths NoV may cause each year among children less than 5 years of age worldwide [26].

1.2.2. Virology

Human caliciviruses, which form the family Caliciviridae (previously referred to as the Norwalk family of viruses or Small Round Structured Viruses) are 27–35 nm nonenveloped icosahedral viruses whose genome is a single-stranded positive RNA molecule. They include the genera Sapovirus and Norovirus (NoV). The NoV genome contains three open reading frames (ORFs), ORF1, 2, and 3. ORF1 encodes viral nonstructural proteins, including an NTPase, a protease and the viral replicase. ORF2 encodes the 58 kD capsid protein, VP1, and ORF3 a smaller capsid protein, VP2. VP1 is divided into N-terminal region, shell (S) domain, protruding domain (P) and C-terminal region. The P domain is in turn divided into P1-1, P2 and P1-2 domains, with P2 bearing the most important antigenic determinants [30] [31]. The virus is uncultivable in cell culture but cloning of either VP1 alone or VP1 and VP2 together in a baculovirus expression system leads to the spontaneous formation of virus-like particles (VLPs) that are antigenically similar to intact virions.

Cloning and sequencing of NoV genomes has allowed genetic characterization of the virus and its repartition into genogroups GI to GVII, each in turn subdivided into genotypes and subgenotypes [32]. Porcine, bovine and murine NoV belong to genogroups II, III and V, respectively. The majority of human NoV outbreaks are caused by viruses belonging to the GII-3 and GII-4 genotypes (genogroup II genotype 3 and genogroup II genotype 4), whose pandemic spread was recognized in the 1990s [33] and which appear to continuously undergo antigenic drift and recombination, leading to the emergence of a multiplicity of new virus variants [34] [35] [36] [37].

These viruses exhibit a restricted tropism for infection of the gastrointestinal tract of humans. Their receptor has been identified as the ABH histo-blood group antigens (HBGAs) on mucosal surfaces [38] [39] [40]. This explains why individuals who have a defective alpha-1,2 fucosyltransferase (FUT2) enzyme and who, for that reason, are unable to express HBGAs on cell surfaces, are resistant to infection [41] [42]. The surface-exposed, carbohydrate-binding domain of the NoV capsid appears to be under heavy immune selection and to evolve by antigenic drift to escape human immune pressure from herd immunity [37].

1.2.3. Vaccines

There are no vaccines available against caliciviruses. However, NoV VLPs produced in a baculovirus system are morphologically and antigenically similar to native virions, as judged from electron microscopy and ELISA. They also are stable at low pH, making them attractive as an oral immunogen. NoV VLPs administered by the intranasal or oral routes to mice with or without a mucosal adjuvant (the *E coli* toxin LT or its detoxified R192G derivative) induced a high serum antibody response as well as faecal IgAs. The safety and immunogenicity of the VLPs was evaluated in a Phase I trial on healthy human volunteers who received two successive 250µg VLP oral doses in bicarbonate buffer. All volunteers showed a >4-fold increase in IgG1 and IgA antibody titers [43]. The question remains of the extent of protection such a vaccine could provide in the field, in view of the natural diversity and variability of NoVs. A broad multivalent mixture of VLPs would most probably be needed to cover a significant number of virus variants and genotypes [44], but the number and serotypes of VLPs to be included is unknown.

The same VLPs were independently used as a test antigen to determine whether immune responses could be generated in volunteers who ingested transgenic potatoes that expressed NoV VLPs. Healthy adult volunteers at the Center for Vaccine Development (CVD), University of Maryland, USA, received 2 or 3 successive 150g doses of transgenic potatoes expressing the 58 kD NoV capsid protein or 3 doses of wild-type potato. Most of the volunteers who ingested the raw transgenic potatoes developed significant increase in the number of specific IgA antibody-secreting cells, 30% developed NoV-specific stool IgAs and 20% specific serum IgGs, but no increase in serum IgG titer was observed after the second dose [45]. Whether the modest antibody titers obtained would be protective against infection is unknown.

1.3. Campylobacter

1.3.1. Disease burden

Campylobacter jejuni ranks as one of the most common bacterial causes of diarrhoea in both industrialized and developing countries, with an estimated 400 million cases worldwide (1.5 million cases in the USA alone) [46]. It also represents the second cause of travellers' diarrhoea and enteric disease in military populations after enterotoxigenic *Escherichia coli* (ETEC). In developing countries, infection is nearly universal in early childhood [47]. It also is an important cause of foodborne illness in young children, including less than 1 year-old infants [48]. Perhaps of greater concern is its reported association with life-threatening cases of Guillain-Barré syndrome (GBS).

Campylobacters are frequent commensals in the intestinal tract of animals – mostly birds – and, as such, are frequently implicated in food-borne diarrhoeal illness [49] [50]

. Transmission generally occurs through consumption of contaminated water, raw milk or undercooked meat, especially poultry meat and meat products. Not washing one's hands and not cleaning kitchen utensils after carving a raw chicken carcass are major risk factors. Transmission also can occur through contaminated recreational waters. Asymptomatic excretion of the pathogen by immune individuals seems to be frequent. Antibiotic resistance is a growing concern.

1.3.2. Vaccines

Immunity to *Campylobacter* appears to be strain-specific and complex, and the antigens conferring immunity are not well understood [51]. No vaccine is available at the moment. Uncertainty regarding the mechanism of GBS is another obstacle to *Campylobacter* vaccine development [52]. A candidate vaccine consisting of heat- and formalin-killed whole bacteria combined with LT as a mucosal adjuvant has been developed by the Navy Medical Research Institute (USA) [53] and shown to provide 87% protection against intestinal colonization in a small number of volunteers challenged post vaccination with a pathogenic *Campylobacter* strain.

Current studies have focussed on the use of flagellin [54] or flagella-secreted protein FspA1 as candidate vaccines to be administered by the nasal route with attenuated LT R192G as an adjuvant. Vaccination of mice with FspA1 resulted in 64% protection against *C jejuni* challenge [55]. The major outer membrane protein (MOMP) from *C jejuni* might be another promising candidate for a subunit vaccine, especially when made into proteoliposomes [56].

An oral live multivalent vaccine expressing antigens from *Campylobacter*, *Shigellas* and ETEC is also currently being developed as a travellers' diarrhoea vaccine (see *Shigella* vaccines below).

1.4. Cholera

Cholera is a substantial health burden in many countries in Africa, Asia, and South and Central America, where it is endemic. The exact scale of the problem is however uncertain because of weaknesses in the existing surveillance system, difficulties to clinically distinguish mild-to-moderate case of cholera from other causes of acute diarrhoea and failures to report cases or even outbreaks to WHO, which acknowledges that only around 5%-10% of cholera cases are actually reported [57].

1.4.1. Disease burden

Cholera is an acutely dehydrating, watery diarrhoeal disease with vomiting, caused by intestinal infection with *Vibrio cholerae*. The acute form of the disease (*cholera gravis*) leads within hours to hypovolemia, acidosis, and potassium deficiency from the loss of fluid and electrolytes [58] [59]. Complications include renal failure, pulmonary oedema, abortion in pregnant women, and profound hypoglycemia and seizures in young children. Before the advent of effective rehydration therapy, cholera epidemics were associated with CFRs exceeding 40% and led to tens of thousands of deaths [60]. Rapid administration of fluid replacement therapy and supportive treatment have reduced mortality to around 4%.

Cholera probably has existed on the Indian subcontinent for thousands of years as judged from ancient manuscripts. The disease was repeatedly one of the most dreaded pandemic diseases in history, being able to spread rapidly to large numbers of people, with a high CFR. Seven cholera pandemics have been recorded since the early nineteenth century. The current "seventh" cholera pandemic started in 1961 and is still continuing. This pandemic, due to the emergence of *V cholerae* biotype El Tor, first appeared in Indonesia and has since spread worldwide, leading to the re-emergence of cholera in Africa in the early 1970s and in Central and South America in the early 1990s. While cholera no longer poses a threat to countries with minimum standards of hygiene, it remains a challenge to countries where access to safe water and proper sanitation are not guaranteed.

Cholera is transmitted via the faecal-oral route, with epidemic outbreaks often occurring after wars or civil unrests or after natural disasters including flooding when water and/or food supplies become contaminated in crowded population settings with limited sanitation. The disease is now endemic in many parts of Africa and Asia. Explosive outbreaks usually occur in areas with inadequate sanitation, poor hygiene, and lack of safe water supplies, whereas in some countries, a seasonal rhythm for cholera epidemics has been observed [61] [62]. Recent outbreaks of cholera in several countries including Iraq, India, and Sudan illustrate the fact that cholera today remains an important threat in almost every developing country in the world.

Vibrios can also persist for long periods of time in environmental waters where they associate with plankton, shellfish and algae, constituting a long-term reservoir. During epidemics, coastal waters become heavily contaminated with *V. cholerae* from infected humans and can be at the origin of cases through drinking of contaminated water or consumption of contaminated seafood, particularly undercooked shellfish [63].

The number of cholera cases reported to WHO annually has remained relatively constant since 1995, varying from 100,000 to 300,000 cases per year, with Africa accounting for >94% of the total. In 2006, a total of 236,896 cases were notified to WHO from 52 countries; 31 out of 46 African countries experienced an outbreak of cholera and reported a total number of cases of 202,407 with 5,259 deaths [64]. These numbers however appear to be grossly underestimated, as many countries in the Indian subcontinent and southeast Asia do not report their cholera cases [65]. As an example, no cholera cases have been reported from Thailand since 1994, although *V. cholerae* was readily isolated from stool samples in 5.4% of cases of bacterial diarrhoeal disease between January 1995 and December 2000 in hospitalized children in Bangkok [66]. Similarly, there is no report of cholera cases from Bangladesh, whereas experts estimate that there might be as many as 1 million cases in the country every year. A recent estimate puts the number of people who die from cholera each year at about 120,000, and the total number of yearly cholera cases worldwide at 3-5 million [67] [68].

The number of imported cholera cases notified to WHO likely also represents a substantial underestimate of the true burden of disease. That number was only 68 during 2005, but many additional cases could readily be identified from sources other than WHO [65]. The overall risk for travellers to contract cholera is often reported to be in the order of 2 to 3 cases per 1 million travellers. Recent estimates have put it at approximately 5 cases per 100,000 travellers [69] and it could be as high as 5 per 1000 for travellers visiting countries in which a cholera outbreak is occurring.

1.4.2. Bacteriology

V. cholerae was discovered by Robert Koch in the early 1880s in the faeces of a patient with the disease. Only enterotoxigenic *V. cholerae* serogroup O1 and new serogroup O139, which emerged in the 1990s in Bangladesh and India, are known to cause epidemics of cholera. Other serogroups of *V. cholerae* (O5, O37, O141) can cause isolated cases of watery diarrhoea, but do not cause epidemics. Isolates of *V. cholerae* serogroup O1 are classified, on the basis of phenotypic characteristics, into two biotypes, El Tor and classical. Currently, the El Tor biotype is responsible for virtually all the cholera cases throughout the world, and classical isolates have not been encountered since the mid-1990s in Bangladesh. *V. cholerae* O1 can be further classified into two serotypes, Inaba and Ogawa, based on serum agglutination. A possible third serotype, Hikojima, has been described, but is very rare. Immunity to *V. cholerae* infection is serogroup-specific, so that vaccines that target serogroup O1 do not protect from infection with serogroup O139.

Steps in the pathogenicity of cholera include colonization of the small intestinal mucosa, production of the pilus structure and elaboration of the enterotoxin cholera toxin (CT), an 84 kD multimeric protein consisting of a central active A subunit bound to five surrounding B subunits [70]. The B subunit is responsible for the binding of the toxin to the GM1 ganglioside receptors on epithelial cell surface, whereas the A subunit, an ADP-ribosylating enzyme, is responsible for the toxicity of the toxin through stimulation of the target cell adenylate cyclase, leading to hypersecretion of fluids and loss of electrolytes.

1.4.3. Vaccines

The historical whole-cell injectable vaccine that was developed by Haffkine in 1894 in India, induced a mediocre 48% protection that lasted for only about three months. The vaccine, which was made of killed *V. cholerae* strains of both Inaba and Ogawa serotypes, never was recommended by WHO, but still may be currently available in some countries.

Two types of cholera vaccines have been developed since then, a killed oral vaccine and a live attenuated oral vaccine; both have been shown to be safe, immunogenic and efficacious [71] [72].

1.4.3.1. Killed oral cholera vaccines

The killed oral vaccine (Dukoral™, licensed by SBL Vaccine, Sweden, to Crucell, Holland), which is recommended since 1999 by WHO as a tool to prevent cholera in populations at risk of an epidemic in emergency situations, consists of a mixture of four preparations of heat- or formalin-killed whole-cell *V. cholerae* O1, representing both serotypes (Inaba and Ogawa) and both biotypes (classical and El Tor), that are then added with purified recombinant cholera toxin B subunit (CTB). Because CT cross-reacts with *E. coli* LT, the vaccine also provides short-term protection against ETEC (see below), which is of added benefit for travellers [73] [74].

The Dukoral™ whole cell/recombinant B subunit (WC/rBS) vaccine – given orally with buffer to neutralize stomach acidity – was found, in field trials in Bangladesh and Peru, to confer 80–90% protection during 6 months in all age groups after administration of 2 doses 1–2 weeks apart. In Bangladesh, protection declined rapidly in young children after 6 months, but was still about 60% in older children and adults after three years [75] [76]. The vaccine was also successfully used for mass vaccination in refugee camps in Uganda [77], Darfour and Indonesia (Aceh) to protect at-risk populations from potential cholera outbreaks. In a field trial in Mozambique, the vaccine demonstrated 89% protection against severe diarrhoea with dehydration and 77% protection against milder forms of the disease [78]. An individually randomized, placebo-controlled trial of killed oral cholera vaccines in 89,596 children more than 2 years of age and women in Bangladesh showed that mass vaccination could provide herd immunity, as protection was also found in children less than 2 years of age [79].

The vaccine is currently administered in a 3-doses schedule to 2-6 years old children, with a boost every 6 months; and as a 2-dose regimen to older children and adults, with boosting every 2 years.

A variant of the Dukoral vaccine containing no recombinant CTB-subunit has been produced and tested in Viet Nam [80] [81]. It is administered in two doses, 1-2 weeks apart. A field trial conducted in Nha-Trang, Viet Nam, showed an efficacy of 66% against *V. cholerae* El Tor after 8 months in all age groups tested. The vaccine (ORC-Vax™) is being used for public health interventions in Viet Nam [82]. A bivalent O1 and O139

whole-cell oral vaccine without CTB has also been developed in Viet Nam and shown to be safe and immunogenic in both adults and children, generating 90% anti-O1 and 68% anti-O139 vibriocidal antibody responses after a two-dose regimen.

Production of the bivalent O1 and O139 ORC-Vax™ vaccine was recently licensed by VaBiotech (Vietnam) to Shantha Biotechnics (India) and BioFarma (Indonesia), which have undertaken a new, complete preclinical and clinical development. A large-scale, randomized, placebo-controlled Phase III trial on 70,000 persons is currently ongoing in urban Kolkata, India, with the help of the International Vaccine Initiative (IVI) in Seoul [83].

1.4.3.2. Attenuated live oral cholera vaccine

This type of vaccine consists of a live attenuated, genetically modified *V. cholerae* O1 Inaba strain (CVD103-HgR), which has been engineered to produce the B subunit (CTB) but not the A subunit of CT. The vaccine, Orochol™ (Berna Biotech, now Crucell, Switzerland) was given orally along with buffer to neutralize stomach acidity. It was available in two formulations, a low dose formulation for developed countries and a 10-fold higher dose formulation for developing countries. Placebo-controlled trials in a number of South American and Asian countries demonstrated the safety and immunogenicity of a single dose of Orochol™ [84]. Protection efficacy against experimental challenge given 3 months after vaccination with *V. cholerae* O1 (of either El Tor or classical biotype) in adult volunteers in the USA was found to be about 80% against all cases of diarrhoeas and 90% against severe diarrhoea. However, in a subsequent large-field trial performed in cholera-endemic Indonesia on 67 000 volunteers, the vaccine failed to demonstrate protection [85], in part due to the limited number of cholera cases recorded during the trial. Although the vaccine retrospectively showed protective efficacy when used for the control of an ongoing outbreak in the Federated States of Micronesia [86], the manufacturer discontinued its production in 2004.

1.4.3.3. Other cholera vaccines in development

Candidate vaccines in development include:

a live attenuated, single-dose, oral vaccine (*V. cholerae* 638) developed in Cuba, already tested in Phase II trials in Mozambique [87]; building on the success of strain 638, Cuban investigators have constructed an analogous attenuated vaccine candidate derived from an O139 strain that should go into clinical trials shortly;

a live attenuated O1 El Tor strain (*Peru-15*) developed as an oral vaccine by AVANT Immunotherapeutics (USA) under the name CholeraGarde™, which elicited a 62% protection against *V. cholerae* challenge in North American volunteers [88] and was found to be safe and immunogenic in a Phase II trial in Bangladesh [89]. Efficacy studies (Phase IIb) were reportedly imminent. Meanwhile, Peru-15 was engineered to express and secrete high levels of CTB by transfection with a recombinant plasmid carrying the CTB gene under the transcriptional control of a strong constitutive promoter. The resulting strain, *Peru-15pCTB*, was shown to secrete approximately 30-fold more CTB than Peru-15, was genetically stable, and elicited high anti-CTB, LT-neutralizing antibody titers and high vibriocidal antibody titers when administered by the oral route to rabbits or by the intranasal route to mice. *Peru-15pCTB* will therefore replace *Peru-15* as an oral, single-dose, bivalent cholera/ETEC vaccine candidate [90]. The vaccine currently is undergoing Phase/II clinical trials;

Bengal 15, similar to Peru-15, and *CVD112*, are live attenuated strains of *V cholerae* O139, which have been shown to be safe and immunogenic in Phase I trials in human volunteers [91];

a parenteral O-antigen-conjugated vaccine, in preclinical development at the Pasteur Institute in Paris;

a parenteral plasmid DNA vaccine, in development at the Putra University in Malaysia and the Malaysia National Biotechnology Directorate;

a rice-based oral vaccine made from transgenic rice seeds that express 30 µg CTB per seed in protein storage organelles where CTB appears to be stable for more than 1.5 year at room temperature. When fed to mice, the transgenic seeds elicited anti-CTB serum IgG and mucosal IgA antibodies that completely blocked LTB-binding to GM1 ganglioside and protected the animals from oral challenge with CT [92].

A proteoliposome based formulation administered by the nasal route that elicits vibriocidal antibodies in mice [93].

The concern remains that live oral cholera vaccines may be less effective among partially immune individuals in cholera endemic areas as pre-existing antibodies could decrease colonization of the gut, as was observed in the case of many other live bacterial oral vaccines [94].

1.5. Enterotoxigenic Escherichia coli (ETEC)

Numerous types of diarrhoeagenic E coli strains have been identified worldwide, including enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), Shiga toxin-secreting (STEC), diarrhoea-associated haemolytic (DHEC), entero-aggregative (EAaggEC), and cytolethal distending toxin-secreting (CDTEC) E coli strains. The prevalence of these strains and the burden of disease they cause are however unequal.

Some enterohaemorrhagic E coli strains have been lysogenized with phages encoding the toxin from *Shigella dysenteriae* [95] thus becoming Shiga toxin-secreting E coli (STEC). Such is the case of the strains belonging to serotype O157:H7, which cause a watery diarrhoea rapidly progressing to frank haemorrhagic colitis, itself leading in about 20% of paediatric patients to serious sequelae, including the haemolytic uremic syndrome (HUS) characterized by thrombocytopenia, hemolytic anemia and renal failure. The natural reservoir of the O157:H7 pathogen is cattle, which harbours the bacterium in its intestines: outbreaks in humans are linked to ingestion of meat or other foods contaminated by bovine faeces. Given the relatively low incidence of serious STEC infections and HUS worldwide, a vaccine against STEC is not thought of as a priority, but the development of Shiga toxoid as a vaccine against *Shigella dysenteriae* is receiving some attention (see *Shigella* vaccines below [46]). As most STEC infections are caused by serogroup O157, immunity to the O157 PS has been tested using a *Pseudomonas aeruginosa* exoprotein A-conjugated O157 PS vaccine, which was used in a Phase II clinical trial in children [96], but protective efficacy remained doubtful.

In contrast with STEC, enterotoxigenic E coli (ETEC) strains remain a major cause of infantile diarrhoea in developing countries and of travellers' diarrhoeas in visitors to these countries. The need to develop a vaccine against ETEC is therefore urgent.

1.5.1. Disease burden

ETEC are an under-recognized but extremely important cause of diarrhoea in the developing world where there is non adequate clean water and poor sanitation [97]. They are the most commonly isolated bacterial enteropathogen in children below 5 years of age in developing countries, and account for several hundred million cases of diarrhoea and several ten of thousand deaths each year [98]. Disease caused by ETEC follows ingestion of contaminated food or water and is characterized by profuse watery diarrhoea lasting for several days that often leads to dehydration and malnutrition in young children. ETEC was thought to account for approximately 200 million diarrhoea episodes and 380 000 deaths annually [99] [100]. A more conservative estimate, about 170,000 deaths every year, was more recently suggested [101]. Repeated ETEC infections and persistent diarrhoeas in children in developing countries are not rare, as observed in infants living in the Nile delta area, who experienced between 4.6 and 8.8 diarrhoeal episodes per year, with ETEC accounting for 66% of these episodes [102]. The peak incidence of ETEC diarrhoea in developing country settings occurs in the first two years of life, with a declining incidence with age thereafter [103]. Surveillance of hospitalized cases of ETEC diarrhoea has shown that a large proportion of cases also occur in individuals over 10 years of age. ETEC are prevalent in surface water sources in developing countries such as Bangladesh, which explains the endemicity of the disease in these countries [104]. In young children, the tendency of ETEC to cause dehydrating diarrhoea is lower (approximately 5% of episodes) than that of rotavirus (approximately 36% of episodes). However, because the incidence of ETEC diarrhoea in children is considerably higher than that of rotavirus diarrhoea, the absolute number of dehydrating diarrhoea episodes due to ETEC is around 70% of that due to rotavirus.

ETEC also are the most common cause of travellers' diarrhoea that affects individuals from industrialized countries travelling to developing regions of the world [105] [106]. Each year, there are an estimated 10 million cases of LT-related ETEC travellers' diarrhoea worldwide [100]. Thus, up to 60% of US visitors to Mexico develop the disease, with travellers genetically predisposed to produce high levels of interleukin-10 (IL-10) being more likely to experience symptomatic ETEC travellers' diarrhoea [107].

1.5.2. Bacteriology

ETEC attach to specific receptors on the surface of enterocytes in the intestinal lumen by virtue of their hair-like fimbriae, which define strain-specific antigenicity. More than 20 types of fimbriae antigens, called E. coli surface antigens or colonization factor antigens (CFAs) have been described. Antibodies targeted to fimbriae are protective but show high serotype-specificity.

Once attached to the intestinal epithelium, ETEC elaborate both the heat-labile (LT) and the heat-stable (ST) toxins, which induce the watery diarrhoea. The heat-labile enterotoxin LT is an 86 kDa protein which, like the cholera toxin, with which it shares 82% amino acid homology, is made of five B subunits that bind to GM1 ganglioside receptors in the intestinal epithelium, and a single enzymatically active A subunit, the ADP-ribosylating activity of which leads to activation of cellular adenyl cyclase, efflux of Cl⁻ ions and watery diarrhoea [108]. The heat-stable toxin (ST) is an 18-amino acid-long, highly folded peptide which also causes disruption of chloride channels in the cell and secretory diarrhoea. LT is expressed in about 66% of ETEC strains, either alone or in combination with ST, and thus is significantly responsible for the worldwide disease burden of ETEC [109]. Efforts are being made to identify major clonal groups among ETEC strains, largely based on O-antigen typing, CFA expression pattern and toxin profile [110].

Because of antigenic mimicry between CTB and LTB, short-term protection against ETEC disease has been documented in individuals immunized with CTB [73] [74] (see Cholera vaccines above).

1.5.3. Vaccines

Natural history studies of ETEC infections in children in developing countries suggest that these infections are immunizing, as reflected by declining rates of ETEC diarrhoea with age, lower ratios of symptomatic to asymptomatic ETEC infections with increasing age, and the protective relationships between initial ETEC infections and subsequent infections that have similar toxin and/or colonization factor phenotypes [111] [112]. These data suggest that immunization against ETEC early in life may be an effective preventive strategy. Pathogenesis of ETEC appears to be linked to CFAs and to the production of LT and/or ST. To provide broad-spectrum protection, an ETEC vaccine should, therefore, contain the most prevalent fimbrial antigens (CFA1 and CS1-CS6) and/or a LT toxoid [113], although this view was recently challenged [114].

1.5.3.1. Killed oral vaccines

The oral killed WC/rBS cholera vaccine (Dukoral™) was found to prevent 23% of all diarrhoea episodes and 52% of episodes due to ETEC in Finnish tourists visiting Morocco. This protection was reported, however, not to last more than a few months [115]. In a retrospective study in Spain, vaccination with Dukoral™ reduced by 43% the risk of travellers' diarrhoea [74]. Vaccination against cholera and ETEC should be recommended to at-risk travellers [116].

The most successful ETEC vaccine approach so far, which was developed by investigators at the University of Göteborg (Sweden), is based on a killed, oral, whole-cell ETEC vaccine containing recombinant CTB together with five strains of formalin-killed ETEC cells that collectively express the colonization factors of greatest epidemiological importance in developing countries (CFA/I and CS1-CS6). Phase II studies of a 2-dose regimen of this vaccine have been conducted in Bangladesh, Egypt, Israel, Nicaragua, the USA and Europe and have found the vaccine to be safe and immunogenic, as manifested by induction of mucosal antibody responses to CTB and to the CFA components of the vaccine. A pilot efficacy trial of this vaccine in European tourists travelling to developing countries found the vaccine to confer about 80% protection against ST-ETEC diarrhoea (the only toxin phenotype detected in this study), although the small number of outcome events precluded statistically significant estimates of efficacy [117]. A 75% protection was reported against severe diarrhoea in US volunteers travelling to Mexico or Guatemala, although the vaccine did not reduce the overall rate of ETEC diarrhoeas in the vaccinees [118]. Trials of the oral killed vaccine efficacy are ongoing in travellers from the USA and Europe, as well as in Israeli military recruits, and Egyptian infants and young children, but vaccine efficacy in young children was found to be disappointingly low [114].

The oral killed vaccine approach is being pursued by several investigators [119]. E coli bacteria K12 over-expressing CFA/I have recently been engineered that could be useful as an oral killed CF-ETEC vaccine [120].

1.5.3.2. Live attenuated oral vaccines

Two live attenuated ETEC strains, PTL002 and PTL 003, which express the colonization factor CFA/II, were tested in a Phase I trial [121]. Based on its superior immunogenicity, PTL003 will be developed further as a component of a live, oral attenuated ETEC vaccine.

Similarly, two nontoxinogenic ETEC strains that express CFA/I have been attenuated by mutagenesis of the *aroC* and *ompR* genes or the *aroC*, *ompC*, *ompF* and toxin genes, respectively. The latter strain, ACAM 2010, was found to be well tolerated and 73% immunogenic when fed to human volunteers. The strain will be developed as a live attenuated oral vaccine by Acambis, UK [122].

Another strategy, which is developed at the Center for Vaccine Development (CVD), University of Maryland (USA), is to use live attenuated *Shigella* vectors for expression of ETEC fimbrial and LT antigens. Such constructs might thereby protect against both *Shigella* and ETEC. Four lots of attenuated *Shigella* vaccine strain CVD 1204 (Δ guaBA) expressing ETEC fimbriae antigens CFA/I, CS2, CS3 and CS4, respectively, were found to be immunogenic in guinea pigs by the intranasal route. An additional strain was constructed that expressed a detoxified version of LT (LThK63). A mixed inoculum containing the five recombinant *Shigella* strains elicited immune responses to the five ETEC antigens plus the *Shigella* vector [123]. In a more recent approach, a combination of three Δ guaBA attenuated vectors, *S flexneri* (CVD 1208), *S sonnei* (CVD1233) and *S dysenteriae* 1 (CVD 1252), was used instead of a single vector [124], eliciting specific immune responses against each of the vectors as well as against each of the five ETEC antigens.

A similar approach is being followed by Microscience, UK, using their spi-VEC oral live attenuated typhoid vaccine as a vector for the delivery of ETEC antigens. The resulting oral vaccine, based on *S typhi* Ty2 derivative TSB7 harboring attenuation deletions in the *ssaV* and *aroC* genes and a chromosomally integrated copy of the *E coli* LT-B subunit, was shown to induce 67% and 97% immune responses to LT-B and *S typhi* lipopolysaccharide (LPS), respectively [125]. It might thus elicit protection against both ETEC diarrhoea and typhoid fever. Another live recombinant oral vaccine is developed that would cover traveller's diarrhoeal diseases due to *Campylobacter*, *Shigella* and ETEC.

The Walter Reed Army Institute of Research has similarly engineered an attenuated *S flexneri* 2a (SC608) vector derived from the well-characterized SC602 live attenuated vaccine strain which has undergone several clinical trials in human volunteers, to express the ETEC fimbriae subunit CfaB (CFA/I structural subunit) in combination with the LT-B subunit. Guinea pigs immunized by the intranasal route were subsequently protected from challenge with wild type *S flexneri* in a keratoconjunctivitis Sereny test [126] and serum antibodies from the vaccinated animals showed antitoxin (anti-ETEC) and agglutination (anti-*S flexneri*) activities.

Finally, the Peru15pCTB live attenuated oral cholera vaccine candidate developed by AVANT will address both *V cholerae* and ETEC diarrhoeas (see Cholera vaccines above)

1.5.3.3. Other ETEC vaccine approaches

A mixture of fimbrial antigen CS6 and LT was administered to human volunteers using a new delivery technology, the transcutaneous immunization patch [127]. An immune response to both antigens was elicited in about 50% of the volunteers. The presence of LT as an adjuvant was required for induction of responses to the CS6 antigen. Transcutaneous immunization with patches of 50 μ g LT alone on days 0 and 21 was shown by IOMAI Corp, USA, to be safe and immunogenic. In a vaccination/challenge study, transcutaneous LT vaccination did not prevent but mitigated illness following high-dose oral challenge with a virulent LT+ ST+ ETEC strain [128]. In a Phase II randomized, double-blind, placebo-controlled field trial, healthy adults travelling to Mexico or Guatemala were 85% protected against severe diarrhoea and 75% protected against moderate-to-severe diarrhoea by vaccination with two LT patches given 2-3 weeks apart [129]. Meanwhile, disruption of the

stratum corneum of the skin before vaccine patch application was found to result in significant increase of the immune neutralizing antitoxin response to LT: the process will likely be used systematically for transcutaneous immunization in the future [112].

Several other approaches are being pursued to develop specific ETEC vaccines including the use of purified colonization factors, of LT-only or LT-ST toxoids, or of edible transgenic plants that express the cholera toxin B subunit (CTB) or the LT B subunit [130].

1.6. Rotaviruses

Rotaviruses (RV) are the leading cause of severe diarrhoeal disease and dehydration in infants and children under the age of 5 worldwide [131]. The global situation recently changed with the advent of new oral RV vaccines [132]. Three oral RV vaccines are currently licensed, a human monovalent live attenuated RV strain, Rotarix™, a pentavalent live bovine-human reassortant vaccine, RotaTeq™, and a lamb-derived monovalent live attenuated strain, LLR, which is only being used in China. Several countries have introduced the Rotarix™ and Rotateq™ vaccines into routine immunization programmes, which hopefully will greatly reduce the burden of gastroenteritis and dehydration worldwide and have a strong impact on infantile diarrhoea mortality in developing countries [133]. A few data on the effectiveness of these vaccines in poor African or Asian countries have recently been made available, but concerns remain regarding the potential effectiveness of any oral live vaccine in these settings in view of prevalence of competing intestinal flora in children, occurrence of mixed infections, high levels of maternally transmitted antibodies and micronutrient malnutrition.

Meanwhile, different, new vaccine approaches such as non-replicating virus-like particles (VLP) vaccines and other routes of administration are being tested in animal models and will soon be evaluated in humans [134].

1.6.1. Disease burden

Rotavirus is currently by far the most common cause of severe diarrhoea in infants and young children worldwide and of diarrhoeal deaths in developing countries [135] with a distinct winter seasonality in temperate climates and year-round exposure in tropical countries. Virtually all children are infected by the time they reach 2 to 3 years of age. Most symptomatic episodes occur between 3 months and 2 years of age with a peak incidence between 7 and 15 months. Symptoms include watery diarrhoea, nausea, vomiting, abdominal pain and dehydration. Outbreaks of RV gastroenteritis in day-care centers and hospitals can spread rapidly among nonimmune children, presumably through person-to-person contacts, airborne droplets, or contact with contaminated toys [136]. Children from low socioeconomic background and low birth weight infants have an increased risk for hospitalization [137].

RV infection can also occur in adults [138], especially in institutionalized or hospitalized elderly patients [139]. Both symptomatic and asymptomatic patients shed RV in their stools for 7-10 days, but shedding can happen to last for several weeks. The virus is highly resistant in the environment and can survive for months in stools at room temperature [140].

Worldwide, RV has been estimated to account for almost 40% of all cases of severe infant diarrhoea [141] [142], which translates into 527 000 deaths each year (range: 475 000-580 000), mostly in children under age 2 [83].[135] Mortality still is the greatest in south

and south-eastern Asia and sub-Saharan Africa, with almost 100,000 deaths each year in India alone and more than 200 000 in African countries [143]. The Asian Rotavirus Surveillance Network, which involves 14 countries working in collaboration with the WHO, PATH and the Centers for Disease Control and Prevention (CDC) in Atlanta, GA (USA) estimated that 73% of hospital admissions of children for diarrhoea in South Korea were RV positive, 58% in Japan, 55% in Vietnam, 53% in Myanmar, 46% in China, 43% in Thailand and 30% in HongKong.

Before the introduction of vaccination, RV gastroenteritis was estimated in the USA to account for over 50 000 hospitalizations, 200 000 emergency department visits, 410 000 physician office visits, and 20 to 40 deaths per year. In 2006 in the region of the Americas there were more than 10 million episodes of RV diarrhoea requiring domiciliary visits, 2 million requiring a clinic consultation, and 75,000 requiring hospitalization, leading to considerable medical costs (more than US\$ 17 million in Mexico alone). In Asia, universal RV immunization would avert about 110 000 deaths, 1.4 million hospitalizations and 7.7 million outpatient visits [83].

1.6.2. Virology

Rotaviruses are 70 nm icosahedral, non-enveloped, double-stranded RNA viruses that belong to the family Reoviridae. The virus is characterized by its three-layer capsid, an outer and an inner capsid and an internal shell that surrounds the 11-segment double-stranded RNA genome. The outer capsid is made of two proteins, VP4, also named “P protein”, and VP7, also known as the “G protein”, which define the “P” and “G” serotypes of the virus, respectively. Both are key neutralization determinants on the surface of the virion. The inner capsid is made of the VP6 protein, the most abundant and immunogenic protein in the virion. Anti-VP6 antibodies do not neutralize virus infectivity but VP6-specific IgAs appear to confer protection *in vivo*, perhaps through inhibition of virus transcytosis through the intestinal epithelium barrier [144].

When mixed infections with distinct RV strains occur, the individual genomic RNA segments can reassort independently, producing progeny “reassortant particles” of mixed parentage, which could theoretically lead to the emergence of up to 110 different G and P combinations. In fact, G serotypes G1–G4 and G9, and P genotypes P[4] and P[8] are predominant worldwide, causing aver 90% infections in industrialized countries and about 68% infections in South American and Asian countries [145]. P[8]-G1 is the globally predominant strain, followed by P[8]-G3, P[4]-G2, and P[8]-G4 [146]. G9 strains have emerged in the early 2000s and have become predominant in some regions of the world, including Europe, Thailand and parts of Eastern Asia. Less usual strains may also be found, such as P[6]-G8 in Africa, P[8]-G5 in Brazil and novel P[11]-G10 and P[6]-G12 in India [147].

Rotavirus surveillance system networks have been constituted with the collaboration of the CDC in Atlanta (USA) and WHO to estimate the hospital-based disease burden of RV gastroenteritis in children less than five years of age, and to constantly update the frequency and characteristics of circulating strains. This aspect is of importance for the development of RV vaccines and for studying the possible vaccine selective pressure leading to emergence of new strains in the vaccinated populations.

1.6.3. Vaccines

Natural RV infection protects partially against reinfections, a first episode in newborns or young infants attenuating the severity of diarrhoea during subsequent episodes. Reinfection

seems to broaden and boost natural immunity. Usually, complete protection against severe gastroenteritis is acquired after the second infection. Therefore, even if vaccination early in life may not prevent all subsequent disease episodes, it should prevent most cases of severe RV disease and their complications such as dehydration, physician visit, hospitalizations and deaths [134]. Because RV remains the most common cause of severe diarrhoea in children in all regions of the world, an RV vaccine will have universal application as part of childhood vaccination programmes [148].

1.6.3.1. Live attenuated RV strains

The first RV vaccines to be tested in humans were the live bovine strains RIT4237 (P[1]-G6) and WC3 (P[5]-G6), and the live simian strain RRV (P[3]-G3), which are attenuated for humans and could be administered by the oral route. The three strains induced neutralizing antibodies in a majority of infants but showed inconstant capacity to protect against RV disease.

In China, a lamb-derived monovalent (P[12]-G10) live-attenuated, 3-dose oral vaccine, was developed by the Lanzhou Institute of Biomedical Products and is used in the private sector. The vaccine is reported to induce neutralizing antibody responses in 60% of vaccinees but its efficacy is not precisely known since it was not tested against placebo in a controlled Phase III trial [135].

A human P[8]-G1 RV strain, RIX 4414, which was isolated from the stools of a sick 15-month old boy in the USA, was attenuated by multiple passages in cell culture, plaque-purified and passaged again in Vero cells. The strain was developed as a 2-dose monovalent oral vaccine by AVANT Immunotherapeutics then licensed to GlaxoSmithKline Biologicals. The vaccine (Rotarix™) showed 70%-85% protective efficacy against severe disease, including that due non-G1 serotypes [149] [150]. It now has been tested in more than 60 countries in Latin America, Africa, Asia and Europe. A large, multicentered safety trial on 63 225 infants between 6 and 14 weeks of age in Latin America and Finland confirmed the initial safety data and indicated no increased attributable risk of intussusceptions (IS) in the high-risk period up to 30 days post any dose [151]. The vaccine was first licensed in 2004 in Mexico and the Dominican Republic and has now been licensed in many countries worldwide. It also has been prequalified by WHO for procurement by UNICEF and the UN Vaccine Fund. Additional Phase IIb and III trials are in progress in South Africa, Malawi and Bangladesh to determine if the vaccine will work well in children from poor settings in developing countries, if it can be administered with the oral polio vaccine without interference, and whether it can safely be administered to HIV positive infants. Final results are due in 2009.

Another human RV strain, RV3 (P2[6]-G3), isolated from newborns at the Royal Hospital in Melbourne, Australia [152] is also developed as a candidate live oral vaccine. A small Phase II study with three doses of 10^5 pfu of the vaccine indicated relatively low immunogenicity in infants as measured by serum IgA levels [153]. However, the vaccine recipients who developed an immune response were protected against clinical disease in the following year. Strategies to increase the potency of the vaccine are under study with a vaccine producer in Indonesia.

1.6.3.2. Live reassortant RV strains

Efforts also were made to develop human-animal reassortant RV strains containing the VP7 or VP4 RNA segment from a human RV strain to provide the required antigenicity and the

other 10 RNA segments from a simian or a bovine strain to provide the attenuated phenotype [154] [155].

A tetravalent rhesus-human reassortant RV vaccine, RRV-TV, was initially developed at the NIH, Bethesda, using the simian RRV strain (G3) mixed with three human-simian reassortant strains of G types 1, 2, and 4, respectively. The vaccine (RotaShield™, Wyeth-Lederle Vaccines, USA) was shown to provide 48–68% protection against any RV disease and 64–91% protection against severe disease [156]. It was introduced in August 1998 on the market in the USA and administered in a three dose schedule to over 600 000 infants within the following year, until an unexpected adverse event, intussusception (IS), was found to occur in a significant number of cases within two weeks after administration of the first two doses of vaccine, leading to its eventual withdrawal [157]. The risk of IS, initially targeted at 1 in 2500 children immunized, has now been reassessed as 1 in 10 000. Its occurrence led to a very thorough safety assessment of the following generation of live oral RV vaccines (viz Rotarix™ and RotaTeq™), with sample sizes in excess of 60 000 subjects. The original Rotashield™ vaccine has now been licensed to a biotech company, BIOVIRx Inc., USA.

A pentavalent human-bovine reassortant vaccine, RotaTeq™, was prepared by Merck Research Co., Pennsylvania, by reassortment between the naturally attenuated bovine RV strain WC3 and five different human RV strains of serotypes G1, G2, G3, G4 and P[8], respectively. The live-attenuated, 3-dose oral vaccine, was tested in a large safety and efficacy trial in Finland and the USA on more than 70 000 children who were carefully monitored for 2 weeks after each immunization for risks of IS. The vaccine was found to be totally safe and to elicit 74% protection against any G1-G4 RV gastroenteritis through the first RV season after vaccination [158]. Vaccination reduced doctor visits for RV diarrhoea by 86% and hospitalizations and emergency department visits by 94.5%. The vaccine was shown not to interfere with the immunogenicity of a combined Hib, DTP, HepB, conjugated pneumococcal and inactivated polio vaccine, nor with concomitant administration of the oral polio vaccine [159]. RotaTeq™ was licensed in February 2006 in the USA and subsequently in many countries worldwide. It officially was recommended for the routine immunization of children in the USA after active surveillance showed only three cases of IS among more than 100 000 vaccinated infants. It also has been included into national vaccination programs in several countries. A Phase III trial is ongoing in African countries (Mali, Ghana, and Kenya). Results are expected end of 2009.

An alternative multivalent bovine-human reassortant oral vaccine was developed by the National Institute of Allergy and Infectious Diseases (NIAID, NIH, Bethesda), based on the attenuated bovine strain UK reassorted with the five most common RV serotypes in humans, G1-G4, G8 and G9 [156]. Phase II data showed a good immune response and no adverse interference with concomitantly administered childhood vaccines. A non-exclusive license for production of the vaccine has been granted to vaccine producers in Brazil, China and India.

Finally, a naturally occurring human-bovine, neonate-derived RV strain, 116E (P[10]-G9), which was isolated from a nosocomial outbreak of asymptomatic infection in New-Delhi, is under development by Bharat Biotech Ltd in India [148] [160]. A similar strain, I321 (P[11]-G10), was found not to be immunogenic [161].

1.6.3.3. Other RV vaccine approaches

New RV vaccine approaches include an inactivated virus vaccine [162], DNA vaccines [163], a VP6 subunit vaccine [164] [165] and virus-like particles (VLPs) expressed in a

baculovirus system [166] [167] [168] Depending on the number of viral proteins expressed, the complexity of the VLPs can vary from mono-layered (VP2-VLPs) to double-layered (VP2/6 VLPs) or triple-layered VLPs (VP2/6/7/4 VLPs).

These candidate vaccines have been found to be immunogenic in mice and rabbits after administration by the parenteral, transcutaneous, oral, nasal or rectal routes. Whether they will be further developed will depend on the outcome of the current live oral RV vaccines, which are facing several key questions in regard with cost, efficacy in developing countries and safety [169] [170] Phase III trials of the Rotarix™ and RotaTeq™ vaccines have been initiated by PATH in partnership with GSK in Malawi and South Africa and with Merck in Kenya, Mali, Ghana, Vietnam and Bangladesh. Even if the two live oral RV vaccines prove to be efficacious in these settings, however, their current price makes them unaffordable to poor countries [171] and new financing mechanisms will have to be set up for vaccination to be implemented on a routine basis in these countries.

Another matter to watch carefully will be that of cross-protection against the full range of RV strains, including serotype G9, which is becoming increasingly important across Asia, and G8, which is gaining prevalence across Africa.

The benefit of vaccination can however be observed in the recent CDC report [172], which indicates a more than 50% reduction in seasonal incidence of RV during 2007-2008 in the USA, coinciding with increased use of RV vaccine. During the first 18 weeks of 2008, only 6% of samples tested positive for RV, compared to 51% in 2006 and 54% in 2007 over the same period.

1.7. [Shigellosis](#)

1.7.1. *Disease burden*

Shigellosis is endemic throughout the world where it is held responsible for some 120 million cases of severe dysentery with blood and mucus in the stools, the overwhelming majority of which occur in developing countries and involve children less than five years of age [101] [173]. About 1.1 million people were estimated to die from Shigella infection each year, with 60% of the deaths occurring in children under 5 years of age. More recent estimates fix the Shigella disease burden at 90 million episodes and 108 000 deaths per year [Lanata, personal communication]. In addition, about 500 000 cases of shigellosis are reported each year among military personnel and travellers from industrialized countries.

The disease is characterized by a short period of watery diarrhoea with intestinal cramps and general malaise, soon followed by permanent emission of bloody, mucoid, often mucopurulent stools. Outbreaks of dysentery due to *S dysenteriae* type 1 are frequent in poor populations living in crowded settings where hygiene is poor and sanitation non-existent. Acute complications may occur that include peritonitis and septicaemia, especially in malnourished children, and the severe Haemolytic Uremic Syndrome (HUS) with renal failure. Since the late 1960s, pandemic waves of Shigella dysentery have hit sub-Saharan Africa, Central America and South and South-East Asia, often striking areas of political upheaval and natural disaster. During the 1994 genocide in Rwanda, approximately 20 000 Rwandan refugees who had fled into the North Kivu region of Zaire died in one month from dysentery caused by a strain of *S dysenteriae* that was resistant to all commonly used antibiotics.

Shigellosis is transmitted from humans-to-humans by the faecal-oral route via contaminated food and water or through person-to-person contact, as often observed in institutionalized

populations. Transmission by house flies has also been documented [174]. Infection is common among travellers and military troops deployed in camps with less than optimal hygiene conditions [46].

In the absence of an existing effective vaccine, the ever increasing frequency of antimicrobial-resistant *Shigella* strains worldwide has become a major source of concern [12] [175]. During a survey of 600 000 persons of all ages residing in Bangladesh, China, Pakistan, Indonesia, Vietnam and Thailand, *Shigellas* were isolated in 5% of the 60 000 diarrhoea episodes detected between 2000 and 2004 and the majority of the bacterial isolates were resistant to amoxicillin and cotrimoxazole [176]. Similarly, during a 36-month surveillance study in a rural district in Thailand, where incidence of shigellosis was measured to be 4/1000/year in less than 5 years-old children, 95% of the *S. sonnei* and *S. flexneri* isolates were resistant to tetracycline and cotrimoxazole, and 90% of the *S. flexneri* isolates were also resistant to ampicillin and chloramphenicol [177]. A similar finding was made in North Jakarta, Indonesia, where a surveillance study done between August 2001 and July 2003 found that children aged 1 to 2 years had a high incidence of shigellosis (32/1000/year) with 73% to 95% of the isolates being resistant to ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol and tetracycline [178].

1.7.2. Bacteriology

Three major species of *Shigella* are responsible for bacillary dysentery: *S. sonnei*, *S. flexneri* and *S. dysenteriae*. A fourth species, *S. boydii*, is responsible for scattered disease foci. These species are further subdivided into subtypes on the basis of the antigen specificity of the O-polysaccharide portion of their LPS [101]. *S. sonnei*, which has a single serotype, is the causative agent of most shigellosis in industrialized countries where it accounts for 77% of cases (compared to 15% in developing countries), but it also has become predominant in Thailand in recent years. *S. flexneri*, which has 14 serotypes and subtypes, is endemic in developing countries (60%) and is the most frequently isolated species worldwide. The predominant serotypes are *S. flexneri* 2a, followed by 1b, 3a, 4a and 6. Untypable *Shigellas* have also recently emerged as a significant cause of diarrhoea. The serotype 1 of *S. dysenteriae* (Sd1) is of particular concern due to its expression of the Shiga toxin. It is the cause of epidemic dysentery and can cause vicious outbreaks in confined populations, especially refugee camps. A major obstacle to the control of Sd1 is its resistance to antimicrobial drugs. However, recent surveillance data from Bangladesh and India show that, for unknown reasons, Sd1 seems to have disappeared from these regions.

Shigella invade the colonic epithelium by transcytosis through M cells and penetration into the epithelial cell layer by the basolateral membrane, then spread laterally from cell-to-cell. This invasive ability is due to several virulence factors encoded by a high molecular weight virulence plasmid, including IpaB, which shows strong affinity for the CD44 hyaluronic acid receptor and IpaC, which forms pores through the basolateral membrane of epithelial cells, allowing the bacterium to penetrate into the cytoplasm of the cell. The bacteria multiply in the cytoplasm and eventually kill the host cell while moving towards adjacent epithelial cells by a process of polymerisation/depolymerisation of the actin tubules mediated by the VirA virulence factor [179]. Their pathogenicity is also due to several enteric toxins, including *Shigella* enterotoxin 1 (ShET-1), which is encoded by chromosomal gene set present in *S. flexneri* strains 2a, enterotoxin 2 (ShET-2), the product of gene *sen*, which is carried by a large virulence plasmid common to most *Shigella* spp, and the Shiga toxin, which is encoded by the *stx* gene from a phage of *S. dysenteriae*. ShET-1 is a classical enterotoxin made of an active A subunit associated with five B subunits. The Shiga toxin inhibits protein synthesis in eukaryotic cells via inactivation of ribosomal RNA, leading to cell death. The toxin is cytotoxic, neurotoxic and enterotoxic. It targets

glomerular epithelial cells and central nervous system microvascular endothelial cells, causing an haemolytic-uremic syndrome (HUS) and seizures [180]. Sd1 also causes a rapid increase in the cell membrane permeability of infected macrophages and destroy their mitochondrial function [181].

1.7.3. Vaccines

Epidemiology and volunteer studies have shown that protective immunity to Shigellas is directed to the O-somatic antigen and is narrowly type-specific, which has hampered the development of an effective Shigella vaccine. Strong mucosal sIgA anti-O-antigen antibody responses follow wild-type infection and experimental challenge [182]. Passive administration of cow's milk immunoglobulin that contained high-titer anti S flexneri 2a antibodies protected volunteers from experimental challenge with wild type S flexneri, whereas cow's milk with low titers of such antibodies did not [183]. In addition, cell-mediated immunity mechanisms, including IFN- γ -secreting T cells, seem to play a role in recovery and immunity [184]. Candidate shigellosis vaccines currently in advanced development include both polysaccharide conjugate and live attenuated vaccines and mostly focus on the most frequently isolated S flexneri 2a and S sonnei, as well as on Sd1, due to the severity of cases [182] [185] [186] [187].

1.7.3.1. Polysaccharide conjugate vaccines

Parenteral conjugate vaccines were produced from purified *Shigella* lipopolysaccharide (LPS) from the relevant bacterial serotypes that were conjugated to tetanus toxoid, recombinant *Pseudomonas aeruginosa* exotoxin A (PsA) or CRM9 mutant diphtheria toxin. These vaccines, which were developed at the NIH, were shown to be 74% efficacious against disease when tested in field trials with Israeli military volunteers [188] [189] and demonstrated safety and immunogenicity in 4–7 year-old children [190] [191]. A Phase III trial of *S flexneri* 2a and *S sonnei* conjugate vaccines was recently completed in young children in Israel [192]. O-antigen-specific IgG antibody elicited by conjugate polysaccharide vaccines prevent *in vitro* invasion of *Shigella* into Caco-2 cells in culture and may be curative [193]. The use of synthetic oligosaccharides that mimic the O-antigen protective epitopes and are conjugated to appropriate protein carriers offers promise for an improved and cheaper future generation of conjugate *Shigella* vaccines [194].

1.7.3.2. Live attenuated vaccines

Definite progress has been made in the field of candidate live oral attenuated shigellosis vaccines, but the small margin that exists between under-attenuation responsible for excessive reactogenicity of the vaccine, especially in children, and over-attenuation leading to insufficient immunogenicity in human subjects, especially in developing countries, remains a major problem.

Serially passaged streptomycin-dependent (SmD) *S. flexneri* 1, 2a, and 3a and *S. sonnei* strains were shown in former Yugoslavia to be attenuated in adults, healthy and institutionalized children and to constitute candidate oral vaccines that were protective in 82–100% of cases [195] [196]. However, side effects due to reversion of the mutation were observed and the development of the vaccine was discontinued.

A live hybrid attenuated *Shigella* strain expressing both *S. flexneri* 2a and *S. sonnei* O-antigens was developed as an oral vaccine (FS) at the Lanzhou Institute of Biological Products, using the T₃₂ attenuated *S flexneri* strain initially developed by Istrati in Romania. Large field studies in China have demonstrated 61–65% protection against *S. flexneri* 2a

and 57–72% protection against *S. sonnei*. A protective efficacy against heterologous *Shigella* species was also claimed [197]. However, the use of a 3-dose vaccination regimen with high doses of a live vaccine strain ($>2 \times 10^{10}$ cfu) remains problematic at this time. Further field studies of the FS vaccine in toddlers and infants may help define the public health application of this vaccine in China.

A live, attenuated *S. flexneri* 2a strain (SC602), and an attenuated *S. dysenteriae* type 1 strain (SC599) carrying mutations in their *icsA*, *iuc*, *iut* and *stxA* genes, were developed at the Pasteur Institute, Paris. IcsA is an outer membrane protein that nucleates cellular actin, thereby allowing the cell-to-cell spread of the bacteria. Iuc iut are involved in the scavenging of Fe^{3+} ions via the production of siderophores (aerobactin). *StxA* encodes the catalytic subunit of the Shiga toxin. SC602 was tested in adult volunteers in the USA and in adults and children in Bangladesh in collaboration with the Walter Reed Army Research Institute (WRAIR) and IVI. A remarkable efficacy against challenge was observed in USA volunteers [198] [199], but results of immunogenicity were disappointingly low in young infants in the field, due to lack of colonization of the gut, perhaps as a consequence of the presence of maternal antibodies from breastfeeding, or due to over-attenuation of the vaccine candidate for this target population.

Numerous approaches at attenuating *S. flexneri* 2a by targeted deletion of virulence genes including the *set* and *sen* genes have similarly been carried out at the Center for Vaccine Development (CVD) in Maryland, USA, resulting in an array of candidate vaccine strains such as CVD 1203, CVD 1204, CVD 1207, CVD 1208 and culminating in strain CVD 1208S, which recently underwent Phase I clinical trials [200] [201] (For a review, see [182]).

Investigators at WRAIR also constructed a series of attenuated *S. sonnei* strains, resulting in strain WRSs1 which showed immunogenicity and clinical acceptability [202]. They also attenuated Sd1 by deletion of its *stxAB* and *virG/icsA* genes, resulting in strain WRSd1 [203], which, unfortunately, was only modestly immunogenic in human volunteers, probably because of insufficient colonization of the gastrointestinal tract. The defect has been corrected in a more recent generation Sd1 vaccine strains by reinserting the *fnr* gene (fumarate/nitrate reductase), a global transcriptional regulator. At the same time, a new *S. flexneri* vaccine candidate strain, WRSf2G11, was constructed by deleting the *icsA*, *set* and *sen* genes [204].

Altogether, this well illustrates the difficulty met in trying to achieve the right balance between robust immunogenicity, especially in young children, optimal colonization and shedding patterns, and clinical tolerance of the attenuated strains, as well as the need to develop a multivalent vaccine to cover a spectrum of *Shigella* spps.

As an alternative strategy, several groups have attempted to express *Shigella* O-antigens in well-tolerated live vectors such as *E. coli*, generating strain EcSf2a-2, or attenuated *S. typhi* strainTy21a, but with only limited efficacy so far (reviewed in [182]).

1.7.3.3. Other candidate *Shigella* vaccines

A formalin-inactivated *S. sonnei* vaccine (SsWC) was developed as an oral, killed, whole-cell vaccine at the Johns Hopkins University in Baltimore, MD, USA, and recently tested in a Phase I trial on a small number of volunteers [205]. Similarly, Antex (USA) is developing a *Shigella* inactivated whole-cell vaccine as well as an oral travellers' diarrhoea vaccine

(Activax™) containing antigens from *Campylobacter*, *Shigella* and ETEC. These candidate vaccines will shortly undergo clinical testing.

Several subunit *Shigella* vaccines, including a parenteral nuclear protein/ribosomal vaccine developed by the International Vaccine Institute (IVI) and the Walter Reed Army Institute of Research (WRAIR), and a nasally administered proteosome vaccine consisting of *Shigella* LPS linked to micelles of the outer membrane protein of group B *Neisseria meningitidis*, still are at a preclinical stage. A novel formulation using a bacterial extract invasion complex named invaplex, which contains IpaB, IpaC, and LPS from *S flexneri* and *S sonnei*, was found to elicit protection against challenge in the guinea pig model [206] [207].

1.8. [Typhoid fever](#)

Salmonella infections in humans are divided into typhoid fever caused by *S typhi* and *S paratyphi* (see below) and a range of diarrhoeal diseases caused by a large number of non-typhoidal Salmonella serovars (NTS). These NTS, which usually have a broad vertebrate host range, show dramatically more severe and invasive presentation in immunocompromised individuals especially HIV carriers, including severe and progressive diseases such as chronic granulomatous disease, blockade of IL-12/ IL-23 /IL-17 and TNF, suppurative foci and bacteremia which may be recurrent. Invasive recurrent NTS bacteremia associated with HIV disease is becoming a huge problem worldwide [208].

Typhoid fever (TF) is a more classical systemic infection caused by the typhoid bacillus, *Salmonella enteritica* serovar Typhi (commonly referred to as *S typhi*), the most common cause of enteric fever, which also includes paratyphoid fever caused by *S paratyphi* A, B and C. These pathogens only infect humans. The disease is transmitted by ingestion of food, including dairy products, or water contaminated by excreta from patients or chronic carriers or handled by infected persons [209] [210]. Highest incidence usually occurs where water supplies serving large populations are contaminated by faecal matter, as existed at the end of the 19th century in many large cities in the USA and Western Europe.

1.8.1. *Disease burden*

TF is spread by the faecal-oral route and closely associated with poor hygiene, lack of clean drinking water and inadequate sanitation. The disease is almost exclusively transmitted by food and water contaminated by the faeces and urine of patients and carriers. Polluted water is the most common source of typhoid transmission. In addition, shellfish taken from sewage-contaminated beds, vegetables fertilized with night-soil and eaten raw, contaminated milk and milk products have been shown to be a source of infection.

Although TF has practically disappeared from industrialized countries, it remains a serious public health problem in several Asian regions of the former USSR and in parts of South and South-East Asia, Africa and South America. In the last outbreak in the Democratic Republic of Congo, between 27 September 2004 and early January 2005, no less than 42 564 cases of typhoid fever were reported, including 214 deaths and 696 cases of peritonitis and intestinal perforations. Also, multiresistant strains of *S typhi* are becoming increasingly common worldwide, further compounding the risk to people living in regions with high endemic disease and to travellers [211] [212]. Strains resistant to chloramphenicol and other recommended antibiotics (ampicillin, cotrimoxazole and even ciprofloxacin) have become prevalent in several areas of the world [213].

TF is characterized by the sudden onset of sustained fever, severe headache, nausea, abdominal pains, loss of appetite, constipation or sometimes diarrhoea. The illness can last for several weeks and even months. The most frequent complications, which arise with a frequency of 1% to 4%, include gastro-intestinal bleeding and intestinal perforation. Severe neurological forms also have been described with mental dullness, stupor, delirium and shock. Hospitalization of TF cases varies from 10% to 40% of cases and usually lasts for 10-15 days or more. Case-fatality rates, which varied from 10% to 30% before the advent of antibiotics, has now been reduced to about 1%-4% with appropriate antibiotic therapy [214]. Paratyphoid fever, which is caused by any of three serotypes of *S. paratyphi* A, B and C, is similar in its symptoms to typhoid fever, but tends to be milder, with a lower fatality rate.

People can transmit TF as long as the bacteria remain in their body; most people are infectious prior to and during the first week of convalescence, but 10% of untreated patients will discharge bacteria for up to 3 months. In addition, 2–5% of untreated patients will become permanent, lifelong carriers of the bacteria in their gall-bladder.

The true burden of TF in developing countries is difficult to estimate. According to recent estimates, 22 million (range 16 million – 33 million) cases occur each year causing 216,000 deaths, predominantly in school-age children and young adults [214]. Asia, with 274 cases per 100,000 persons has the highest incidence of TF cases worldwide, especially in Southeast Asian countries and on the Indian subcontinent, followed by sub-Saharan Africa and Latin America with 50 cases per 100,000 persons. In an urban slum in Dhaka, incidence of bacteremic TF was found to be 390/100,000 population, with a 9-fold higher risk for pre-school children than for older persons [215].

Recent prospective population-based disease-surveillance studies supported by the Bill and Melinda Gates Foundation and conducted by the Diseases of the Most Impoverished (DOMI) Program at five sites in China, India, Indonesia, Pakistan and Vietnam revealed high rates of TF among children in urban slums, including children below 5 years of age. In three urban slums in Karachi, Kolkata and North Jakarta, incidence of blood-confirmed TF cases among children 5 to 15 years of age ranged from 180 cases to 494 cases per 100,000 [216].

The introduction of TF vaccines in routine vaccination programs in Asia would be highly beneficial in view of the burden of disease and cost of illness to governments and individuals [217]. But, so far, only two countries, China and Vietnam, have incorporated typhoid vaccination into their routine immunization programs, and only in a limited fashion. The Dehli State, India, also introduced vaccination in 2004 in 2-5 years old children through community-based campaigns. The reason why these efforts have not be more generalized lies in part in the fact that most developing countries are uncertain of their true TF disease burden, due to lack of rapid diagnostic tools, infrequency of laboratory testing and poor reporting system [218].

Most cases of TF in industrialized countries are imported cases from endemic countries [219].

1.8.2. Bacteriology

Taxonomy within the genus *Salmonella* has been the source of great confusion. The most recent classification, based on DNA sequences, has left only two species, *S. enteritica* and *S. bongori*, further subdivided into subspecies and serovars. To avoid confusion, *S. enteritica* serovar Typhi continues to be referred to as *S. typhi*. The bacteria is characterized

by its flagellar antigen, H, its lipopolysaccharidic (LPS) O antigen, and, in addition, its polysaccharide (PS) capsular virulence (Vi) antigen, found at the surface of freshly isolated strains. The complete sequence of the 4 809 037-bp genome has been determined. In addition to the plasmid encoding antibiotic resistance, a virulence plasmid was found that shows homology with the virulence plasmid of *Yersinia pestis*.

Upon ingestion, typhoid bacilli rapidly penetrate the small intestinal mucosa by transecytosis through M cells and enterocytes, and are taken up by macrophages or diffuse into mesenteric lymph nodes. A primary bacteraemia follows and the pathogen rapidly attains intracellular haven throughout the reticuloendothelial system. This is followed by a sustained secondary bacteraemia associated with clinical illness. *S. typhi* also shows remarkable predilection for the gall-bladder where infection tends to become chronic, especially in individuals with a pathologic gall-bladder condition.

1.8.3. Vaccines

The heat-killed, phenol-preserved, injectable whole-cell *S. typhi* vaccine that was utilized as far back as 1896 in England and Germany, is still licensed today in several countries in spite of its high reactogenicity. Two new vaccines are currently licensed and widely used worldwide, a subunit (Vi PS) vaccine administered by the intramuscular route and a live attenuated *S. typhi* strain (Ty21a) for oral immunization [220] [221]. Several typhoid vaccination programs that involve annual children vaccination campaigns using the injectable Vi vaccine have been carried out in Asia, resulting in a marked reduction or near disappearance of the disease, including in age groups not targeted for vaccination, thus suggesting a possible herd protective effect of vaccination [222].

1.8.3.1. The Vi polysaccharide vaccine

The A subunit of *S. typhi* PS was developed as a vaccine in the 1980s in the laboratory of John Robbins at the NIH and licensed to Sanofi-Pasteur. The vaccine is based on purified Vi antigen, a linear homopolymer of galacturonic acid that is purified from the bacteria by treatment with Cetavlon, the detergent used for the preparation of the meningococcal PS vaccine. First licensed in 1994, the vaccine (Typhim™, Sanofi-Pasteur; Typbar™, Bharar) is administered as one dose of 25 µg by the IM or SC route. It now is in the public domain and is produced by several manufacturers including developing country manufacturers. The vaccine is progressively introduced into school attending children vaccination programs in Asian countries [223] [224] [225].

Its efficacy was demonstrated in multiple randomized trials. One of the first trials in Nepal involved persons aged 5-44 years and showed a 75% protection against TF during 20 months of active surveillance. Another study in South Africa demonstrated a 64% protective efficacy after 21 months in children aged 5-16 years, declining to 55% at 3 years after vaccination [226] [227]. A locally produced Vi vaccine prepared by the Shanghai Institute of Biological Products, which was evaluated in a randomized placebo-controlled, double-blind trial on about 60 000 5-19 years old children in China showed a 69% efficacy against blood culture-confirmed TF over 19 months [228]. The Vi vaccine is considered to be protective for at least three years; it also has demonstrated a remarkable safety profile. Several high-quality producers from developing countries have acquired the technology to produce it at low cost, including two producers in China.

As is the case with other PS vaccines, Vi PS is poorly immunogenic in infants and cannot be used to vaccinate children less than 2 years of age. This has prompted the development of a Vi conjugate vaccine at the National Institutes of Health, USA, using recombinant

Pseudomonas aeruginosa exotoxin A as the protein carrier (Vi-rEPA). In a Phase IIb 2-dose trial among 5525 2-5 year old children in the Mekong Delta of Vietnam, where TF is highly endemic, the Vi-rEPA vaccine demonstrated 91% efficacy over 27 months and 89% over 46 months of follow-up [229]. Similar results were obtained in Cambodia [230]. The NIH prototype vaccine is not being commercially developed, partly due to the complexity introduced by the exotoxin A carrier, but a diphtheria toxoid-based conjugate Vi vaccine has been developed at the International Vaccine Institute (IVI) in Seoul and transferred to Shantha, Indonesia. A Phase I clinical trial is planned to begin soon. Another Vi conjugate vaccine is in development at the All India Institute of Medical Sciences in New Delhi using the OmpC protein from *S typhi* as carrier.

1.8.3.2. The live attenuated Ty21a vaccine

The attenuated *S. typhi* strain Ty21a was generated in Switzerland by chemical mutagenesis of wild-type strain Ty2 and developed as the first live oral typhoid fever vaccine. The strain is characterized as lacking both a functional galactose-epimerase (*galE*) gene and the Vi antigen, although other mutations in the genome probably are responsible for the attenuated phenotype. The vaccine (Vivotif™) is currently manufactured by Crucell (formerly Berna Biotech) as enteric-coated capsules to be swallowed every other day for one week. A liquid formulation of the vaccine is no longer manufactured. The vaccine can be taken simultaneously with the attenuated CVD103-HgR *V. cholerae* vaccine. Ty21a is licensed in 56 countries in Africa, the Americas Asia and Europe.

The vaccine was extensively tested in Alexandria, Egypt, where the liquid formulation showed 96% protection for 3 years, and in Santiago, Chile, where it was found to elicit a 67% protection against blood culture-confirmed TF over 3 years and 62% over 7 years [231]. The liquid formulation was shown to be 78% protective at 5 years. Ty21a is therefore considered to provide protection for at least 5-7 years. The vaccine was less efficacious when tested in Indonesia, where protective efficacy was found to be only 33%-53%, suggesting that in-the-field effectiveness in TF hyper-endemic areas, such as Indonesia, were quite lower than in areas with lower incidence of the disease, such as Egypt or Chile. In Chile, Ty21a also showed 42%-56% cross-protection against paratyphoid fever caused by serovar Paratyphi B [232].

A head-to-head comparison of the Ty21a and Vi vaccines has been proposed by WHO in order to make future recommendations for countries severely affected by typhoid.

1.8.3.3. Other live attenuated *S typhi* vaccines

Several live attenuated *S typhi* strains are being developed to be used as oral TF vaccines. In preliminary clinical trials, these strains seem to be even more immunogenic than Ty21a [233].

Perhaps the most advanced of these live attenuated candidate vaccines is Ty800, a *phoP/phoQ* deletion mutant of Ty2, which is developed by AVANT Immunotherapeutics [234] and has passed Phase II trials. The vaccine has been shown to stimulate vigorous IgA and serum O antibody responses in volunteers.

Another attenuated strain is CVD909, an *aroC/aroD/htrA* deletion mutant which was engineered to constitutively express the *S typhi* Vi antigen. The strain, which induces anti-Vi antibodies in orally vaccinated subjects [235], is developed as a live attenuated TF vaccine by Acambis and Crucell.

The third live attenuated vaccine candidate is ZH09, a *Salmonella typhimurium* strain deleted of the Salmonella pathogenicity island, which is developed by Emergent Biosolutions and is in Phase II trial [236]. A killed but metabolically active *S typhimurium* strain, CKS362, is also being developed as a candidate Salmonella vaccine [237].

In addition, an attenuated *S. typhi* Ty2 strain with deletions in *ssaV* and *aroC* genes has been developed by Microscience (UK) as a live vector (spi-VEC) for oral vaccines against TF and ETEC diarrhoea [125] (see ETEC vaccines above).