



Review

# Polysaccharide Vaccines: A Perspective on Non-Typhoidal *Salmonella*

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**Abstract:** Polysaccharides are often the most abundant antigens found on the extracellular surfaces of bacterial cells. These polysaccharides play key roles in interactions with the outside world, and for many bacterial pathogens, they represent what is presented to the human immune system. As a result, many vaccines have been or currently are being developed against carbohydrate antigens. In this review, we explore the diversity of capsular polysaccharides (CPS) in *Salmonella* and other selected bacterial species and explain the classification and function of CPS as vaccine antigens. Despite many vaccines being developed using carbohydrate antigens, the low immunogenicity and the diversity of infecting strains and serovars present an antigen formulation challenge to manufacturers. Vaccines tend to focus on common serovars or have changing formulations over time, reflecting the trends in human infection, which can be costly and time-consuming. We summarize the approaches to generate carbohydrate-based vaccines for *Salmonella*, describe vaccines that are in development and emphasize the need for an effective vaccine against non-typhoidal *Salmonella* strains.

**Keywords:** carbohydrate; bacterial vaccines; *Salmonella*; typhoid fever; gastroenteritis; T-cell-independent antigen; capsule; capsular polysaccharide



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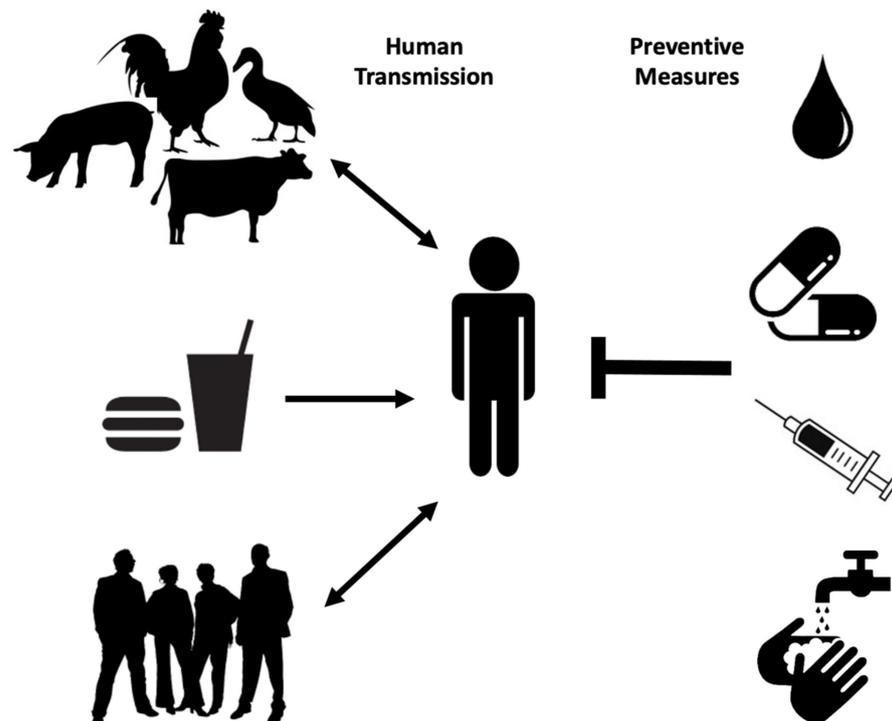
## 1. Salmonellosis

Salmonellosis is one of the most common foodborne diseases worldwide [1]. Symptoms of Salmonellosis include abdominal cramps, diarrhea, nausea, vomiting, chills, fever, and headache, and typically develop 6–72 h after ingestion. These symptoms can last up to 2–14 days depending on the initial concentration of bacteria ingested, serotype, age and the immune status of the host [2,3]. Salmonellosis in humans is primarily caused by non-typhoidal *Salmonella* (NTS), consisting of >1000 different serovars, with serovars Enteritidis and Typhimurium being the most common [4]. NTS infections can cause bacteremia in rare cases (i.e., 3–10%), which can lead to further complications, such as endocarditis, septic shock, infection of the liver, spleen, biliary tract and urinary tract, mesenteric lymphadenitis, pneumonia, osteomyelitis, pulmonary and brain abscess, empyema, meningitis, CNS infections and death [3]. In addition, perforation of the gut and necrosis of Peyer's patches can result in toxic encephalopathy [5]. The risk of bacteremia is higher in immunosuppressed individuals, infants, and the elderly, and the number of infections has risen in recent times in correlation with increased incidences of the human immunodeficiency virus (HIV) and malaria.

The global surveillance of Salmonellosis is primarily conducted by the US Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO). In the United States, *Salmonella* strains cause 1.35 million reported infections, 26,500 hospitalizations and 420 deaths annually [6]. According to the United States Department of Health and Human Services, *Salmonella* infections are the leading cause of hospitalizations and

death among foodborne pathogens. For Canadians, travel to endemic regions was the most common factor for reported NTS infections [4], specifically, travel to Central and South America, the Caribbean, Mexico and the Dominican Republic have seen the highest rates of infection [4]. Between 2009 and 2013, a yearly average of 6500 infections was reported throughout Canada, and in 2018, 7300 infections were reported, accounting for 48% of all notifiable isolates reported within the year [4]. In developing countries, *Salmonella* is responsible for 20% of childhood diarrheal infections and the mortality rate can reach as high as 24%, with certain serotypes having higher mortality rates than others [3]. However, 60–80% of Salmonellosis cases are thought to be either not diagnosed at all or are classified as sporadic cases [2,4].

Human transmission occurs through ingestion of *Salmonella*-contaminated food (i.e., raw or undercooked poultry) or drinks, contact with infected animals, especially food animals and pets, and animal feed (Figure 1). Person-to-person transmission through the fecal–oral route is also common and is facilitated by inadequate handwashing [3]. Preliminary diagnosis of salmonellosis in Canada is mainly based on symptoms and travel history and can be confirmed by serotyping (i.e., whole genome sequencing) each specimen from stool, rectal swabs, vomit, urine, deep tissue wounds, or sterile sites at the public health laboratories [4,7]. Treatment is based on symptoms and typically includes electrolyte replacement and rehydration, due to fluid loss that occurs through diarrhea and vomiting [2]. Commonly used first-line antibiotics include fluoroquinolones for adults and azithromycin for children. Ceftriaxone is used as an alternative first-line treatment [8]. However, antimicrobial therapy is usually reserved for immunocompromised patients, infants, children, the elderly and for severe cases of gastroenteritis, as unnecessary administration of antibiotics can select for resistant strains and contributes to prolonged shedding [2].



**Figure 1. Transmission and prevention of *Salmonella* infections.** Human transmission of *Salmonella* primarily occurs through ingestion of contaminated food or water, contact with infected animals or animal feed and person-to-person transmission. Dual arrows are shown between humans and animals because transmission can occur in both directions. The best preventive measures to reduce the incidence of human infections include proper sanitation, treatment of infected individuals, vaccination (when possible) and access to clean water.

Multidrug-resistant *Salmonella* strains have emerged as far back as 1989 [3]. As of 1997, more than 70% of clinical isolates showed resistance to ampicillin, tetracycline, sulphonamides, streptomycin, and chloramphenicol, with few strains even resistant to nalidixic acid, trimethoprim, sulfamethoxazole, and kanamycin [9]. Ceftriaxone resistance is considered significant because it is often correlated with resistance to other antibiotics commonly used, such as ceftriaxone, cefixime, amoxicillin-clavulanic acid, and ampicillin. As extra-intestinal Salmonellosis in young children and pregnant women is treated with Ceftriaxone, it is recognized as a drug of very high importance to human medicine. Thus, patients infected with even moderately Ceftriaxone-resistant *Salmonella* strains (i.e., *Salmonella* serovar Heidelberg) are at elevated risk of ceftriaxone therapy failure [10]. Proper sanitation, access to clean water supplies and treatment of infected individuals are among the best prophylactic measures to prevent the spread of Salmonellosis especially in endemic regions (Figure 1) [3]. A vaccine against common NTS serovars would also represent a big step in reducing the global incidence of Salmonellosis and bacteremia.

## 2. Capsular Polysaccharides

Extracellular polysaccharides are carbohydrate-based polymers synthesized and secreted into the external environment by bacteria [11]. They include lipooligosaccharides (LOS), lipopolysaccharides (LPS), capsular polysaccharides (CPS), teichoic acids, lipoteichoic acids, glycoproteins, and peptidoglycans [11]. They can be present on the cell surface as slime with no visible attachment (often called exopolysaccharides; EPS), or as CPS covalently linked to the bacterial cell surface or anchored to the cell surface via lipid A (LOS and LPS) [12]. In rare cases, CPS can be released from the cell due to poor stability of the phosphodiester interactions between the CPS and the cell surface phospholipid molecules. Conversely, at times, EPS can also remain tightly associated with the cell surface [13]. Therefore, the nomenclature does not always match the biological reality.

CPS are highly hydrated molecules containing 95–99% water and are composed of regularly repeating units of one or more monosaccharides linked via glycosidic bonds. They can take the form of homo- or heteropolymers. Homopolymers are composed of identical monosaccharides, while heteropolymers are composed of at least two different monosaccharides [14]. CPS are incredibly diverse in terms of their structure and composition because they can be composed of different monosaccharides connected by varying glycosidic linkages and modified with a diverse array of organic and inorganic molecules [12,14,15]. The structural diversity and the presence of immunological epitopes on CPS depend on a multitude of factors, including the type of monosaccharides, variations in the glycosidic linkages, the presence of organic and inorganic molecules and the introduction of side chains, configuration of the anomeric center of the sugar molecule, and enantiomeric form of the sugar [12,16].

During capsule biosynthesis, activated precursor molecules (monophosphate and diphosphate sugars) are assembled by inner membrane enzymes to form the growing polysaccharide chain. Following this, in the case of LPS assembly, the seven transport proteins Lpt A–G move the newly synthesized polysaccharide through the periplasm and across the outer membrane to the cell surface [17,18]. Studies have indicated the presence of transenvelope assembly complexes that coordinate simultaneous biosynthesis, export and translocation of the polysaccharide [19]. In some instances, identical translocation pathways are used for certain capsular polysaccharides and LPS O-Antigens [20].

CPS producing bacteria are highly diverse and include both animal and plant pathogens such as *Escherichia coli*, *Salmonella* serovar Typhi, non-typhoidal *Salmonella* serovars, *Shigella* species, *Neisseria* species, *Vibrio* species, *Streptococcus* species, *Cryptococcus neoformans*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Bacteroides fragilis*, *Haemophilus influenzae*, *Aeromonas salmonicida*, *Acetobacter methanolicus*, *Burkholderia mallei*, and *Francisella tularensis* [16,21–28].

### 2.1. Capsular Polysaccharides—Classification

Historically, the classification of CPS started with *E. coli*, which has over 80 different CPS, known as K antigens [29]. Certain K antigens have identical polysaccharide chains and differ only in the modifications of the polysaccharides [30]. According to the classification system introduced by Whitfield and Roberts (Table 1), a CPS can be classified into four categories [19]. Group 1 CPS are acidic polysaccharides containing uronic acid and tend to be similar in structure and are often inaccurately classified as colanic acid [19]. The most prominent feature distinguishing group 1 CPS and colanic acid is that most wild-type bacteria are unable to produce colanic acid when grown at 37 °C on common lab media, whereas they can produce CPS [19].

Group 2 capsules are linked to the cell surface through  $\alpha$ -glycerophosphatidic acid. In some bacteria, cytidine 5'-monophospho-3-deoxy-d-manno-2-octulosonic acid (CMP-KDO) provides a link between the phospholipid and the polysaccharide. The expression of group 2 capsules is significantly influenced by the growth temperature, and they are not typically expressed below 20 °C. Group 2 capsules harbour an  $\alpha$ -glycerophosphate moiety at the terminus [31]. Group 3 capsules are largely similar to group 2 capsules but differ in not being regulated by growth temperature. Both group 2 and 3 capsules are co-expressed with colanic acid and many different types of O-serogroups. Although there is only a hypothetical  $\alpha$ -glycerophosphate moiety at the terminus of group 3 capsules, genetic data support the presence of a common anchor for both group 2 and group 3 capsules. Despite the lack of information regarding the group 3 capsule biosynthesis pathway, functionally interchangeable CPS homologs have been identified between groups 2 and 3 capsules [31].

When bacteria produce a group 4 capsule as the only serotype-specific polysaccharide, it is classified as the O-Antigen of that isolate [19]. Group 4 CPS were previously classified as group 1 CPS and as a result, became known as the 'O-Antigen capsules' [31]. Group 4 CPS, despite many similarities, are far more diverse than group 1 CPS. One example is the group 4 representative K40 antigen that was assigned to group 1 due to the presence of acetamido sugars in its repeat unit. K40 antigen was then shown to be surface expressed as smooth LPS and as an unlinked O-Antigen capsule and was reclassified into group 4 [12,32]. This confusion in classification has arisen since the K40 antigen is co-expressed with a neutral LPS linked polymer such as O8 or O9 antigen [33]. Group 1 and 4 antigens are subgrouped into  $K_{LPS}$  and capsular K antigens, where  $K_{LPS}$  is made up of low molecular weight K antigenic oligosaccharides containing few repeat units that are linked to the cell surface through lipid A.  $K_{LPS}$ , however, are different from LPS with the serological O-Antigen found on the same cell [19]. Capsular K antigens are made up of high molecular weight antigens that form the capsule structure responsible for masking O-Antigen in serotyping. These capsular K antigens are not linked to lipid A core as in LPS molecules [33].

During translocation, group 1 and 4 capsule assembly occurs at the periplasmic face of the plasma membrane and uses the Wzx pathway, while the assembly of group 2 and 3 capsules occurs at the cytoplasmic face of the plasma membrane and an ABC transporter is used for translocation. During the assembly of capsules from all four groups, the sequential action of glycosyltransferase enzymes joins individual repeat units together to elongate the polysaccharide [33].

**Table 1. Classification of capsules.** Classification is based on Whitfield and Roberts 1999 research article [34]. Reprinted with permission from ref [34]. Copyright obtained 2021, John Wiley and Sons.

Characteristic	Group 1	Group 2	Group 3	Group 4
Thermostability	Yes	No	No	Yes
Thermoregulated (expressed below 20 °C)	No	Yes	No	No
Co-expressed with colanic acid	No	Yes	Yes	Yes
Co-expressed with O serogroups	Limited	Many	Many	Mainly O8, O9. Can be none

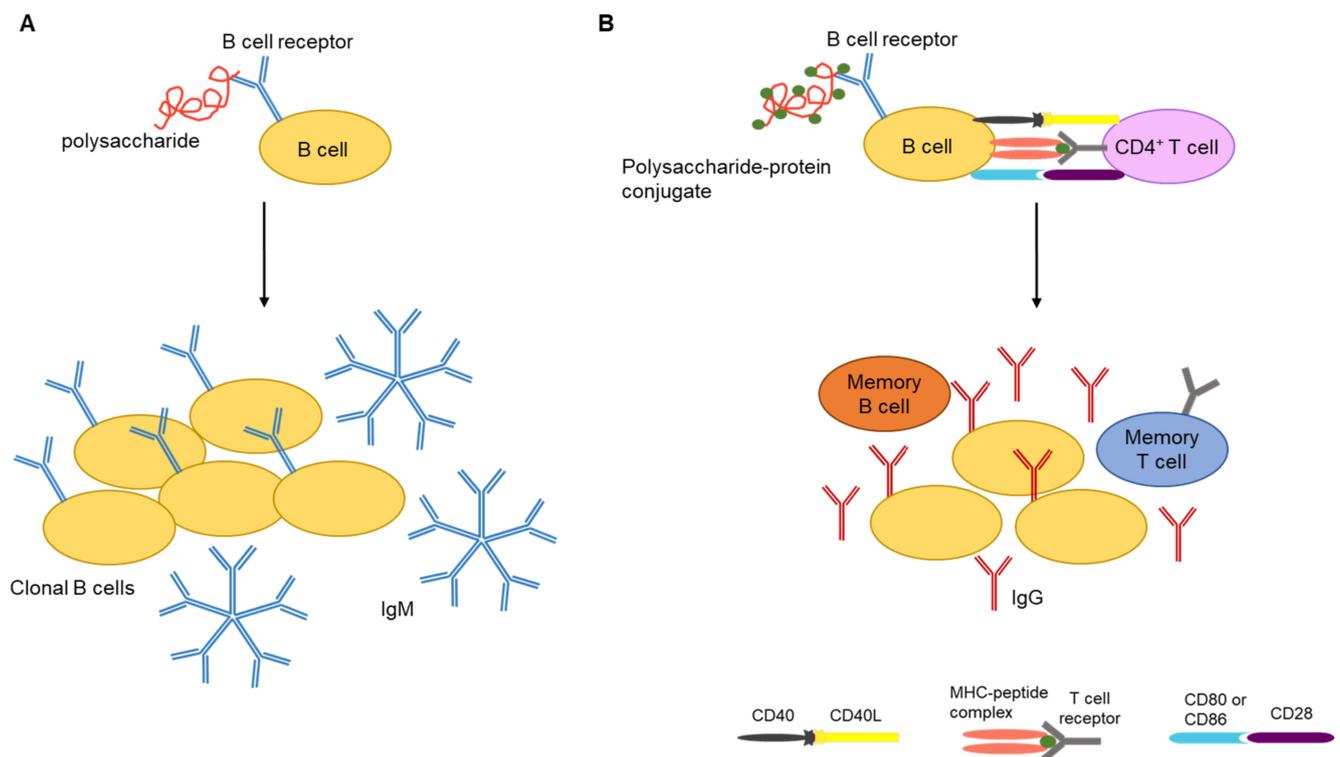
Table 1. Cont.

Characteristic	Group 1	Group 2	Group 3	Group 4
Former K antigen group	1A	II	I/II or III	IB (O-Antigen capsules)
Capsule assembly	Periplasmic face of the plasma membrane	Cytoplasmic face of the plasma membrane	Cytoplasmic face of the plasma membrane	Periplasmic face of the plasma membrane
Export system	Wzx pathway	ABC transporter	ABC transporter	Wzx pathway
Translocation proteins	Wza, Wzc	KpsD, KpsE (may be KpsF)	KpsD (may be KpsE)	Unknown
Polymerization system	Wzy dependent	Processive	Processive	Wzy dependent
Direction of polymer chain growth	Reducing terminus	Non-reducing terminus	Non-reducing terminus (hypothetical)	Reducing terminus
Terminal lipid moiety	Lipid A core in K <sub>LPS</sub> ; unknown for capsular K antigen	α-Glycerophosphate	α-Glycerophosphate (hypothetical)	Lipid A core in K <sub>LPS</sub> ; unknown for capsular K antigen
Elevated levels of CMP-Kdo synthetase	No	Yes	No	No
Positively regulated by the Rcs system	Yes	No	No	No

## 2.2. Capsular Polysaccharides—Function

As a major cell-surface component of many bacterial species, CPS can provide diverse functions. CPS often contribute to virulence, with innate and adaptive immune evasion; they also provide barrier protection, desiccation resistance, and act as lubricating agents and reduce friction over solid surfaces. For example, CPS facilitate swarming by *Proteus mirabilis* on solid surfaces [14]. It was observed that capsulated *E. coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* strains are more resistant to desiccation than non-capsulated strains [34]. In addition, CPS can allow bacterial adherence to host cells, surfaces, and to each other, thereby facilitating biofilm formation and colonization [35]. Biofilm formation is ubiquitous and can provide bacteria with nutritional advantages as well as protection from bacteriophage and phagocytic protozoa [12,36].

Studies have shown that pure carbohydrate antigens are T-cell-independent activators of B cells. Consequently, CPS antigens often induce immunoglobulin M (IgM) responses without a detectable level of IgG production (Figure 2A). The absence of antibody class switching from IgM to IgG and the inability to produce higher titers of antibody upon subsequent exposure to the antigen are hallmarks of T-cell-independent antigens [16]. However, conjugation of polysaccharides to carrier proteins can lead to T-cell activation, memory B-cell production, and the development of polysaccharide-specific immune responses (Figure 2B) [37].



**Figure 2. Antibody production in response to polysaccharide antigens.** (A) Polysaccharide antigens alone lead to IgM production, while (B) polysaccharide–protein conjugated antigens lead to IgG production and immune memory.

### 2.3. Capsular Polysaccharides—Regulation

Despite the lack of concrete evidence in the mechanisms behind the regulation of CPS expression, it is hypothesized that reduced external osmolarity can act as a signal to increase CPS production [12]. In support of this argument, it has been shown that reduced external osmolarity leads to increased expression of Vi antigen in *S. Typhi* and expression of anionic polysaccharide alginate by *Pseudomonas aeruginosa* [38,39]. In *E. coli*, the expression of genes for the biosynthesis of EPS colanic acid is increased in the face of harsh environments and desiccation [34]. In general, the expression of a given type of CPS is associated with a unique infection. For example, K1 antigen-expressing *E. coli* are the primary cause of neonatal meningitis [12]. Remarkably, chemically identical capsular polysaccharides can be produced by different genera of bacteria. For example, *E. coli* K1 CPS is identical to *Neisseria meningitidis* group B CPS, and *E. coli* K18, K22, and K100 CPS are identical to *Haemophilus influenzae* serotype b CPS [12,40].

### 2.4. Capsular Polysaccharides—*Salmonella* and Other Selected Bacterial Species

There are two types of CPS found in *Salmonella*—the Vi capsule (also known as Vi antigen) produced by *Salmonella* serovar Typhi, and the O-Antigen capsule produced by NTS. The Vi antigen is composed of O-acetylated  $\alpha$ -1,4-linked *N*-acetylgalactosaminuronic acid residues and is attached to the cell surface via a reducing terminal lipid structure [41]. The Vi capsule increases virulence and disease severity of *S. Typhi* infection by interfering with complement deposition and subsequent neutrophil chemotaxis and bacterial clearance [5,42]. Deletion of genes for Vi capsule biosynthesis can remarkably enhance neutrophil chemotaxis in vitro. In addition, the presence of the Vi capsule prevents complement activation through the alternative pathway, as C3b is unable to deposit on the capsule surface and promotes opsonophagocytosis [42,43]. It has also been demonstrated that the Vi capsule promotes immune evasion by masking LPS molecules and preventing recognition through pathogen recognition receptors (PRR). As a consequence, Vi-positive *S. Typhi* does not induce neutrophil influx and can disseminate systemically and cause a

persistent infection [5]. Furthermore, the capsule-mediated suppression of TNF- $\alpha$  production by human monocytes leads to low serum concentrations of pyrogenic cytokines and thereby prevents the development of septic shock [44,45].

The O-Antigen capsule produced by NTS is co-regulated with cellulose and fimbriae to be part of the extracellular matrix (ECM). The group 4 O-Antigen capsules were shown to be important for attachment to surfaces, colonization, and desiccation resistance [23]. *S. Typhimurium* O-Antigen capsule was shown to be expressed both at lower and higher temperatures indicating that the capsule may play a role in bacterial survival inside and outside the host, as described for other bacterial species, such as *Hyphomonas* strain MHS-3 [46,47]. *Salmonella* O-Ag capsule was initially hypothesized to be a CPS that was distinct from very long chain LPS O-Ag and assembled by the *yih* operons [23]. However, in our recent study, we hypothesize that O-Ag capsule and very-long-chain LPS O-Ag are the same CPS and demonstrated that the *yihUTSRQP* operon is not directly involved in capsule assembly [47].

*Campylobacter jejuni* produces LOS and CPS that are genetically and biochemically similar to polysaccharide capsules in other Gram-negative bacteria [26]. Due to the presence of hypervariable polysaccharide biosynthesis genes, the structure of these polysaccharide monomers can vary between *C. jejuni* strains. Groups 2 and 3 CPS are part of the Penner antigen used for *C. jejuni* serotyping. It has been shown that *C. jejuni* CPS can be released to the surrounding environment in either naïve or lipid-free form depending on the presence or absence of deoxycholate; a component of bile salt. Even though the specific purpose of released CPS is unknown, it is regarded as important for the survival and pathogenesis of *C. jejuni* in foreign environments [26]. Treatment of *C. jejuni* CPS with phospholipase release a lipid-free product to the medium indicating that these CPS are substituted with a phospholipid in place of lipid A [26].

Another well-characterized CPS is produced by *Staphylococcus aureus*. Clinical isolates of *S. aureus* are classified into eight groups based on their CPS. The type 8 CPS is the most prevalent among bacteremic isolates and confers resistance to complement-mediated opsonization and phagocytosis [28]. *Bacteroides fragilis* produced zwitterionic CPS (contains both positive and negative charge) that can activate CD4+ T cells [16]. It has also been shown that administration of *B. fragilis* CPS alone protects Wistar rats against subsequent infection with *B. fragilis* [48].

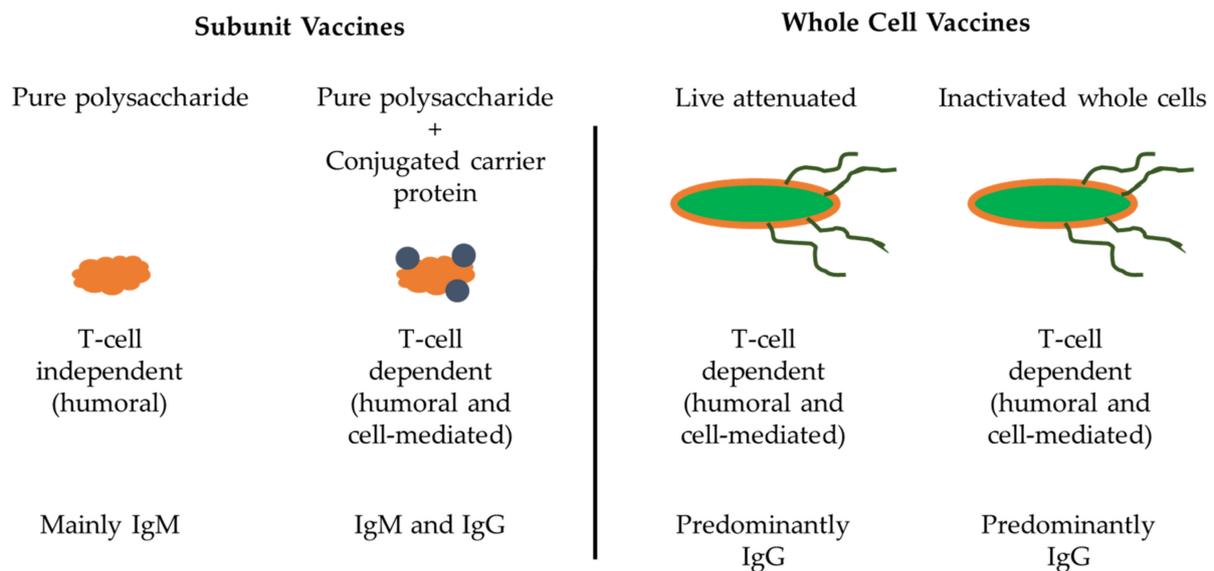
Polysaccharide capsules, associated with the cell surface, can regulate interactions between the bacterium and the environment. Although CPS are often associated with Gram-negative bacteria, many Gram-positive bacteria synthesize lipoteichoic acids or teichoic acids containing sugar alcohols with phosphodiester linkages. These sugar alcohols play a key role in the attachment of bacteria to surfaces and their interactions with host innate and adaptive immune systems [49].

### 3. Polysaccharide Vaccines

Since the late 20th century, polysaccharide vaccines have helped save the lives of millions of people worldwide. However, there are several limitations associated with pure polysaccharide vaccines. Mainly these include hyporesponsiveness and poor immunogenicity in children under the age of two [50]. In addition, the response for polysaccharide antigens among two to five-year-old children, the elderly, and the immunocompromised individuals are not optimal, and these populations remain at an elevated risk of contracting bacterial diseases [37]. Furthermore, due to hyporesponsiveness, pure polysaccharide vaccines are unable to provide long-lasting herd immunity, which is a key element in the prevention of invasive diseases. Herd immunity is achieved when the majority of a community is immunized against a given infectious disease, such that even those who have not received immunization are protected as the spread of the disease is contained [51].

Polysaccharide vaccines are classified as inactivated subunit vaccines (Figure 3). To date pure polysaccharide vaccines have been produced for four encapsulated bacteria: *Streptococcus pneumoniae* (pneumococcus), *Neisseria meningitidis* (meningococcus), *Haemophilus*

*influenzae* type b (Hib) and *S. Typhi* [52]. The adaptive immune response to polysaccharide antigens is usually T-cell independent, implying that polysaccharides are capable of activating B cells in the absence of T helper cells [53]. However, the lack of T-cell induction and in turn failure of B-cell stimulation by T cells results in an absence of immunoglobulin class switching, induction of memory B cells, affinity maturation, prolonged antibody response, which often leads to hyporesponsiveness to subsequent vaccination and/or to natural infection.



**Figure 3. General concepts for development of vaccines.** Pure polysaccharide vaccines are T-cell-independent activators of B-cells and their use usually results in an antibody only response (i.e., IgM). Polysaccharide–protein conjugate vaccines typically generate both humoral and cell-mediated immune responses. Both live-attenuated (i.e., mutagenesis) and inactivated whole-cell (i.e., heat or chemically inactivated) vaccines mount a mixed cell-mediated and humoral response dominated by IgG antibodies.

Polysaccharide capsules can have numerous identical epitopes near each other and can crosslink immunoglobulin receptors on the surface of B cells for B-cell activation. However, these polysaccharides are not processed and presented on MHC class II molecules for T helper cell activation [54]. Although polysaccharide antigens are unable to induce memory B-cell production, they can activate previously formed memory cells, leading to the terminal differentiation of memory cells into plasma cells. However, this depletes the reservoir of memory B cells specific for a given polysaccharide antigen and could result in a condition called hyporesponsiveness, which is the inability to respond to subsequent exposure to the same antigen due to lack of memory B cells [55]. Repeated administration of the same polysaccharide antigen (s) can exacerbate this situation. In contrast, repeated administration of most protein vaccines leads to a booster effect resulting in progressively higher antibody titers. In addition, antibodies produced in response to polysaccharide antigens are predominantly IgM, which have less functional activity than IgG antibodies [55].

Marginal zone (MZ) B cells, which are produced in the spleen, B1 cells, and naïve B cells to a lesser extent, are the most important at recognizing polysaccharide antigens. B1 and MZ B cells express a large number of CD21 receptors and can bind to complement coated polysaccharides [55,56]. Individuals with congenital asplenia, splenic hypofunction or who have undergone splenectomy are highly susceptible to encapsulated bacterial diseases, as they have a depleted number of functional MZ B cells and are defective at removing opsonized bacteria [55]. Furthermore, the immune response to polysaccharide antigens is age dependent as children under the age of two are also susceptible to encapsulated bacteria since the MZ is not fully developed prior to this age and is unable to facilitate the development of B cells [57]. In addition, the presence of maternal antibodies

and the effect of suppresser T cells also contribute to the lack of response to polysaccharide antigens in infants [37]. However, some zwitterionic polysaccharides such as capsules from *B. fragilis*, *S. pneumonia* serotypes 1 and 3, and *N. meningitides* serogroup A possess characteristics of T-cell-dependent antigens and are presented in an MHC class II-dependent manner. These antigens are immunogenic in even infants and young children [58,59].

To address the concerns of T-cell-independent immunogenicity of pure polysaccharide vaccines, polysaccharide antigens were chemically conjugated to carrier proteins in the late 1980s, resulting in polysaccharide–protein conjugates that were T-cell dependent and highly immunogenic [60]. The first polysaccharide–protein conjugate vaccine was produced for *H. influenzae* type-b. A conjugate vaccine for *S. pneumonia* was produced in 2000 and for *N. meningitides* in 2005. When these vaccines were introduced to the immunization schedules of multiple countries, it resulted in a significant reduction in the disease burden associated with encapsulated bacteria [61,62]. Conjugate vaccines have the advantage of inducing memory B-cell production, induction of immunity in children under the age of 2, and the capacity to overcome hyporesponsiveness [62,63].

Although immunogenic, the magnitude of the antibody response to conjugate vaccines and their persistence is age dependent. It was observed that in infants and toddlers, the serum antibody concentration following immunization with conjugate vaccines declined rapidly even though the immunological memory lasted much longer [64,65]. Despite multiple doses being administered to infants, the effectiveness of MenC and Hib vaccines declined within one year from initial immunization in the absence of a booster dose after the age of one [61,66]. The importance of persistent serum antibodies was demonstrated in vaccine failures where despite being immunized and having memory B cells, some children succumbed to meningococcal and Hib infections [67,68]. This is explained by the fact that encapsulated bacteria can invade and cause disease within a few hours, while memory B cells require several days to mount a protective antibody response [65,69]. Thus, protection against encapsulated bacterial infections requires high titers of persistent serum antibodies as well as induction of immune memory.

### 3.1. Polysaccharide Vaccines in Use

Current vaccines often have bi-, tri-, tetra-, and/or multivalent formulations, where the given number of separate protein or polysaccharide antigens are combined in a single vaccine. Although pure polysaccharide vaccines have been effective at saving lives for years, conjugation of polysaccharide antigens from *N. meningitidis*, *S. pneumoniae*, *H. influenzae* type b, and *S. Typhi* have generated T-cell-dependent antigens that are effective even in very young children [70].

#### 3.1.1. *Neisseria meningitidis*

The first polysaccharide vaccine against *N. meningitidis* was used in the United States in 1984 and was given to all the new military personnel upon entering basic training. Upon the administration of this tetravalent polysaccharide vaccine, no cases of meningococcal infections related to serogroup Y or W135 were reported in the United States military [37]. However, this vaccine was poorly immunogenic among infants and young children [37]. A quadrivalent vaccine containing polysaccharide antigens from *N. meningitidis* serogroup A, C, Y, and W135 was initially licensed in the United States in 1981. A bivalent vaccine containing polysaccharide antigens from serogroup A and C is also available in some countries [52].

Serogroup C (MenC) conjugate vaccine containing either tetanus toxoid or inactivated diphtheria toxin, CRM<sub>197</sub>, was first licensed in the United Kingdom in 1999, while a quadrivalent A, C, Y, and W135 diphtheria toxoid conjugate vaccine became first available in North America in 2005 [71]. In addition, several other vaccine candidates have been tested, including A, C, Y, and W135 antigens conjugated to tetanus toxoid or conjugated to CRM<sub>197</sub> [52]. As the serogroup B capsular polysaccharide is highly similar to cell surface glycoproteins on fetal brain tissue, which results in tolerance and reduced

immunogenicity, the development of a pure or conjugate polysaccharide-based vaccine remains a significant hurdle in vaccine development against *N. meningitidis* serogroup B [71]. One study compared the effectiveness between the meningococcal serogroups A and C pure polysaccharide vaccine (MACP) with the conjugate vaccine against serogroup C (MCC) since the use of MACP vaccine had raised concerns that this vaccine is leading to hyporesponsiveness to serogroup C polysaccharide antigen [72]. In this study, individuals who received a primary immunization of MACP were given a second immunization of either MACP or MCC. Those who received MCC produced significantly higher titers of IgG antibody than the individuals who received MACP as the second dose. In addition, the response to the second MACP vaccine was considerably lower than that following the first immunization. This supported the observation that repeated vaccination with the MACP vaccine is ineffective and leads to hyporesponsiveness towards the meningococcal serogroup C polysaccharide antigens in adults. However, it was possible to overcome the MACP induced hyporesponsiveness with subsequent vaccination with MCC [72].

Antibody titers induced in infants after immunization with *N. meningitidis* serogroup C (MenC) vaccine fell below the threshold of protection in 50% of vaccinees by one year of age and only 12% of vaccinees maintained protection at four years of age [73]. There is very little evidence that immunization of infants with the MenC vaccine confers protection beyond one year, as antibody titers decrease rapidly following infant immunization [74]. When administered after one year of age, the MenC vaccine induced a more persistent antibody response, but sustained levels of protection were not observed until much later in childhood in many children [66]. In addition, the decline in protective immunity following infant immunization was also noted for the *H. influenzae* type b vaccine [52].

### 3.1.2. *Streptococcus pneumoniae*

A pure polysaccharide hexavalent vaccine against *S. pneumoniae* was first licensed in 1947 but was eventually withdrawn due to low sales and lack of acceptance [37]. A polyvalent vaccine containing 14 polysaccharide antigens became available in the United States in 1977 and was recommended for at-risk populations including the elderly, immunocompromised patients, and individuals with chronic cardiac, pulmonary, or renal diseases [75]. The vaccine had 93% efficacy following three years from initial immunization among immunocompetent individuals under 55 years of age, but only a 46% efficacy in individuals over 85 years of age [75]. Furthermore, the vaccine's efficacy was shown to decline over time. However, this can be explained by the waning immunity and the exposure to additional pneumococcal serotypes over the years [75,76].

The current polyvalent pneumococcal vaccine was first licensed in 1983, is made of 23 immunologically distinct polysaccharide antigens and is effective in preventing disease in immunocompetent individuals [75]. Nonetheless, upon the introduction of the Hib conjugate vaccine in 1988, the disease burden among children in the United States declined by 50% per year from 1988 to 1991 [75]. In addition, several other pneumococcal vaccines are also in use. These include the seven-valent conjugate vaccine (PCV7) that contains the serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, and the ten-valent conjugate vaccine additionally containing serotypes 1, 5, and 7F. The eleven-valent and thirteen-valent conjugate vaccines have also been tested and the thirteen-valent vaccine is licensed in the United States [52,71]. As there are multiple pathogenic *S. pneumoniae* serotypes, the development and conjugation of each polysaccharide to carrier proteins remains a complex and challenging process [71].

### 3.1.3. *Haemophilus influenzae*

A pure polysaccharide vaccine for *Haemophilus influenzae* type b (Hib) was first used in the United States in 1985, but due to poor efficacy in children younger than 18 months of age and immunological variability in older children, the vaccine was withdrawn from use in 1988 [77]. The pure polysaccharide Hib vaccine elicited an age-dependent antibody response, where older children and adults developed protective levels of antibodies with long-lasting efficacy [78,79], while younger children generated protective, but lower levels

of antibody titers that decreased after one year from initial immunization. In addition, infants, being the most susceptible to Hib infection, were non-responsive to the pure polysaccharide Hib vaccine [77,79]. In clinical trials, a reduction in the nasal carriage with vaccine-specific *S. pneumoniae* serotypes in children was also observed following immunization with two multivalent pneumococcal conjugate vaccines [37,80]. Furthermore, in older children and adults, even a single dose of a conjugate vaccine leads to highly persistent anti-capsule antibodies [65,66].

In 1989, the pure Hib polysaccharide vaccine was replaced by a polysaccharide–protein conjugate vaccine linking the Hib polysaccharide polyribosylribitol phosphate to diphtheria toxoid, tetanus toxoid, or meningococcal outer membrane protein [52]. Subsequent immunizations with pure polysaccharide Hib or Hib conjugate vaccines do not generate booster responses in adults. However, multiple immunizations with Hib conjugate vaccine elicit booster responses in infants with elevated antibody titers [77,81,82]. The United Kingdom introduced the Hib conjugate vaccine to the infant immunization schedule in 1992 [52]. These vaccines are administered either alone or in combination with other vaccines for the protection of preschool children [52]. As a result, meningitis and other infections caused by Hib have been eliminated in countries that have incorporated the Hib conjugate vaccines into the infant immunization schedules [77,83].

#### 3.1.4. *Shigella*

The *Shigella* polysaccharide conjugate vaccine is based on the O-specific polysaccharide (O-SP) domain of LPS. It has been shown that serum IgG to O-SP induced protective immunity to Shigellosis [84]. To increase immunogenicity in young children, O-SP was covalently linked to carrier proteins. In one clinical study, it was shown that conjugation of *Shigella sonnei* and *Shigella flexneri* 2a O-SP to succinylated *P. aeruginosa* exotoxin A or Diphtheria toxin mutants was highly immunogenic, and resulted in high titers of serum IgG against O-SP within one week of immunization [84]. Out of the different vaccine candidates, the *P. aeruginosa* conjugate vaccine resulted in the highest IgG titers and the highest efficacy was observed for *S. sonnei* conjugate vaccine at 74% [84,85]. Following immunization with *S. sonnei* conjugate vaccine, IgG and secretory IgA antibody was detected in urine, implying that this vaccine was able to induce mucosal immunity [86]. In addition, a single immunization with *S. sonnei* conjugated to *P. aeruginosa* exoprotein A (rEPA) conferred specific protection against shigellosis in young adults [87].

#### 3.1.5. *Staphylococcus aureus* and *Vibrio cholera*

*S. aureus* type 5 and type 8 capsular polysaccharides are of high clinical importance. Immunization with *S. aureus* capsular polysaccharide conjugate vaccines generated high titers of specific antibodies. These antibodies were able to recognize variations in capsular polysaccharides that occur among clinically significant *S. aureus* isolates [88]. In addition, the *V. cholera* O1 serotype Inaba polysaccharide and cholera toxin conjugate vaccine were shown to induce serum anti-LPS IgG with bactericidal activity. *V. cholera* specific antibodies, whether acquired through natural immunity following colonization by the bacteria, or after immunization, are all directed against the LPS molecules [89]. Furthermore, antibodies generated against the O-specific polysaccharides of *V. cholera* serotype Inaba were able to cross recognize polysaccharides from *V. cholera* serotype Ogawa [89].

#### 3.1.6. Typhoidal *Salmonella* Vaccines

The development of an effective *Salmonella* vaccine for humans remains a pressing issue. *Salmonella* can survive both extracellularly and intracellularly within monocytes and macrophages. This is a significant challenge for vaccine development, as both humoral and cell-mediated immunity are needed to efficiently target and clear both extracellular and intracellular bacteria, respectively [90,91]. Antibodies mediate pathogen killing through opsonization, thereby facilitating phagocytosis and preventing the spread of the disease [92]. T cells, on the other hand, are involved in destroying *Salmonella* residing within phagocytic

cells [93,94]. The requirement of both arms of the immune system has been demonstrated through animal studies, where mice lacking T cells were unable to control *Salmonella* infections [95]. In other studies, passive transfer of specific antibodies provided significant clearance of *Salmonella*, in vivo [90,96].

The first inactivated whole-cell vaccine against *S. Typhi* was introduced to British and United States military personnel in 1896 [53]. During the next 100 years, this vaccine dramatically reduced the number of deaths associated with typhoid fever [53,97]. Although the inactivated whole-cell vaccine had an efficacy of 73% over three years post-immunization, it also had high reactogenicity and therefore it was discontinued from use [98–100]. In recent years, several clinical trials have tested the efficacy and type of immune response mounted following immunization with inactivated whole-cell *Salmonella* vaccines and had failed to find a significant cellular response. Additionally, individuals with pre-acquired natural immunity expressed a transient suppression of cellular immunity, questioning the usefulness of whole-cell vaccine administration in endemic regions [101,102].

Currently, there are three vaccine types against *S. Typhi*: (1) live attenuated, (2) pure Vi polysaccharide capsule-based, and (3) Vi-protein conjugates (Table 2). Non-specific chemical mutagenesis of the *S. Typhi* Ty21 strain was used to generate the live-attenuated vaccine Ty21. Ty21 does not express Vi capsule and as a result, the immune response to live-attenuated Ty21 strain is not directed against the Vi capsule [103]. The Ty21 vaccine requires three doses to induce the best protective immunity and in combination, the three doses have an efficacy of 51% [98,104]. This vaccine is not thermostable over long periods and requires a cold chain. This is a setback as the vaccine is most needed in developing countries where access to refrigeration is difficult [53]. It has been shown that freeze-drying can increase the thermal stability of the vaccine, but this measure is not employed widely [105]. Despite the Ty21 vaccine being licensed for use with adults and children over five years of age, seroconversion was greatly reduced in young children compared to adults [106]. Nonetheless, the Ty21 vaccine can induce T-cell immunity and has shown cross-protection against *S. Paratyphi B* in clinical studies. In addition, in vitro cross-protection has been shown for *S. Paratyphi A* and *B* [107,108]. As the antibody response is mostly directed against the O:9 O-Antigen epitope of *S. Typhi*, it was shown that the Ty21 vaccine can elicit cross-protective immunity against invasive non-typhoidal *Salmonella* (iNTS) and NTS serovars also expressing the O:9 or O:9,12 epitopes [53,109,110].

**Table 2.** Human vaccines currently licensed and vaccine candidates being developed for *S. Typhi*, *S. Paratyphi A*, and non-typhoidal *Salmonella*<sup>a</sup>.

Description	Developer	Academic/Commercial	Phase
<b>Vaccines Licensed for <i>S. Typhi</i></b>			
Neotyf, Typhoral, Vivotif (Ty21a live attenuated)	Johnson & Johnson	Commercial	Marketed
Bio Typh (Vi capsular polysaccharide)	BioMed	Commercial	Marketed
Typhim Vi, Typhyvax (Vi capsular polysaccharide)	Sanofi (Pasteur Merieux)	Commercial	Marketed
Typherix (Vi capsular polysaccharide)	GlaxoSmithKline	Commercial	Marketed
Peda-Typh (conjugate)	BioMed	Commercial	Marketed
Typbar-TCV (conjugate)	Bharat Biotech	Commercial	Marketed
Typhoid vaccine	Microgen	Commercial	Marketed
Vi Polysaccharide typhoid vaccine	China National Pharmaceutical (Beijing Tiantan Biological)	Commercial	Marketed
Vi-rEPA (Vi conjugate)	Lanzhou Institute (China)	Commercial	Marketed
Tyvac VI plus	VHB Life Sciences	Commercial	Marketed

Table 2. Cont.

Description	Developer	Academic/Commercial	Phase
Typhoid vaccine	Zydus Cadila (Zydus Vaccicare)	Commercial	Marketed
Tyrix Vi	SK Holdings	Commercial	Marketed
ShantypH	Sanofi (Shantha Biotechnics)	Commercial	Marketed
Typhobox, Typhovax	Green Cross	Commercial	Marketed
ZerotypH	Boryung	Commercial	Marketed
vax-TyVi	Finlay Institute	Commercial	Marketed
Typhoid-Kovax	Sanofi	Commercial	Marketed
Hepatyrix	GlaxoSmithKline	Commercial	Marketed
VIVAXIM	Sanofi	Commercial	Marketed
Biovac Typhoid	Wockhardt	Commercial	Marketed
Typho-Vi	Bio-Med	Commercial	Marketed
<b>Vaccines in Development in Recent Years for <i>S. Typhi</i></b>			
Vi-DT typhoid conjugate vaccine	Bio Farma	Commercial	Phase I
Typhetec	Prokarium	Commercial	Phase I
OmpC and OmpF	Institute Mexicano del Seguro Social	Academic	Phase 1 in Mexico
Enteric fever vaccine	Prokarium	Commercial	Phase II
M01ZH09 (live attenuated)	Emergent BioSolutions	Commercial	Phase II
CVD 909 (live attenuated)	University of Maryland	Academic	Phase II
Ty800 (live attenuated)	Avant Immunotherapeutics	Commercial	Phase II
Vi-rEPA (Vi conjugate)	National Health Institute	Academic	Phase III
Vi-CRM (Vi conjugate)	Biological E	Commercial	Phase III
Vi conjugated to fusion protein PsaA-PdT	Harvard Medical School	Academic	Preclinical
O:9-DT (conjugate)	International Vaccine Institute (IVI)	Academic	Preclinical
Salmonella typhi + paratyphi vaccine	Prokarium	Commercial	Research/Preclinical
Salmonella Vaccine Project	Affinivax	Commercial	Research/Preclinical
Vi conjugated to fusion protein PsaA-PdT	Harvard Medical School	Academic	Research/Preclinical
Ty21a typhoid vaccine expressing <i>Shigella</i> LPS	Protein Potential LLC.	Commercial	Research/Preclinical
O:2,12-DT + Vi-DT	International Vaccine Institute (IVI)	Academic	Research/Preclinical
O:2,12-CRM197 + Vi-CRM197	Biological E	Commercial	Research/Preclinical
<b>Vaccines in Development in Recent Years for <i>S. Paratyphi</i></b>			
CVD 1902 (live attenuated)	University of Maryland, Bharat Biotech	Commercial	Phase I
Paratyphoid A conjugate vaccine	Lanzhou Institute (China)	Academic	Phase II
O:2-TT (conjugate)	Technology transfer from NIH to Lanzhou Institute (China)	Commercial	Phase II
O:2,12-DT + Vi-DT	International Vaccine Institute (IVI)	Academic	Research/Preclinical
O:2,12-CRM197 + Vi-CRM197	Biological E	Commercial	Research/Preclinical

Table 2. Cont.

Description	Developer	Academic/Commercial	Phase
O:2-TT (conjugate)	Technology transfer from NIH to Chengdu Institute (China)	Academic	Preclinical
O:2-TT (conjugate)	Changchun Institute of Biological Products	Commercial	Preclinical
<b>NTS Vaccines in Development in Recent Years</b>			
WT05— <i>aroC</i> and <i>ssaV</i> mutants (live attenuated)	Microscience, Wokingham Berkshire	Academic	Phase 1
NTS Vaccine	University of Maryland	Academic	Research/Preclinical
CVD 1921 and CVD 1941 NTS Project (live attenuated)	University of Maryland, Bharat Biotech, Wellcome Trust	Commercial	Research/Preclinical
Bivalent iNTS-GMMA	GlaxoSmithKline (Novartis Vaccine Institute for Global Health; NVGH)	Commercial	Research/Preclinical
Bivalent Conjugate (O:1,4[5],12-CRM197 + O:1,9,12-CRM197)	GlaxoSmithLine (Novartis Vaccine Institute for Global Health; NVGH NVGH)	Commercial	Research/Preclinical
OmpD	University of Birmingham	Academic	Research/Preclinical
O:4,5/O:9-flagellin (conjugate)	University of Maryland	Academic	Preclinical
O:4, 12-TT (conjugate)	National Institutes for Health NIH)	Academic	Preclinical
Os-po (O:4 porin conjugate)	National Bacteriology Laboratory, Stockholm		Preclinical
<i>S. Typhimurium ruvB</i> mutant (live attenuated)	Seoul National University	Academic	Preclinical
<i>Salmonella hfq</i> deletion mutant (live attenuated)	Indian Institute of Science Bangalore	Academic	Preclinical
SA186 (live attenuated)	Istituto Superiore di Sanita Roma	Academic	Preclinical
MT13 (live attenuated)	KIIT University Odisha	Academic	Preclinical
DNA adenine methylase mutants (live attenuated)	University of California, Santa Barbara	Academic	Preclinical
live attenuated, regulated delayed attenuation	Arizona State University	Academic	Preclinical

<sup>a</sup> This is not a comprehensive list of all the vaccine candidates that are being tested, especially since this list does not reflect vaccine candidates tested in research studies. This information was compiled from the Boston Consulting Group—Vaccine pipeline information web page ([https://vaccinesforamr.org/review-of-pathogens/vaccine-pipeline-information/#Salmonella\\_non-typhoidal](https://vaccinesforamr.org/review-of-pathogens/vaccine-pipeline-information/#Salmonella_non-typhoidal), accessed on April 2021), National Institute of Health—U.S. National Library of Medicine ClinicalTrials.gov web page (<https://clinicaltrials.gov/ct2/home>, accessed on April 2021), and the MacLennan et al., 2014 research article [53].

The pure Vi polysaccharide capsule-based vaccine generates primarily an antibody response directed towards the Vi capsule. However, recent evidence indicates that the immune response could also be due to other *Salmonella* components in the vaccine [110]. Unfortunately, the vaccine is considered to be non-immunogenic in infants, due to the Vi capsule being a T-independent antigen and the serum antibody response being mostly IgM driven. Therefore, the pure Vi vaccine is only licensed for children over 2 years of age [53]. In contrast to the Ty21 vaccine, the Vi capsule-based vaccine requires a single dose and has an efficacy of 55% over three years. In one study only 2 out of 32 subjects generated a sufficient amount of IgG towards the Vi polysaccharide capsule-based vaccine [111]. However, in typhoid fever endemic regions, immunization with this vaccine resulted in 55% and 75% efficacy rates among adults and children over the age of 5 years, respectively [37].

The Vi polysaccharide capsule-based vaccine also requires a cold chain to prolong stability and effectiveness [53]. Additionally, vaccination with the Vi capsule-based vaccine leads to the production of circulating plasmablasts (immature plasma cells) expressing systemic homing receptors (L selectin), while vaccination with the Ty21 vaccine leads to the expression of mucosal homing receptors ( $\alpha_4\beta_7$ ), as with natural infection [110]. Thus, vaccination with the Vi capsule will not direct immune cells specifically to the intestinal mucosal region, rather they will be distributed systemically, and consequently, the immune cells will not have a targeted response at the intestinal site of infection. No clinical trials have been performed to date to examine the efficacy and protection of these two types of vaccines combined [53].

In recent years, several different Vi protein conjugate vaccines have been licensed, especially in Asia, to counter the low immune response mounted to the pure polysaccharide vaccine (Table 2). The importance of antibodies in clearing *S. Typhi* infections was demonstrated by efficacy data for Vi capsule-based vaccine and Vi protein conjugate vaccine candidate phase III studies [98,104,112,113]. A phase III study of a glycoconjugate vaccine combining *S. Typhi* Vi capsule with *P. aeruginosa* exoprotein A (Vi-rEPA), conducted over a decade ago in Vietnamese children between two and five years of age, found a 91% efficacy after 27 months and 89% efficacy after 46 months from primary immunization [53]. Thus, protein conjugation holds promise in developing an effective vaccine against *S. Typhi* infections.

Although several vaccines against *S. Typhi* are being developed (Table 2), no new vaccine against *S. Typhi* has been introduced to the market in recent times. The delay in seeing new vaccines in the market is mainly due to a lack of commercial interest in developing vaccines against typhoid fever and iNTS disease since these are largely diseases of low-income countries, which have little money to invest in vaccines [114]. New vaccines are expected to provide improved protection and efficacy, especially in young children, and to reduce the cost of delivery; these vaccines are to be administered as part of the national Expanded Programs on Immunization [53]. Vaccines for *S. Typhi* and/or *S. Paratyphi* are to be given at nine months, as disease peaks at two years of age, and vaccines for iNTS are to be given between two and four months of age, prior to the disease peak at one year of age [115–117].

#### 4. Non-Typhoidal *Salmonella* Vaccines

Although a humoral response is sufficient to clear most *S. Typhi* infections, both antibody and cell-mediated immunity are required for proper clearance of NTS. This has been shown in field studies in sub-Saharan Africa, where the age-related prevalence of iNTS disease in children declined with the acquisition of specific antibodies [116]. In addition, serum antibodies have shown in vitro bactericidal activity and oxidative killing of iNTS [116,118]. It has also been demonstrated that O-Antigen-based conjugate vaccine candidates induce sufficient protection against otherwise lethal doses of *Salmonella* in mice [119,120]. Passive transfer of monoclonal antibodies specific for O-Antigen was also able to confer protection against lethal doses of *Salmonella* challenge [121,122]. Immunization of animals with a heat-killed invasive African *S. Typhimurium* sequence type 313 strain led to the majority of antibodies mounted against the O-Antigen [123]. On the other hand, the importance of cell-mediated immunity in clearing NTS is apparent through the association between HIV infection and predisposition to NTS, especially when the CD4<sup>+</sup> count is below 200 cells/uL [124]. HIV has been shown to increase susceptibility to NTS through dysregulation of cytokine response, humoral immunity, and disruption of the integrity of the gastrointestinal mucosa [94,125]. Therefore, vaccine candidates that induce protective mucosal immunity, preventing gastroenteritis and NTS invasion from the gastrointestinal tract, would be highly beneficial for HIV-infected individuals [53].

#### 4.1. Glycoconjugate Vaccines for NTS

There are several different types of NTS vaccine candidates currently being tested, which fall into three main categories: glycoconjugate, live attenuated, and subunit vaccines (Table 2). Glycoconjugate vaccines covalently link a poorly immunogenic antigen, such as a bacterial surface polysaccharide, to a protein carrier molecule to activate CD4<sup>+</sup> T cells. For glycoconjugation, most *Salmonella* vaccine strategies use the common carrier proteins tetanus toxoid, diphtheria toxoid, nontoxic recombinant diphtheria toxoid, or *P. aeruginosa* exoprotein A (rEPA) [53]. In one study, *S. Typhimurium* O-Antigens 4 and 12 covalently linked to bovine serum albumin (BSA) were administered with Freund's adjuvant, leading to the production of high O4 specific antibody titers in rabbits, comparable to those elicited by immunization with heat-killed bacteria [126].

It was proposed that glycoconjugate vaccines using *Salmonella* carbohydrates linked to *Salmonella* proteins could be more effective at inducing *Salmonella*-specific antibodies since antibodies can be produced against both *Salmonella* proteins and carbohydrates [127,128]. In addition, conjugation to *Salmonella* proteins could lead to the generation of a *Salmonella*-specific T-cell response. Conjugation of *S. Typhimurium* O:4 polysaccharide to outer membrane (OM) porins led to better protection of mice upon challenge, as compared to using porins alone or O:4 conjugated diphtheria toxoid [128]. As flagellin is the only cell-surface protein antigen typically used for typing *Salmonella*, it was investigated for the ability to generate protective immunity. Immunization of mice with flagellin alone, or in conjugation with the O-Antigen leads to the induction of protection [119,129,130]. For example, immunization with O:4,5 and O:9 conjugated to *Salmonella* flagellin in iNTS vaccine candidates led to enhanced protective immunity, as compared to either antigen alone [119,131]. Flagellin is the primary ligand for TLR5 activation and can signal to the innate immune system and lead to immunomodulatory effects in mice [130,132]. One concern is that in some *Salmonella* serovars, such as *S. Typhimurium*, flagellin can have phase variable expression and is not constitutively expressed during infection [53]. Another concern is due to the expression of multiple types of flagellin, immunization against one flagellin may not provide cross-protection against infection with a strain expressing a different type of flagellin.

#### 4.2. Live-Attenuated Vaccines for NTS

Live-attenuated vaccines can induce both humoral and cellular immunity, and thereby facilitate *Salmonella*-specific T-cell responses. These vaccines are the easiest to deliver (orally) and can induce strong mucosal immune responses [110]. In addition, the availability of numerous *Salmonella* antigens on live bacteria can yield broad protective immune responses and even allow cross recognition between *Salmonella* serovars. The primary challenge with developing live-attenuated vaccines is in balancing the level of immunogenicity with a proper and sufficient level of attenuation [53]. Currently, several live-attenuated *S. Typhimurium* strains are being tested for their efficacy in preclinical and Phase I clinical studies (Table 2). Most of these strains contain mutations or deletions in genes coding for secretion systems and virulence.

Additionally, recombinant attenuated *Salmonella* vaccines (RASV) have been used as vectors to deliver vaccine antigens for various other bacterial pathogens. RASV expressing pneumococcal surface protein A (PspA) elicited an IgG response with a predominant rPspA specific IgG1 (Th2-type) and *Salmonella* LPS and OMP specific IgG2a (Th1-type) responses [133]. Similarly, oral immunization of mice with RASV vectors expressing genes from seven different serotypes of CPS from *S. pneumoniae* elicited a Th1-type immune response and subsequent in vitro clearance of the *S. pneumoniae* expressing the corresponding CPS serotype [134]. Immunization with RASV vectors expressing *Salmonella* and *M. tuberculosis* effector protein SopE resulted in similar observations [135]. Furthermore, RASVs with regulated delayed attenuation have seen significantly higher anti-PspA responses (i.e., IgG, IL-4, and INF- $\gamma$ ), compared to the controls [136]. These vaccines express wild-type phenotypes at immunization and are attenuated after colonization of the host tissues, by

inactivating the virulence genes [136]. Thus, RASVs not only facilitate the induction of an immune response against the vaccine antigen from other bacteria but also induce protective responses against *Salmonella*-specific antigens [137–139].

#### 4.3. Subunit Vaccines

Protein-based subunit vaccines are composed of multiple antigenic epitopes and can have broad coverage. A subunit approach is currently in use for multivalent pneumococcal and meningococcal conjugate vaccines. In another strategy to develop broad coverage, cross-protective vaccines can use highly conserved protein antigens produced through recombinant technology [140–142]. Proper antigens can be selected using bioinformatics analysis of whole-genome sequences and reverse vaccinology [143]. Examples of highly conserved *Salmonella* protein antigens include flagellin and porins (i.e., OmpC, F, and D). Both recombinant and purified protein vaccine candidates have been tested against conserved protein epitopes [2]. Subunit vaccines can induce both T-cell responses and antibody production; a balanced Th1-Th2 approach is vital to proper clearance of *Salmonella*. One of the downsides of subunit vaccines developed through recombinant technology is that it is difficult to maintain and preserve the proper conformation of proteins, especially when there are several membrane-spanning domains. This can result in the induction of a poor antibody response. One approach is to purify proteins from whole *Salmonella* cells rather than relying on recombinant proteins [142].

#### 4.4. Other Vaccines Currently Being Developed for NTS

As glycoconjugate vaccines primarily rely on the production of antibodies specific for surface carbohydrate moieties, it may not be sufficient to efficiently deal with the growing iNTS disease. The development of new live-attenuated, protein-based, or Generalized Modules for Membrane Antigens (GMMA)-based vaccines will serve to provide broader protection by activating *Salmonella*-specific T-cell responses [53,144–146]. Vaccine development has been hindered due to a lack of understanding regarding specific antigenic epitopes of *Salmonella* and preservation of proper protein conformations. GMMA technology (i.e., outer membrane blebs) is used to deliver outer membrane proteins and surface polysaccharides in the correct orientation and conformation to induce protective immunity. In addition, the membrane components of GMMAs have adjuvant activity by co-delivering multiple pathogen-associated molecular patterns (PAMPs) along with the target antigens [2]. In our hands, immunization of mice with GMMAs isolated from colanic acid overproducing strains was sufficient to significantly reduce *Salmonella* colonization [47]. It is possible that the development of a GMMA-CPS-based vaccine against NTS can be explored in the future.

### 5. Looking Ahead

The primary goal of this review was to provide a perspective of the current state of NTS vaccine development and to encourage vaccine candidate testing and clinical development. Despite the type of vaccine developed, in terms of delivery, time and the associated cost of administration, it will be advantageous to develop vaccines that require only one or two doses to mount an optimal immune response, compared to the required three doses of the current Ty21 vaccine [53]. Although the current vaccine pipeline is looking weak for NTS, there are candidates not listed in official databases, therefore the prospects are stronger than they appear. In our opinion, there needs to be a greater commitment of scientific expertise and availability of resources to accelerate vaccine candidates, especially taking *S. Typhi* as a proof of principle for vaccine development. Less expensive conjugate methods and technological advancements should be considered to facilitate vaccine accessibility in low-income countries. Subunit vaccines, especially those using glycoconjugate technologies, are costly to produce and since most vaccines are to be used in low-income countries, affordability remains a key factor in vaccine development. To balance the cost-effectiveness, it has been proposed that multivalent vaccines composed of 5 to 6 antigens could provide

cross-protection against the majority of the iNTS serovars [127]. Furthermore, a better understanding of the regional burden of NTS/iNTS infections will enhance informed policy decisions.

An NTS vaccine would be both beneficial as a vaccine for travelers in developed countries, and as an early childhood vaccine in endemic regions. Although an NTS vaccine would require no new implementation provided the vaccine is introduced to childhood vaccination schedules, vaccination in high-disease-burden regions may require additional infrastructure for storage. One of the biggest hurdles is that commercial attractiveness for NTS vaccines remains low, given the demand remains relatively low in middle- and high-income countries. Thus, it should be noted that the limited candidates in the pipeline are a reflection of a lack of interest and resources to drive clinical testing, rather than a reflection of the feasibility of developing an NTS vaccine.

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