# Annex 3

Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines

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Guidelines published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.
Introduction

These Guidelines are intended to assist NRAs in evaluating the scientific issues connected with the quality, safety and efficacy of typhoid conjugate vaccines that use Vi polysaccharide covalently linked to a carrier protein. The available guidelines for Vi polysaccharide typhoid vaccine (1) and for live, attenuated Ty21a vaccines (2) are not applicable to typhoid conjugate vaccines consisting of Vi polysaccharide – derived from Salmonella enterica subspecies enterica serovar Typhi (S. Typhi), Citrobacter freundii sensu lato (C. freundii s.l.) or other bacterial sources – conjugated with a carrier protein, such as diphtheria toxoid (DT), tetanus toxoid (TT), recombinant Pseudomonas aeruginosa exoprotein A (rEPA), the nontoxic mutated form of diphtheria toxin – such as cross-reactive material 197 (CRM197) – or another suitable protein.

These Guidelines are based on experience gained during the development of experimental typhoid conjugate vaccines as well as relevant information obtained from the evidence for other types of bacterial polysaccharide–protein conjugate vaccines, such as Haemophilus influenzae type b (Hib), meningococcal and pneumococcal conjugate vaccines. The evidence gathered thus far indicates that typhoid conjugate vaccines may overcome several limitations of unconjugated Vi polysaccharide vaccines. Conjugate vaccines may demonstrate: (a) greater efficacy and effectiveness; (b) longer persistence of immunity; (c) immunogenicity across all age groups, including infants and toddlers aged younger than 2 years; (d) perhaps some degree of herd immunity; and (e) induction of immune memory with initial dosing, leading to anamnestic responses to a subsequent dose or doses.

Part A of these Guidelines sets out guidance on manufacturing and quality control, while Part B addresses the nonclinical evaluation of these vaccines and Part C addresses their clinical evaluation. Part D provides guidance for NRAs.

General considerations

This section provides a brief overview of the scientific knowledge that underpins the guidance given in Parts A, B and C. A comprehensive review of the immunological basis for typhoid vaccines is also available from WHO (3).

Typhoid fever is an acute generalized infection of the mononuclear phagocyte system (previously known as the reticuloendothelial system), intestinal lymphoid tissue and gall bladder caused by S. Typhi. Paratyphoid fever is a clinically indistinguishable illness caused by S. enterica subspecies enterica serovar Paratyphi A or B (or, more rarely, C) (4, 5). Typhoid and paratyphoid fevers are often referred to collectively as enteric fever. In most endemic areas, typhoid accounts for approximately 75–80% of cases of enteric fever. However,
in some regions, particularly in Asia, S. Paratyphi A accounts for a relatively larger proportion of all enteric fevers (6, 7).

**Pathogen**

S. Typhi is a member of the family *Enterobacteriaceae*. It is a Gram-negative, non-lactose fermenting bacillus that produces trace amounts of hydrogen sulfide. Its antigens include an immunodominant lipopolysaccharide (LPS) O9, flagellar H and capsular polysaccharide Vi.

Vi acts as a virulence factor by preventing anti-O antibody from binding to the O antigen, and also inhibits the C3 component of the complement from fixing to the surface of S. Typhi. The Vi antigen is not unique to S. Typhi – it is also expressed by S. Paratyphi C, *C. freundii* s.l. and S. *enterica* subspecies *enterica* serovar Dublin. The genes responsible for the biosynthesis of Vi polysaccharide are located in a locus (*viaB*) within Salmonella pathogenicity island 7 (SPI-7) in the S. Typhi chromosome. Several other loci participate in the complex regulation of Vi expression. Almost all S. Typhi isolates from blood cultures can be shown to express Vi. Nevertheless, Vi-negative strains have been identified occasionally, both in sporadic cases as well as during outbreaks (8). Some of these strains are regulatory mutants that can revert to a Vi-positive state (9). However, some Vi-negative isolates from blood have been shown to harbour deletion mutations in critical genes (e.g. *tviB*) within the *viaB* locus that render the strains unable to synthesize Vi. This raises the theoretical concern that large-scale usage of Vi-containing vaccines (either polysaccharide or conjugate) could lead to selective pressure that creates a biological advantage for the emergence of Vi-negative strains (10).

**Pathogenesis**

Typhoid infection begins with ingestion of S. Typhi in contaminated food or water. In the small intestine, the bacteria penetrate the mucosal layer, and ultimately reach the lamina propria. Translocation from the intestinal lumen mainly occurs by S. Typhi targeting M cells overlying gut-associated lymphoid tissue. Within this lymphoid tissue and in the lamina propria, S. Typhi invokes an influx of macrophages and dendritic cells that ingest the bacteria but fail to destroy them. Thus some bacteria remain within macrophages in the lymphoid tissue of the small intestine and flow into the mesenteric lymph nodes where there is an inflammatory response mediated by the release of various cytokines. Bacteria enter the bloodstream via lymphatic drainage, thereby seeding organs of the mononuclear phagocyte system (such as the spleen, liver and bone marrow) and gall bladder by means of a silent primary bacteraemia. After a typical incubation period of 8–14 days the clinical illness begins, usually with the
onset of fever, abdominal discomfort and headache. An accompanying low-level secondary bacteraemia occurs.

Before the availability of fluoroquinolone antibiotics, clinical relapses were observed in 5–30% of patients treated with antibacterial agents such as chloramphenicol and sulfamethoxazole/trimethoprim. These post-treatment relapses occurred when typhoid bacilli re-emerged from their protected intracellular niches within the macrophages of the mononuclear phagocyte system, where the antibacterial agents could not penetrate.

In a small proportion of patients infected with S. Typhi who have premorbid abnormalities of the gall bladder mucosa, such as occurs consequent to gallstones, gall bladder infection becomes chronic (i.e. excretion lasts for longer than 12 months) (11). Such chronic carriers, who are not clinically affected by the presence of typhoid bacilli in their system, may excrete the pathogen in their faeces for decades (12). They serve as a long-term epidemiological reservoir in the community, and can transmit typhoid wherever there is inadequate sanitation, untreated water supplies or improper food handling.

**Epidemiology**

Typhoid is restricted to human hosts, and chronic carriers constitute the reservoir of infection. In the late nineteenth century and early twentieth century, typhoid was endemic in virtually all countries in Europe and the Americas. Subsequently, the widespread use of chlorination, sand filtration, and other means of water treatment drastically reduced the incidence of typhoid fever despite the high prevalence of chronic carriers (11). Typhoid remains endemic in most developing countries, mainly because large segments of the population lack access to safe water and basic sanitation services. In addition, there are limited programmes for detecting carriers and restricting them from handling food.

**Disease burden**

Variable estimates of typhoid fever have been published in the scientific literature. The true incidence of typhoid fever in most regions of developing countries is not known. A study published in 2004 estimated that 22 million cases occur each year, causing 216,000 deaths, predominantly in school-age children and young adults; the annual incidence was estimated to be 10–100 per 100,000 population (13). A systematic review of population-based studies from 1984 through 2005 reported an annual incidence of 13–976 per 100,000 population each year based on diagnosis by blood culture (14).

Several factors affect the calculation of the burden of typhoid disease. In the absence of a rapid, affordable and accurate diagnostic test, blood culture is recognized as the gold standard. However, blood culture alone identifies only 60–70% of the cases that are detectable using bone marrow culture or bile fluid
culture (14). Prior treatment with antibacterial agents also affects culture results. However, relying on clinical diagnosis alone may overestimate the burden because several febrile syndromes caused by other microorganisms, such as malaria, dengue and leptospirosis, can be confused with typhoid, particularly in children.

The incidence of typhoid, its age-specific distribution and the severity of clinical disease gleaned from passive surveillance implemented at health facilities often appears quite different from data acquired through active surveillance, during which households are visited systematically once or twice weekly to detect fever among their members. In 2008, a study by Ochiai and colleagues reported the incidence of typhoid detected through passive surveillance (and modified passive surveillance in two countries where additional health clinics were introduced into the community) in five Asian countries (15). The incidence of typhoid fever ranged from 15.3 per 100 000 person-years among people aged 5–60 years in China, to 451.7 per 100 000 person-years among children aged 2–15 years in Pakistan (15). Incidence data from the placebo control groups in vaccine trials have also provided information on the incidence of typhoid fever in multiple geographical areas and venues. However, because vaccine efficacy trials are typically carried out in areas with high endemicity, caution must be taken when extrapolating these incidence rates to other populations.

In general, there is less information on the burden of disease in children aged younger than 2 years than in older age groups. In the surveillance study conducted at sites in five Asian countries, two sites (Kolkata, India, and North Jakarta, Indonesia) included surveillance of children aged younger than 2 years (15). In Kolkata the recorded annual incidence among children aged younger than 2 years was 89 cases per 100 000 child-years (15); in North Jakarta the annual incidence was 0 cases per 100 000 child-years. In Kolkata only 1 of 145 blood cultures from febrile children in this age group was positive for S. Typhi (0.69%) (16); in Jakarta 0 of 404 blood cultures were positive for febrile children in this age group. By comparison, the incidence of culture-confirmed typhoid fever in Kolkata was 340.1 cases per 100 000 child-years in children aged 2–4 years; it was 493.5 cases per 100 000 child-years in children aged 5–15 years. In North Jakarta the annual incidence of typhoid was 148.7 cases per 100 000 child-years among 2- to 4-year-olds, and 180.3 cases per 100 000 child-years among children aged 5–15 years.

Prior to the availability of antibacterial agents, typhoid resulted in a case-fatality rate of approximately 10–20% (17). The 2008 WHO position paper on typhoid fever estimated that 216 000–600 000 deaths occurred annually (18). Most of the mortality occurs in developing countries, and 80% of deaths occur in Asia. A review by Crump and colleagues reported community-based mortality ranging from 0–1.8% across five studies in developing countries; hospital-based mortality ranged from 0–13.9% (across all ages in 12 studies); and in children younger than 15 years mortality ranged from 0–14.8% (across 13 studies) (14).
Few studies have estimated the prevalence of chronic carriers of typhoid and paratyphoid in developing countries. A survey in Santiago, Chile, conducted when typhoid fever was highly endemic there in the 1970s, estimated a crude prevalence of 694 typhoid carriers per 100,000 population (19). In Kathmandu, Nepal, among 404 patients (316 females and 88 males) with gall bladder disease undergoing cholecystectomy, S. Typhi was isolated from 3.0% of bile cultures and S. Paratyphi A from 2.2% (20). Since the overall prevalence of cholelithiasis in the population of Kathmandu was not known, the overall prevalence of chronic carriage in that population could not be calculated.

**Clinical features**

S. Typhi infection results in a broad spectrum of clinical features, most often characterized by persisting high-grade fever, abdominal discomfort, malaise and headache. Important clinical signs in hospitalized patients include hepatomegaly (41%), toxicity (33%), splenomegaly (20%), obtundation (2%) and ileus (1%) (21). Before antibacterial agents became available, gross bleeding from the gastrointestinal tract and perforations occurred in 1–3% of untreated patients, but these are now rarely observed except in settings with poor access to health care.

Typhoid fever has the potential to cause serious complications. Hospital-based reports suggest that more than 50% of patients may have complications. In 2005, Huang and colleagues analysed in which systems various complications were likely to occur – the central nervous system (3–55%), the hepatobiliary system (1–26%), the cardiovascular system (1–5%), the pulmonary system (1–6%), bones and joints (less than 1%), and haematological system (rarely) (22). Intestinal perforations leading to peritonitis and death continue to be reported in some settings today, although this is rare.

**Immune responses to natural infection**

Natural typhoid infection is usually associated with the detection of serum antibodies and mucosal secretory immunoglobulin A intestinal antibody against various S. Typhi antigens; cell-mediated immune responses are also measurable (23–27). In areas where typhoid is endemic, there is an age-related increase in the prevalence and geometric mean titre of anti-Vi antibodies (28). Antiflagella (H antigen) serum IgG antibodies following natural infection are long-lived, and have been studied for seroepidemiological surveys (29).

While serological responses to LPS and flagella antigens tend to be fairly strong and are commonly found in patients with culture-confirmed acute typhoid fever, only about 20% of such patients exhibit significant levels of anti-Vi antibody (30, 31). In contrast, high concentrations of anti-Vi serum IgG antibody are detected in 80–90% of chronic carriers (30, 31).
Cell-mediated immunity also appears to play a part in protection – it has been observed that peripheral blood mononuclear leukocytes of otherwise healthy adults residing in typhoid-endemic areas, who have no history of typhoid, proliferate on exposure to S. Typhi antigens (32).

**Disease control**

Similar to other enteric and diarrhoeal diseases, typhoid fever exists predominantly in populations with inadequate access to safe water and basic sanitation. Effective typhoid control requires a comprehensive approach that combines immediate measures, such as accurate and rapid diagnostic confirmation of infection and timely administration of appropriate antibiotic treatment, as well as sustainable long-term solutions such as providing access to safe water and basic sanitation services.

Other interventions, such as treating household water, ensuring that food is handled properly, washing hands with soap, and discouraging defecation in the open may also be effective control measures (33–35). The most effective strategy for improving the health of typhoid-affected populations is to implement and maintain municipal water and sanitation systems.

Vaccination against typhoid has proved to be an effective preventive intervention, especially when coupled with hand washing, the treatment of household water, and the provision of adequate sanitation and other preventive measures. A detailed review of the immunological basis for typhoid vaccination has been published (36).

**Typhoid vaccines**

**Inactivated whole cell vaccine**

Inactivated S. Typhi bacteria (heat-inactivated and phenol-preserved) were first utilized to prepare parenteral vaccines more than 100 years ago. In the 1960s, WHO sponsored field trials that evaluated the efficacy of inactivated parenteral whole-cell vaccines in several countries (37, 38), and documented a moderate level of efficacy lasting up to 7 years (39). Data from studies of human immune responses and immunogenicity studies in rabbits suggested that anti-H antibodies might represent an immune correlate of protection (40); later extrapolation from the results of mouse protection studies suggested that responses to Vi antigen may correlate with protection (41). However, these vaccines were associated with considerable rates of systemic adverse reactions (42) and are no longer used.

**Live, attenuated Ty21a oral vaccine**

In the early 1970s, an attenuated strain of S. Typhi was developed through chemical-induced mutagenesis of pathogenic S. Typhi strain Ty2 (43). The resultant mutant strain lost the activity of the epimerase enzyme encoded by the
galE gene, and was also no longer capable of expressing Vi polysaccharide. The vaccine was found to be stable, safe and efficacious in adults as well as children (44–48), but the level of protective immunity achieved varied according to the formulation of the vaccine, the number of doses administered and the interval between doses.

For example, three doses of a provisional formulation of vaccine or placebo administered to about 32,000 children (aged 6–7 years) in Alexandria, Egypt, gave a point estimate of efficacy of 95% (95% confidence interval (CI), 77–99%) during 3 years of follow-up (49). Three doses of enteric-coated capsules administered to Chilean schoolchildren aged 6–19 years using two different dose intervals (either alternate days or 21 days between doses) demonstrated a point estimate of efficacy of 67% (95% CI, 47–79%) during 3 years of follow-up; for the group receiving doses on alternate days, the point estimate of protection over 7 years was 62% (95% CI, 48–73%) (44, 50). The estimate of protection was 49% (95% CI, 24–66%) with the 21-day interval between doses. Another trial used four doses administered within 7 days to Chilean schoolchildren and demonstrated even greater protection (51). Only 5% of children aged 6–7 years had difficulty swallowing the capsules (51). As of 2013, almost all countries where Ty21a is licensed utilize a three-dose course of enteric-coated capsules taken on alternate days, except the United States and Canada, which recommend a four-dose course.

Two other field trials in Chile (48) and Indonesia (47) compared the enteric-coated capsules with three doses of the liquid formulation. The liquid formulation conferred greater efficacy than the capsules in both trials. In Chile, where doses were given on alternate days, results with the liquid formulation were superior to Indonesia where the doses were administered 1 week apart (the point estimate of efficacy in Chile was 77%; in Indonesia it was 53%). In Chile, 78% protection was documented up to 5 years after vaccination with the liquid formulation (50). There is also indirect evidence that large-scale vaccination with Ty21a may provide some degree of protection against typhoid to people who have not been vaccinated through the mechanism of herd immunity.

**Vi polysaccharide vaccine**

Technological advances have made it feasible to purify Vi polysaccharide and to prepare vaccines that are almost totally free of contaminating LPS (52); these vaccines are associated with low rates of febrile reactions (1–2%).

The immunological basis of purified Vi polysaccharide parenteral vaccines is the generation of serum anti-Vi IgG antibodies in 85–90% of vaccine recipients older than 2 years.

Clinical trials with the vaccine showed a rise in anti-Vi antibody titres in adults and children (53–55). However, subsequent inoculations with Vi did not boost the antibody response. Although a single dose has been associated with the persistence of antibodies for up to 3 years in some recipients, many adult
recipients in non-endemic areas showed a marked drop in antibody levels after 2 years (56, 57). An epidemic of typhoid fever among French soldiers deployed in Côte d’Ivoire showed that the risk of typhoid fever was significantly higher in persons vaccinated more than 3 years previously (58).

Field trials in children and adults in Nepal given a single (25-µg) dose showed 72% vaccine efficacy during 17 months of follow up (53); a field trial in schoolchildren in South Africa also using a single (25-µg) dose showed 60% protection during 21 months of follow-up (54). In South Africa, protection declined to 55% at 3 years (59). Another field trial in China in people aged 3–50 years given a single 30-µg dose showed 69% efficacy during 19 months of follow-up (60). Thus the main advantage of the Vi vaccine is that a single dose provides moderate protection. The disadvantage is that no data suggest that protective efficacy lasts beyond 3 years, so revaccination is necessary within that time.

Most data suggest that children who are younger than 5 years respond poorly to Vi polysaccharide vaccines (61). However, one cluster-randomized trial in Kolkata, India (62), showed that protective efficacy among young children (aged 2–4 years) was 80%, which was higher than that observed in children aged 5–14 years (56%) and in older persons (46%). In contrast, a cluster-randomized field trial of similar design and using the same Vi vaccine in Karachi, Pakistan, reported an adjusted total protective effectiveness of −38% (95% CI, −192% to 35%) for children aged 2–5 years compared with 57% (95% CI, 6% to 81%) for children aged 5–16 years (61).

Thus, a single dose of Vi vaccine can provide moderate protection for a limited duration, but the vaccines have the usual limitations associated with polysaccharide vaccines, including poor immunogenicity in infants and young children, short-lived immunity and lack of anamnestic immune responses to subsequent doses (56, 63, 64).

Vi polysaccharide–protein conjugate vaccine

Experience with several polysaccharide–protein conjugate vaccines (such as Hib, meningococcal and pneumococcal vaccines) has shown that conjugation overcomes many of the limitations associated with unconjugated bacterial polysaccharides. On the basis of this experience and to try to address the limitations of the various typhoid vaccines described above, several Vi polysaccharide–protein conjugate vaccines have been developed.

A preparation of Vi polysaccharide conjugated to rEPA (Vi-rEPA) was evaluated in a series of studies in endemic and other areas. Schoolchildren and preschool children from highly endemic areas who received the Vi conjugate vaccine achieved and maintained higher levels of anti-Vi IgG serum antibodies compared with those who received the Vi polysaccharide vaccine (65–67). The immunogenicity of this Vi conjugate vaccine was observed to be dose
dependent (67). Following the administration of a single dose, detectable antibody levels were maintained for as long as 10 years in adults and 8 years in children.

A placebo-controlled, randomized, double-blind study in Vietnamese children aged 2–5 years in the highly endemic area gave an estimated vaccine efficacy of 89% after nearly 4 years (65, 67).

Vietnamese infants who received Vi-rEPA at age 2 months, 4 months and 6 months showed a rise in anti-Vi level from a geometric mean concentration (GMC) of 0.66 enzyme-linked immunosorbent assay (ELISA) units in cord blood to 17.4 ELISA units at 7 months (i.e. 1 month after the third dose) (68). By 12 months of age, the GMC had declined to 4.76 ELISA units. An additional dose at this age resulted in a boosting effect, with a GMC of 50.1 ELISA units 1 month later; 1 month after the booster dose more than 95% of infants had levels higher than 3.5 ELISA units, which is a putative antibody concentration associated with protection using the assay described in the study. Antibody responses to the routine vaccines used in the Expanded Programme on Immunization (administered simultaneously at age 2 months, 4 months and 6 months) were comparable in all groups.

A typhoid conjugate vaccine that uses Vi prepared from \textit{C. freundii} s.l. and CRM197 as the carrier protein has been demonstrated to elicit a significantly higher level of anti-Vi IgG in European adults who had never been exposed to typhoid fever (69). Vi preparations from \textit{C. freundii} s.l. have been shown to be immunologically indistinguishable from and structurally similar to those from \textit{S. Typhi} (70, 71), although size differences have been observed for Vi polysaccharide from \textit{S. Typhi} and \textit{C. freundii} s.l. by size-exclusion high-performance liquid chromatography (HPLC).

**Animal challenge studies**

In the 1950s and 1960s, WHO encouraged research to evaluate inactivated typhoid vaccines in various passive and active mouse-protection models to assess whether a model could be identified that predicted and correlated with the results of large-scale field trials of the vaccines in humans (72–74).

A more recent evaluation of Vi conjugate vaccines in bacterial challenge models has been reported (71). Hale and colleagues used a transformed Vi-producing \textit{S. enterica} subspecies enterica serovar Typhimurium strain (C5.507) in a challenge model with BALB/c mice. Vaccination with Vi polysaccharide conjugated to the \textit{Klebsiella pneumoniae} outer membrane 40-kD protein (rP40) provided partial protection from infection against C5.507. Opsonization assays demonstrated post-vaccination enhancement of Vi-positive bacterial uptake by macrophages derived from cultured murine bone marrow. Rondini and colleagues also showed protection in BALB/c mice against challenge with Vi-positive C5.507 subsequent to vaccination with Vi derived from \textit{C. freundii} s.l. conjugated with CRM197 (75).
Historically, animal models could not closely mimic the disease process of human typhoid. In 2010, Libby and colleagues (76) reported results with engrafted human haematopoietic stem cells into (NOD)-SCID-IL12rγnull diabetic mice. A 10-fold increase in liver bacterial burden was reported subsequent to intraperitoneal infection with S. Typhi. In other studies with engrafted immunocompromised Rag2−/−γc−/− mice with human fetal liver haematopoietic stem cells and progenitor cells, or with human umbilical cord blood cells, a more human-like disease was observed that included dissemination and replication of bacteria in liver and spleen (77–79). Other murine typhoid models are in development, such as those in mice deficient in toll-like receptor 4 (TLR4) (80) and TLR11 (81, 82). The TLR mouse models may provide an advantage over human immune system mice since variability due to engraftment is not present.

Part A. Guidelines on manufacture and control

A.1 Definitions

A.1.1 International name and proper name

The international name of the vaccine should be typhoid conjugate vaccine. The proper name should be the equivalent of the international name in the language of the country of origin. The use of the international name should be limited to the vaccines that satisfy the specifications formulated below.

A.1.2 Descriptive definition

A typhoid conjugate vaccine is a preparation of S. Typhi or C. freundii s.l. Vi polysaccharide covalently linked to a carrier protein. It may be formulated with a suitable adjuvant. It should be presented as a sterile, aqueous suspension or as freeze-dried material. The preparation should satisfy all of the specifications given below.

A.1.3 International reference materials

There are no international reference materials that can be used to measure the polysaccharide content, molecular mass or size distribution, or animal immunogenicity of the Vi polysaccharide-based typhoid conjugate vaccines being developed. Working standards for Vi polysaccharide, either from S. Typhi or C. freundii s.l., are being developed.

An international reference material to standardize antibody responses to Vi polysaccharide conjugate vaccines against typhoid is being developed, and is expected to be available in the future. A national reference preparation of purified human anti-Vi polysaccharide IgG is available for standardizing ELISAs to evaluate the immune response to experimental vaccines in clinical studies (83) (see section C.2.1).
A.1.4 Termination
The definitions given below apply to the terms used in these Guidelines. They may have different meanings in other contexts.

Carrier protein: the protein to which the Vi polysaccharide is covalently linked for the purpose of eliciting a T cell-dependent immune response to the Vi polysaccharide.

Final bulk: the homogeneous preparation in a single container from which the final containers are filled, either directly or through one or more intermediate containers.

Final lot: a number of sealed, final containers that are equivalent with respect to the risk of contamination that may have occurred during filling and freeze-drying (if performed). Therefore, a final lot should have been filled from a single container and freeze-dried in one continuous working session.

Master-seed lot: bacterial suspensions for the production of Vi polysaccharide or the carrier protein should be derived from a strain that has been processed as a single lot and is of uniform composition. The master-seed lot is used to prepare the working-seed lots. Master-seed lots should be maintained in the freeze-dried form or be frozen below –45 °C.

Modified carrier protein: a carrier protein that has been chemically or physically modified and prepared for conjugation to the polysaccharide.

Modified polysaccharide: purified polysaccharide that has been modified by a chemical reaction or a physical process in preparation for conjugation to the modified carrier protein.

Purified bulk conjugate: a bulk conjugate prepared from a single lot or pool of lots of modified polysaccharide and a single lot or a pool of lots of carrier protein. This is the parent material from which the final bulk is prepared.

Purified polysaccharide: the material obtained after final purification of polysaccharide. The lot of purified polysaccharide may be derived from a single harvest or a pool of single harvests that have been processed together.

Single harvest: the material obtained from one batch of culture that has been inoculated with the working-seed lot (or with the inoculum derived from it), harvested and processed together during one production run.

Working-seed lot: a quantity of live strains for the production of Vi polysaccharide or the carrier protein that are of uniform composition and that have been derived from the master-seed lot by growing the organisms and maintaining them in freeze-dried aliquots or frozen at or below –45 °C. The working-seed lot is used to inoculate the production medium.

A.2 Guidelines on general manufacturing
The general manufacturing recommendations contained in Good manufacturing practices: main principles for pharmaceutical products (84) and Good
manufacturing practices for biological products (85) should be applied at establishments manufacturing Vi polysaccharide conjugate vaccines.

Details of the standard operating procedures for preparing and testing Vi polysaccharide conjugate vaccines that have been adopted by the manufacturer, together with evidence that each production step has been appropriately validated, should be submitted for approval to the NRA. All assay procedures used for quality control of the conjugate vaccine and vaccine intermediates should be validated. When they are required, proposals to modify the manufacturing process and quality control methods should also be submitted for approval to the NRA before they are implemented.

Production strains for Vi polysaccharide and the carrier proteins should be handled according the specifications for their biosafety level, and depend on the requirements of the NRA (86). Standard operating procedures should be developed to deal with emergencies arising from accidental spills, leaks or other accidents. Personnel employed by the production and control facilities should be adequately trained. Appropriate protective measures, including vaccination, should be implemented if available.

A.3 Control of starting material
A.3.1 Certification of bacterial strain
A.3.1.1 Bacterial strain for preparing Vi polysaccharide

The bacterial strain used for preparing Vi polysaccharide should be from single, well characterized stock that can be identified by a record of its history, including the source from which it was obtained and the tests used to determine the characteristics of the strain.

The strain should be capable of stably producing Vi polysaccharide. S.Typhi and C.freundii s.l. have been shown to be suitable sources for Vi polysaccharide. 1H nuclear magnetic resonance (NMR) spectroscopy and immunochemical tests are suitable methods for confirming the identity of the polysaccharide.

A.3.1.2 Bacterial strain for preparing the carrier protein

The bacterial strains used for preparing the carrier protein should be identified by their history, including the source from which they were obtained and the tests used to determine the characteristics of the strains.

A.3.2 Bacterial-seed lot system

The production of both Vi polysaccharide and the carrier protein should be based on a seed-lot system involving a master seed and working seed. Cultures derived from the working seed should have the same characteristics as the
cultures of the strain from which the master-seed lot was derived (see sections A.3.1.1 and A.3.1.2).

Each new seed lot prepared should be characterized for Vi production using appropriate methods. For example, if materials of animal origin are used in the medium – whether for seed-lot preparation, for the preservation of strain viability, for freeze-drying, or for frozen storage – they should comply with the *WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products* (*87*), and should be approved by the NRA.

Manufacturers are encouraged to avoid the use of materials of animal origin wherever possible.

**A.3.3** **Bacterial culture media**

Basal medium must be sterilized, and manufacturers are encouraged to use semi-synthetic or chemically defined media that do not have ingredients of animal origin.

The liquid culture medium used to prepare bacterial-seed lots and to produce polysaccharide antigen should be free from ingredients that form a precipitate when hexadecyltrimethylammonium bromide (CTAB) is added at a concentration subsequently used in the manufacturing process.

Culture media should be free from substances likely to cause toxic or allergic reactions in humans. If materials of animal origin are used they should comply with the *WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products* (*87*) and should be approved by the NRA.

**A.4** **Control of vaccine production**

**A.4.1** **Control of polysaccharide antigen production**

The Vi polysaccharides that are used in licensed vaccines are defined chemical substances if they are prepared to similar specifications, for example as described in Requirements for Vi polysaccharide typhoid vaccine (Requirements for biological substances No. 48) (*1*). As a result, it is expected that they will have comparable potencies independent of the manufacturing process.

**A.4.1.1** **Single harvests for preparing Vi polysaccharide antigen**

The consistency of the production process should be demonstrated by monitoring the growth of the organisms and the yield of Vi polysaccharide.

**A.4.1.1.1** **Consistency of microbial growth for antigen production**

The consistency of the growth of production strains should be demonstrated by monitoring the growth rate, pH and the final yield of Vi polysaccharide, although monitoring should not be limited to these parameters.
A.4.1.2 Bacterial purity

If required, samples of the culture should be taken before inactivation and examined for microbial contamination. The purity of the culture should be verified by using suitable methods; these should include inoculation on appropriate culture media. If contamination is found, the culture and any product derived from it should be discarded.

A.4.1.2 Bacterial inactivation and antigen purification

Generally, S. Typhi is inactivated by formaldehyde or by a suitable inactivating agent, or by alternative methods (e.g. heating). The inactivation process should be adequately validated.

The biomass of S. Typhi or C. freundii s.l. is removed by centrifugation or tangential flow filtration. The Vi polysaccharide is purified from the supernatant by precipitation with CTAB. All reagents should be pharmaceutical grade and sterile. Controls should be in place to monitor the bioburden during purification. Methods used for further purification of this intermediate should be agreed with the NRA. To ensure stability, purified Vi polysaccharide in powder form should be stored at 2–8 °C, and purified Vi polysaccharide in solution should be stored below –20 °C. The duration during which the polysaccharide will remain stable should be validated.

A.4.1.3 Control of purified Vi polysaccharide antigen

Each lot of purified Vi polysaccharide should be tested for identity and purity, as well as the additional parameters described below. All tests should be validated by and agreed with the NRA.

A.4.1.3.1 Identity

Vi polysaccharide is a linear homopolymer composed of (1→4)-2-acetamido-2-deoxy-α-D-galacturonic acid that is O-acetylated at carbon-3 (88).

A test should be performed on the purified polysaccharide to verify its identity. NMR spectroscopy (89) or a suitable immunoassay is appropriate and convenient.

A.4.1.3.2 Molecular size or mass distribution

The molecular size or mass distribution of each lot of purified polysaccharide should be estimated to assess the consistency of each batch. The distribution constant (KD) should be determined by measuring the molecular size distribution of the polysaccharide at the main peak of the elution curve obtained by a suitable chromatographic method. The KD value or the mass distribution limits, or both, should be established and shown to be consistent from lot to lot for a
given product. For gel filtration, typically at least 50% of the Vi polysaccharide should be eluted at a KD value lower than a predefined value, depending on the chromatographic method used.

An acceptable level of consistency should be agreed with the NRA. Alternatively, calculation of the peak width at the 50% level can be used to analyse the distribution of molecular weight (MW). Suitable methods for this purpose are gel filtration using: (a) a refractive index detector (90); (b) a colorimetric assay; or (c) a light scattering detector (91). Manufacturers are encouraged to produce Vi polysaccharide that has a consistent distribution of molecular size.

A.4.1.3.3 Polysaccharide content

The concentration of the Vi polysaccharide in its fully O-acetylated, acid form in eluted fractions can be measured using Hestrin’s method (92) or another suitable method, such as NMR (89). The acridine orange staining method (88, 93) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) (93) have been reported to produce comparable results for Vi polysaccharide in a range of 15–200 µg/ml. A suitable immunoassay, for example rocket immunoelectrophoresis or ELISA, may also be considered. A suitable reference preparation of Vi polysaccharide should be used. These methods should be validated, and agreed with the NRA.

A.4.1.3.4 O-acetyl content

The O-acetyl content of the purified Vi polysaccharide is important for the immunogenicity of Vi; it should be at least 2.0 mmol/g polysaccharide (52% O-acetylation) (88, 90, 94).

Hestrin’s method (92) or NMR (89, 95) may also be used to quantitatively determine O-acetylation. The methods used and the acceptance criteria should be agreed with the NRA.

A.4.1.3.5 Moisture content

If the purified polysaccharide is to be stored as a dried form, the moisture content should be determined using suitable, validated methods, and the results should be within agreed limits; the methods used and the acceptable limits should be agreed with the NRA.

A.4.1.3.6 Protein impurity

Each lot of purified polysaccharide should contain no more than 1% (weight/weight) of protein as determined by a suitable, validated assay that uses bovine serum albumin as a reference (96).

Sufficient polysaccharide should be assayed to detect accurately 1% protein contamination.
A.4.1.3.7 Nucleic acid impurity

Each lot of purified polysaccharide should contain no more than 2% of nucleic acid by weight as determined by ultraviolet spectroscopy, on the assumption that the absorbance of a 10-g/l nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 200 (90); other validated methods may be used.

Sufficient polysaccharide should be assayed to detect accurately 2% nucleic acid contamination.

A.4.1.3.8 Phenol content

If phenol has been used to prepare the Vi polysaccharide antigen, each lot should be tested for phenol content using a validated method that has been approved by the NRA. The phenol content should be expressed in µg per mg of purified Vi antigen, and shown to be consistent and within the limits approved by the NRA.

A.4.1.3.9 Endotoxin

To ensure an acceptable level of pyrogenic activity in the final product, the endotoxin content of each lot of purified Vi polysaccharide should be determined, and shown to be within limits agreed with the NRA.

A.4.1.3.10 Residues of process-related contaminants

The residues of process-related contaminants in the purified polysaccharide (e.g. CTAB, formaldehyde and antifoaming agents) should be determined, and shown to be within limits agreed with the NRA. The routine testing of each lot before release for residual process-related contaminants may be omitted once consistency has been demonstrated on a number of lots; this number should be agreed with the NRA.

A.4.1.4 Modified polysaccharide preparations

Several registered and candidate polysaccharide-conjugate vaccines use chains of modified polysaccharides. Subsequent modification or truncation of Vi may be considered for use if the strain has been adequately characterized.

A.4.1.4.1 Chemical modification

Several methods are satisfactory for the chemical modification of polysaccharides prior to conjugation. The method that is chosen should be approved by the NRA. As part of the in-process control procedures, the processed polysaccharide that will be used in the conjugation reaction may be assessed to determine the number of functional groups introduced.
A.4.1.4.2 Molecular size or mass distribution

The degree of reduction in the size of the polysaccharide depends upon the manufacturing process. The average size or mass distribution (i.e. the degree of polymerization) of the processed polysaccharide should be measured using a suitable method. The size or mass distribution should be specified for each type of conjugate vaccine; appropriate limits for consistency should be specified since the size may affect the reproducibility of the conjugation process.

A.4.2 Control of carrier-protein production

A.4.2.1 Consistency of microbial growth for the carrier protein

The consistency of the growth of the microorganisms used should be demonstrated using methods such as pH and the final yield of the appropriate protein or proteins; other methods may also be used.

A.4.2.2 Characterization and purity of the carrier protein

Proteins that have been used as carriers in licensed conjugate vaccines include TT, DT and CRM197, but carriers could also include other proteins if these are approved by the NRA, such as rEPA. Manufacturers may choose other carrier proteins for conjugation provided that the vaccine is safe and immunogenic.

The test methods used to characterize such proteins to ensure that they are nontoxic and to determine their purity and concentration should be agreed by the NRA.

TT and DT should be of high purity and satisfy the relevant recommendations published by WHO (97, 98).

Either classical CRM197 or recombinant CRM197 produced by genetically modified micro-organisms may be used. CRM197 with a purity not less than 90% as determined by high-performance liquid chromatography (HPLC) should be prepared by column chromatographic methods.

For carrier proteins already in use, a higher level of purity may already have been specified and may be required. The content of residual host DNA should be determined, and results should be within the limits that have been approved for the particular product by the NRA. When CRM197 is produced in the same facility as DT, methods should be used to distinguish the CRM197 protein from the active toxin.

The identity of the carrier protein should be determined serologically, and characterized using a combination of the following physicochemical methods as appropriate: (a) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); (b) isoelectric focusing; (c) HPLC; (d) amino acid analysis; (e) amino acid sequencing; (f) circular dichroism; (g) fluorescence spectroscopy; (h) peptide mapping; (i) or mass spectrometry (99). Outcomes should be consistent with the reference material.
A.4.2.3 Degree of activation of the modified carrier protein

Adipic acid dihydrazide (ADH) or other appropriate linkers, such as N-Succinimidyl 3-(2-pyridyldithio)-propionate, can be used to modify the carrier protein. The level of protein modification should be monitored, quantified and be consistent. The use of an in-process control may be required. The reproducibility of the method used for modification should be validated.

The level of modification of the carrier protein by ADH can be assessed by determining the amount of hydrazide; this is done by using colorimetric reactions with 2,4,6-trinitrobenzenesulfonic acid and with ADH as a standard (100–102). Other suitable methods include fluorescent tagging followed by HPLC, or quadrupole time-of-flight mass spectrometry.

A.4.3 Conjugation and purification of the conjugate

A number of methods of conjugation are in use; all involve multistep processes (93, 100–102). Prior to demonstrating the immunogenicity of the Vi polysaccharide conjugate vaccine in clinical trials, both the methods of conjugation and the control procedures should be established to ensure the reproducibility, stability and safety of the conjugate.

The derivatization and conjugation processes should be monitored and analysed for unique reaction products. Residual unreacted functional groups or their derivatives are potentially capable of reacting in vivo, and may be present following the conjugation process. The manufacturing process should be validated, and the limits for unreacted activated functional groups (those that are known to be clinically relevant) at the conclusion of the conjugation process should be agreed with the NRA.

After the conjugate has been purified, the tests described below should be performed in order to assess the consistency of the manufacturing process. The tests are critical for ensuring consistency from lot to lot.

A.4.4 Control of the purified bulk conjugate

Tests for releasing purified bulk conjugate should be validated.

A.4.4.1 Identity

A suitable immunoassay should be performed on the purified bulk conjugate to verify its identity.

Depending on the buffer used, NMR spectroscopy may be used to confirm the identity and integrity of the polysaccharide in the purified bulk conjugate (95, 103–105).
A.4.4.2 Endotoxin
The endotoxin content of the purified bulk conjugate should be determined unless otherwise justified, and shown to be within limits agreed with the NRA.

A.4.4.3 O-acetyl content
The O-acetyl content of the purified bulk conjugate should be determined by NMR or by other appropriate methods. The O-acetyl content of the purified bulk conjugate should be agreed with the NRA.

A.4.4.4 Residual reagents
The purification procedures for the conjugate should remove any residual reagents that were used for conjugation and capping. The removal of reagents, their derivatives and reaction by-products, such as ADH, phenol and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (known as EDC, EDAC or EDCI), should be confirmed using suitable tests or by validation of the purification process.

The specifications of the process and the quantifiable methods to be used should be agreed upon in consultation with the NRA.

The process should also demonstrate that no significant covalent modification of the Vi itself has occurred (e.g. less than 5% of the Vi monosaccharides should have been modified). For example, many common conjugation procedures use EDC, and a frequent side reaction can result in Vi carboxylates being covalently modified to form an N-acylurea. Such modification may alter the structure of the Vi, and this modification is known to be immunogenic, leading to antibodies that cross-react with other EDC-modified polysaccharides, such as those in Hib, pneumococcal and meningococcal conjugate vaccines; thus this modification may interfere with these vaccines. The N-acylurea content can be readily measured using NMR.

A.4.4.5 Polysaccharide content
The content of Vi polysaccharide should be determined using an appropriate validated assay. Methods that have been used to determine the Vi polysaccharide content include the colorimetric assay with acridine orange, or HPAEC–PAD (93), which has superior reproducibility.

A.4.4.6 Conjugated and unbound (free) polysaccharide
A limit for the presence of unbound (free) Vi polysaccharide relative to total Vi polysaccharide should be set for each purified bulk conjugate; this limit should be agreed with the NRA. The upper limit should be specific for the
polysaccharide conjugate formulation, and the limit should not be exceeded during the shelf-life of the batch. Methods that have been used to assay unbound polysaccharide include gel filtration; ultrafiltration and hydrophobic chromatography; ultracentrifugation followed by HPAEC–PAD, or colorimetric detection (90, 101); other suitable methods may be developed and validated.

A.4.4.7 Protein content

The protein content of the purified bulk conjugate should be determined using an appropriate validated assay. Each batch should be tested for conjugated and unbound protein. The unconjugated protein content of the purified bulk conjugate should comply with the limit for the product that has been agreed with the NRA.

Appropriate methods for determining unbound protein include HPLC or capillary electrophoresis.

A.4.4.8 Conjugation markers

The success of the conjugation process can be assessed by characterizing the conjugate using suitable methods. For example, an increase in the MW of the protein component of the conjugate compared with the carrier protein should be determined using the Coomassie blue stain with SDS–PAGE; an increase in the MW of the conjugate compared with both the Vi polysaccharide and the protein components should be evidenced by the gel filtration profile. The conjugate should retain antigenicity for both Vi and the carrier protein as demonstrated by dot blot or western blot.

Where the chemistry of the conjugation reaction results in the creation of a unique linkage marker, such as a unique amino acid, the validation batch should be assessed to quantify the extent of the covalent reaction of the Vi polysaccharide with the carrier protein, so that the frequency of the covalent bond is given as a function of the number of polysaccharide repeating units or overall polysaccharide content.

A unique linkage marker could be assessed for the validation batch or, alternatively, the manufacturing process should be validated to demonstrate that it yields conjugate with a level of substitution that is consistent from batch to batch.

A.4.4.9 Absence of reactive functional groups

The validation batch should be shown to be free of reactive functional groups or their derivatives that are suspected to be clinically relevant on the polysaccharide and the carrier protein.

Where possible, the presence of reactive functional groups – for example, those derived by ADH treatment – should be assessed for each batch. Alternatively,
the product of the capping reaction may be monitored, or the capping reaction can be validated to show that reactive functional groups have been removed.

A.4.4.10 Ratio of polysaccharide to carrier protein
The ratio of polysaccharide to carrier protein in the purified bulk conjugate should be calculated. For this ratio to be a suitable marker of conjugation, the content of each of the conjugate components prior to their use should be known. For each purified bulk conjugate, the ratio should be within the range approved by the NRA for that particular conjugate, and should be consistent with the ratio in vaccine that has been shown to be effective in clinical trials.

A.4.4.11 Molecular size or mass distribution
It is important to evaluate the molecular size or mass of the polysaccharide–protein conjugate to establish the consistency of production, product homogeneity and stability during storage.

The relative molecular size of the polysaccharide–protein conjugate should be determined for each purified bulk conjugate using a gel matrix appropriate to the size of the conjugate (106). The method should be validated and should have the specificity to distinguish the polysaccharide–protein conjugate from other components that may be present (e.g. unbound protein or polysaccharide). The specification of molecular size or mass distribution should be vaccine-specific and consistent with that of lots shown to be immunogenic in clinical trials.

Typically the size of the polysaccharide–protein conjugate may be examined by methods such as gel filtration using high-performance size-exclusion chromatography (HPSEC) on an appropriate column. Since the ratio of polysaccharide to protein is an average value, characterization of this ratio over the molecular size or mass distribution (e.g. by using dual monitoring of the column eluent) can provide further proof of the consistency of manufacturing (99, 107).

A.4.4.12 Bacterial and mycotic bioburden
The purified bulk conjugate should be tested for bacterial and mycotic bioburden according to the methods described in Part A, section 5.2, of the revised General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973) (108), or using methods approved by the NRA. If a preservative has been added to the product, appropriate measures should be taken to prevent it from interfering with the test.

A.4.4.13 Specific toxicity of the carrier protein
When appropriate, the bulk conjugate should be tested to confirm the absence of specific toxicity in the carrier protein.
A.4.4.14  pH
If the purified bulk conjugate is a liquid preparation, the pH of each batch should be tested, and the results should be within the range of values shown to be safe in clinical trials and stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.4.4.15  Appearance
The appearance of the bulk purified conjugate should be examined. It should be clear to moderately turbid, and colourless to pale yellow.

A.4.5  Preparation and control of the final bulk
A.4.5.1  Preparation
The final bulk is prepared by mixing a preservative or stabilizer (if used), or both, with a suitable quantity of the bulk conjugate to meet the specifications of vaccine lots that have been shown to be safe and efficacious in clinical trials. If an adjuvant is used, it should be mixed with the final bulk at this stage.

A.4.5.2  Test for bacterial and mycotic sterility
Each final bulk should be tested for bacterial and mycotic sterility according to the requirements of Part A, sections 5.1 and 5.2, of the revised General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973) (108), or using methods approved by the NRA. If a preservative has been added to the final bulk, appropriate measures should be taken to prevent it from interfering with the test.

A.4.5.3  Sterile filtration
The final bulk conjugate should be sterile-filtered just before the final bottling. The concentration of both Vi and carrier protein, and the integrity of the conjugate, should be verified in the final filtrate.

A.5  Filling and containers
The recommendations concerning filling and containers given in Good manufacturing practices for biological products (85) should be applied.

A.6  Control of the final product
A.6.1  Inspection of the final containers
Each container of a final lot should be inspected visually (manually or with automatic inspection systems), and those showing abnormalities – such as improper sealing, lack of integrity, clumping or the presence of particles – should be discarded.
A.6.2  **Control tests on the final lot**
The tests used before releasing the final lot should be validated.

A.6.2.1  **Identity**
Identity tests on the Vi polysaccharide and the carrier protein should be performed on each final lot. An immunological test or a physicochemical assay may be used for the Vi polysaccharide and the carrier protein.

A.6.2.2  **Bacterial and mycotic sterility**
The contents of the final containers should be tested for bacterial and mycotic sterility according to the requirements of Part A, sections 5.1 and 5.2, of the revised General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973) (108), or using a method approved by the NRA. If a preservative has been added, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.6.2.3  **Polysaccharide content**
The amount of Vi polysaccharide conjugate in the final containers should be determined and shown to be within the limits agreed with the NRA.

The formulations of conjugate vaccines produced by different manufacturers may differ. A quantitative assay for the Vi polysaccharide should be carried out. The specification is likely to be product-specific. The following types of tests may be used: (a) colorimetric methods; (b) chromatographic methods (including HPLC or HPAEC–PAD); or (c) immunological methods (including rate nephelometry).

A.6.2.4  **Unbound (free) polysaccharide**
A limit for the presence of free Vi polysaccharide should be set for each type of conjugate vaccine. Assessing the level of unconjugated polysaccharide in the final lot may be technically demanding; as an alternative, the molecular size of the conjugate could be determined for the final lot to confirm the integrity of the conjugate. An acceptable value should be consistent with the value seen in batches used for clinical trials that showed adequate immunogenicity; the value should be approved by the NRA.

A.6.2.5  **O-acetyl content**
The O-acetyl content of the Vi polysaccharide conjugate in the final container should be determined for each final lot by NMR (89) or by other appropriate methods, such as Hestrin’s method (92). Routine release testing of each lot for
O-acetyl content in the final product may be omitted if the NRA agrees and if the O-acetyl content is measured at the level of conjugate bulk, and data validating the process that were obtained during the product's development confirmed that formulation and filling do not alter the integrity of the functional groups. A limit for the O-acetyl content of the Vi polysaccharide conjugate should be approved by the NRA (94).

A.6.2.6 Molecular size or mass distribution
The molecular size or mass distribution of the polysaccharide conjugate should be determined for each final lot using a gel matrix appropriate to the size of the conjugate; for example, HPSEC multiple angle laser light scattering (MALLS) may be used (106). The analysis of molecular size or mass distribution for each final lot may be omitted provided that the NRA agrees and the test has been performed on the conjugate bulk (see section A.4.4.11).

A.6.2.7 Endotoxin or pyrogen content
The pyrogenic activity of the vaccine in the final container should be tested in rabbits. The endotoxin should be tested using a validated Limulus amoebocyte lysate test or a suitable in vitro assay. The pyrogen content and the endotoxin content should be within the limits agreed with the NRA.

A.6.2.8 Adjuvant content and degree of adsorption
If an adjuvant has been added to the vaccine, its content should be determined using a method approved by the NRA. The amount and nature of the adjuvant should also be agreed with the NRA. If aluminium compounds are used as adjuvants, the amount of aluminium should not exceed 1.25 mg per single human dose.

The consistency of adsorption of the antigen to the adjuvant is important; the degree of adsorption should be tested in each final lot and should be within the range of values measured in vaccine lots shown to be clinically effective. The methods used and the specifications should be approved by the NRA.

A.6.2.9 Preservative content
If a preservative has been added to the vaccine, its content should be determined using a method approved by the NRA.

The amount of preservative in each dose of the vaccine should be shown not to have any deleterious effect on the antigen, or to impair the safety of the product in humans. The efficacy of the preservative should be demonstrated. The preservative used and the concentration should be approved by the NRA.
A.6.2.10  **General safety (innocuity)**

The need to test the final lots of the Vi polysaccharide conjugate vaccine for unexpected toxicity (also known as abnormal toxicity) should be agreed with the NRA. This test may be omitted from routine lot release once the consistency of production has been established to the satisfaction of the NRA, and when reliable good manufacturing practices are in place.

A.6.2.11  **pH**

If the vaccine is a liquid preparation, the pH of each final lot should preferably be near 7.2; liquid preparations should be tested, and the results should be within the range of values shown to be safe and effective for vaccine lots in clinical trials and stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.6.2.12  **Moisture content**

If the conjugate is dried, the acceptable level of residual moisture should be established, and the limit should be agreed with the NRA.

A.6.2.13  **Osmolality**

The osmolality of the final lots should be determined and shown to be within the limits agreed with the NRA.

A.6.3  **Control of diluents**

The recommendations in Good manufacturing practices: main principles for pharmaceutical products (84) should apply to the manufacture and quality control of the diluents used to reconstitute conjugate typhoid vaccines. An expiry date should be established for the diluents based upon stability data. For lot release of the diluent, tests should be done to assess the appearance, identity, volume, sterility and content of key components.

A.7  **Records**

The recommendations in Good manufacturing practices for biological products (85) should be followed as appropriate for the level of development of the candidate vaccine.

A.8  **Samples**

A sufficient number of lot samples of the product should be retained for future studies and needs. Vaccine lots that are to be used for clinical trials may serve as reference materials in the future, and a sufficient number of vials should be reserved and stored appropriately for that purpose.
A.9 Labelling
The recommendations in Good manufacturing practices for biological products (85) that are appropriate for a candidate vaccine should be applied, and the following additional information should also be included.

The label on the cartons enclosing one or more final containers, or the leaflet accompanying each container, should include:

- a statement that the candidate vaccine fulfils Part A of these Guidelines;
- the information that if the vaccine is a lyophilized form it should be used immediately after reconstitution; if data have been provided to the licensing authority to indicate that the reconstituted vaccine may be stored for a limited time then the length of time should be specified;
- information on the volume and nature of the diluent to be added to reconstitute the lyophilized vaccine; this information should specify that the diluent approved by the NRA should be supplied by the manufacturer.

A.10 Distribution and shipping
The recommendations appropriate for candidate vaccines given in Good manufacturing practices for biological products (85) should be followed.

Shipments should be maintained within specified temperature ranges, and packages should contain cold-chain monitors (109).

A.11 Stability, storage and expiry date
The recommendations appropriate for candidate vaccines given in WHO Good manufacturing practices for biological products (85) and Guidelines on stability evaluation of vaccines (110) should be followed. The statements concerning storage temperature and expiry date that appear on primary or secondary packaging should be based on experimental evidence, and should be submitted to the NRA for approval.

A.11.1 Stability testing
Stability testing should be performed at different stages of production, namely on stored intermediates (such as the purified polysaccharide, the carrier protein and the purified bulk conjugate) and on the final product. Parameters that indicate stability should be defined or selected appropriately, according to the stage of production. A stability protocol should be established for intermediates and for the final product; the protocol should include release assays that have been agreed
with the NRA. During production, it is advisable to assign a shelf-life duration to all in-process materials, in particular to intermediates that are stored.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three lots of the final product manufactured from different bulk conjugates.

In addition, a real-time real-condition stability study should be conducted on at least one final container lot produced each year.

The formulation of vaccine and adjuvant (if used) should be stable throughout the shelf-life. Acceptable limits for stability should be agreed with the NRA.

The polysaccharide component of conjugate vaccines may be subject to gradual hydrolysis at a rate that may vary depending upon the type of conjugate, the formulation or adjuvant, the excipient, and conditions of storage. The hydrolysis may result in a reduced molecular size of the Vi polysaccharide component, a reduction in O-acetyl content, a reduction in the amount of polysaccharide bound to the carrier protein or in a reduced molecular size of the conjugate, or some combination of these.

The O-acetyl content should be monitored quantitatively for stability testing and release testing. The quantity of free protein should be monitored for stability testing and release testing. The molecular size or mass distribution should be monitored for stability testing and release testing.

If applicable, the residual moisture should be monitored for stability testing and release testing.

Tests should be conducted before licensing to determine the extent to which the stability of the product is maintained throughout the proposed validity period. The free saccharide content should be determined as a percentage of total saccharide, and should meet recommendations for the final product until the expiry date as established by the manufacturer and defined in section A.6.2.

Where applicable, the level of adsorption of the conjugate to the adjuvant should be shown to be within the limits agreed with the NRA, unless data show that the immunogenicity of the final product does not depend on the adsorption of the antigen to the adjuvant.

Accelerated stability studies may provide additional supporting evidence of the stability of the product or consistency in manufacturing, or both, but are not recommended for establishing the shelf-life of the vaccine under a defined storage condition.

When any changes are made in the production process that may affect the stability of the product, the vaccine produced by the new method should be shown to be stable.

If manufacturers consider incorporating a vaccine vial monitor (VVM) into the label, they should provide appropriate data to justify a correlation between the stability kinetics of the vaccine and the selected VVM (111).
A.11.2 Storage conditions
Before being distributed by the manufacturer or before being issued from a storage site, the vaccine should be stored at a temperature that has been shown by the manufacturer to be compatible with a minimal loss of titre. The maximum duration of storage and the optimal storage conditions should be defined based on the findings of stability studies; these should be agreed with the NRA and should ensure that all quality specifications for the final product, including the minimum titre specified on the label of the container or package, will be maintained for the duration of the shelf-life.

A.11.3 Expiry date
Expiry dates should be based on the findings of stability studies and the determination of shelf-life, and approved by the NRA. The expiry dates for vaccines and diluents may be different from one another.

A.11.4 Expiry of reconstituted vaccine (if applicable)
For single-dose containers, the reconstituted vaccine should be used immediately. For multidose containers, the container should be stored in a dark place at 2–8 °C unless photostability studies have shown that this is unnecessary. The expiry time for an opened container should be defined by stability studies and approved by the NRA, but it should not exceed 6 h.

Part B. Nonclinical evaluation of new typhoid conjugate vaccines

B.1 General principles
Detailed guidelines from WHO on the design, conduct, analysis and evaluation of nonclinical studies of vaccines are available separately (112), and they should be read in connection with Part B of these Guidelines. Specific issues to be considered in relation to candidate Vi conjugate vaccines are considered in section B.3. Plans for nonclinical studies to be conducted during the development of the vaccine should be discussed with the NRA early in the review process.

B.2 Product characterization and process development
It is critical that vaccine production processes are appropriately standardized and controlled to ensure consistency in manufacturing and the collection of nonclinical data that may suggest safety and efficacy in humans.

Candidate formulations of Vi conjugate vaccines should be characterized to define the critical structural and chemical attributes that indicate the polysaccharide, the conjugating protein and the conjugate product are sufficiently
pure and stable, and their properties are consistent. The extent of product characterization may vary depending on the stage of development. Vaccine lots used in nonclinical studies should be adequately representative of those intended for use in clinical investigations – that is, the safety data should support the initiation of clinical studies in humans. Ideally, the lots should be the same as those used in the clinical studies. If this is not feasible, then the lots should be comparable with respect to physicochemical data, stability and formulation.

B.3 Nonclinical immunogenicity studies

Immunogenicity studies in animal models should be conducted because they provide valuable proof-of-concept information that can be used to support a clinical development plan. In addition, immunogenicity data derived from appropriate animal models are useful in establishing the immunological characteristics of the Vi polysaccharide conjugate product, and may guide the selection of doses, schedules and routes of administration that will be evaluated in clinical trials. To ensure immunogenicity in nonclinical testing weaning mice (younger than 6 weeks) should receive intramuscularly two injections 2 weeks apart of the conjugate vaccine and Vi should be used for a control group. Anti-Vi IgG should then be measured. The conjugate should induce a response that is at least four times higher than the response induced by Vi, and a booster response should occur after the second dose (100). Immunogenicity studies of Vi polysaccharide conjugates have been conducted in mice (71, 93, 113–115); in humans, correlation has been made between the level of anti-Vi IgG and protection against clinical disease (53, 116). Therefore, the primary end-point for nonclinical studies of the immunogenicity of Vi conjugate vaccines should be the level of anti-Vi elicited.

Nonclinical studies of immunogenicity may include an evaluation of seroconversion rates or geometric mean antibody titres, or both. When possible, nonclinical studies may be designed to assess relevant immune responses, including the functional immune response (e.g. by evaluating serum bactericidal antibodies, opsonophagocytic activity and serum-dependent opsonophagocytic killing) (see section C.2.2). These studies may also address the interference that can occur among antigens when multiantigen vaccines are used (see section C.2.3). In such cases, the response to each antigen should be evaluated.

Although there have been advances in animal models (see the section on General considerations), no ideal animal model exists that establishes direct serological or immunological correlates of clinical protection. In the absence of such a model, it is important to ensure that the production batches have the same protective efficacy as those used and shown to be protective in clinical trials. Therefore, the emphasis is increasingly being placed on ensuring consistency in manufacture through the use of modern physical, chemical and immunological quality-control methods.
B.4 Nonclinical toxicity and safety

WHO guidelines on nonclinical evaluation of vaccines (112) should be followed when assessing toxicity and safety. Toxicity studies for Vi polysaccharide conjugate typhoid vaccines may be performed in an appropriate animal model. These studies should entail careful analysis of all major organs, as well as of tissues proximal to and distal from the site of administration, to detect unanticipated direct toxic effects; these effects should be assessed for a wide range of doses, including those exceeding the intended clinically relevant dose. If novel proteins are used to manufacture conjugate vaccines, toxicity studies should be performed on these proteins first. Nonclinical safety studies should be conducted in accordance with the GLPs that have been described elsewhere (117, 118). For ethical reasons, it is desirable to apply the 3Rs concept of “Replace Reduce Refine” to minimize the use of animals in research where scientifically appropriate.

Part C. Clinical evaluation of new typhoid conjugate vaccines

C.1 General principles

C.1.1 General considerations for clinical studies

In general, clinical trials should adhere to the principles described in the WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (119).

The general principles described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (120) apply to Vi polysaccharide conjugate vaccines, and should be followed. Some issues specific to conjugate vaccines or to the clinical development programme for Vi conjugate vaccines, or both, are discussed below and should be read in conjunction with the general guidance mentioned above.

In particular, the methodological and statistical considerations described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations, sections B.2 and B.3 (120) should be taken into account.

Additional data on the safety, immunogenicity, efficacy and effectiveness of Vi conjugate vaccines may emerge, including insights into correlates of protection, and the suggestions for clinical development programmes that are provided in this section should be read with this in mind. Clinical programmes are expected to change once licensed Vi polysaccharide conjugate vaccines become widely available for use in various age groups.
C.1.2  Outline of the clinical development programme

In accordance with the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (120), early clinical development programmes should identify an appropriate dose of conjugated Vi antigen and suitable immunization schedules for the target age groups. These initial studies should also provide a preliminary assessment of the vaccine’s safety. Studies to determine an adequate dose and regimen are necessary for each candidate Vi conjugate vaccine that is developed, since it is not possible to extrapolate the antigen content and schedule identified for one conjugate vaccine to another. This consideration applies even if the same carrier protein is used for two different Vi conjugate vaccines, since experience with other conjugated polysaccharide vaccines has indicated that differences in conjugation chemistry can affect immunogenicity.

It is recommended that the major part of the pre-licensure clinical development programme is conducted in subjects who are representative of the intended target population.

The minimum acceptable content of the pre-licensure clinical programme for each candidate conjugate vaccine, and the expectations for the data to be generated during the post-licensure period, should be discussed between sponsors and the relevant NRA. Factors expected to have an important influence on the pre-licensure programme include the intended target age range and the availability of licensed unconjugated Vi vaccines or conjugated Vi vaccines, or both, for each age group.

Although data on antibody persistence and responses to booster doses are considered important, the collection and submission of these data usually occur during the post-approval period. Therefore, sponsors and NRAs should agree on the minimum duration of follow-up that will be required before the initial application dossier is submitted.

C.1.3  Evidence to support efficacy

C.1.3.1  Subjects aged at least 2 years

Protective efficacy studies against typhoid can be conducted only in endemic areas with relatively high rates of disease. In endemic areas a prospective comparison of subjects aged 2 years or older immunized with a Vi conjugate vaccine and an unvaccinated control group is not appropriate because there are licensed vaccines that have documented efficacy against typhoid in certain age groups. Also, a study of the relative protective efficacy (e.g. comparing a candidate Vi conjugate vaccine with an unconjugated Vi vaccine) is not likely to be feasible due to the large number of subjects that would be required to derive robust statistical conclusions.
Taking these issues into account, as well as evidence supporting the role of anti-Vi IgG antibody in protecting against Vi-expressing S. Typhi, it is not considered necessary to estimate the protective efficacy of candidate Vi conjugate vaccines against typhoid in subjects who are aged 2 years or older. In this age group the pre-licensure assessment of the likely protective efficacy of conjugated Vi vaccines could be based on appropriate studies of comparative immunogenicity (see section C.3).

Nevertheless, successful typhoid challenge studies conducted in healthy adults using an appropriate and validated model (i.e. one in which some protective efficacy of unconjugated Vi vaccines is detectable) could provide considerable supporting evidence of the efficacy of a Vi conjugate vaccine. Human challenge studies may also provide at least limited information on the relationship between the immune response and various efficacy parameters. If, in consultation with the NRA, sponsors decide to conduct typhoid challenge studies in humans, they should be undertaken only by physicians with appropriate expertise, and in a carefully controlled setting, to ensure the safety of the volunteers. Healthy adults should be screened to detect underlying pre-existing conditions and to exclude risk factors for complications, including gall bladder disease. The challenge strain should be well characterized and there should be complete information on its susceptibility to antibacterial agents.

C.1.3.2 Subjects younger than 2 years

There is no information on the protective efficacy or effectiveness against typhoid of any Vi conjugate vaccine in children who are younger than 2 years old when first vaccinated. Therefore, there is a need to carefully consider the potential value and feasibility of conducting a prospective, randomized study of protective efficacy in a region where background rates of proven typhoid disease have been documented in this age group. Whether such a study is required, or whether it can be replaced by an appropriate assessment of immunogenicity that is followed by post-approval effectiveness studies, can only be decided on a case-by-case basis after discussions between sponsors and NRAs. Since conducting a pre-licensure study of protective efficacy would likely prolong the time until the vaccine is approved, the decision regarding the requirement for a protective efficacy study should take into account factors such as the regional burden of typhoid disease in this age group.

If a pre-licensure protective efficacy study is conducted, it should compare rates of febrile illnesses associated with a positive blood culture for S. Typhi between a group that receives the candidate Vi conjugate vaccine and an appropriate control group. A double-blind design is recommended but this would require that the control group is randomized to a non-typhoid vaccine from which they may derive some benefit that is indistinguishable in appearance from the candidate conjugate vaccine and is administered in the same way (i.e.
route and schedule). If a suitable non-typhoid vaccine cannot be identified then the control subjects could be unvaccinated (i.e. avoiding the use of a placebo injection). In this case a double-blind design would not be possible but it would be important to make every effort to ensure that investigators are unaware of the treatment assignment.

Further information on designing and conducting studies of protective efficacy, and on assessing the effectiveness of vaccines is provided in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (120).

C.1.3.3 Vaccine effectiveness

Whether or not a pre-licensure study of protective efficacy against typhoid is performed, it is recommended that efforts be made to estimate the vaccine’s effectiveness during the post-licensure period (see section C.5).

C.2 Assessment of the immune response

C.2.1 Total anti-Vi IgG in serum

The primary parameter for assessing the humoral immune response to a vaccine is usually based on a measure of functional antibody. However, there are no well established or standardized assays for assessing functional antibody responses to Vi-containing vaccines, and it is not known how the results of such assays correlate with vaccine efficacy. A correlation between total serum antibody (59) or total anti-Vi IgG in serum (61, 65, 67, 68, 121) and protection against typhoid has been described, although there is no established cut-off value that clearly predicts prevention of clinical disease. Thus, it may be acceptable to base the primary assessment of the immunogenicity of candidate Vi conjugate vaccines on the total concentration of anti-Vi IgG.

In recent years, the assessment of immune response to licensed unconjugated Vi vaccines has predominantly used ELISA to measure total anti-Vi IgG in serum (62, 66, 122). Older assays, such as radioimmunoassay (53) and passive haemagglutination (55), are now rarely used (57). However, several ELISAs have been used in studies of different vaccines (69, 123). In 2013, when these Guidelines were prepared, there was no international standard available. However, reagents and a software analysis tool for a Vi antibody ELISA are available free of charge from the United States Food and Drug Administration and the United States Centers for Disease Control and Prevention (contact information is listed in Table 3.1).

It is essential that the assays used to report data from the clinical studies that are considered to be pivotal for an application dossier should be fully validated. Once an international standard becomes available, all sponsors should use the standard to calibrate the assays used to determine concentrations of anti-Vi IgG.
Table 3.1  
**Contact details for materials for Vi antibody ELISA**

<table>
<thead>
<tr>
<th>Name</th>
<th>Provider address</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhi</em> anti-Vi (human) and <em>S. typhi</em> Vi polysaccharide, lot 05</td>
<td>Center for Biologics Evaluation and Research, United States Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD, 20903, USA</td>
<td><a href="http://www.fda.gov/BiologicsBloodVaccines/ScienceResearch/BiologicsResearchAreas/default.htm">http://www.fda.gov/BiologicsBloodVaccines/ScienceResearch/BiologicsResearchAreas/default.htm</a></td>
</tr>
<tr>
<td>ELISA calculation programme</td>
<td>United States Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA</td>
<td><a href="http://www.cdc.gov/ncird/software/elisa/index.html">http://www.cdc.gov/ncird/software/elisa/index.html</a></td>
</tr>
</tbody>
</table>

C.2.2 **Other immune-response parameters**

As part of the overall characterization of the immune response to candidate Vi conjugate vaccines, sponsors may consider evaluating one or more of the following parameters, at least for subsets of serum obtained from different age groups and at different time points:

- serum bactericidal antibody (SBA);
- opsonophagocytic antibody (OPA);
- antibody avidity; following an initial T cell-dependent immune response in individuals naive for Vi antigen it would be expected that antibody avidity would increase over time, and there should also be differences between postprimary doses and postbooster doses;
- IgG subclass responses;
- evidence of T cell-dependent immune response with memory B-cell recruitment – e.g. an anamnestic response to a booster dose of vaccine, or detection of memory B cells using an in vitro cultured enzyme-linked immunosorbent spot assay (ELISPOT).

C.2.3 **Characterization of the immune response**

C.2.3.1 **Antibody kinetics**

The anti-Vi antibody kinetic should be assessed in recipients of the candidate Vi conjugate vaccine group and in subjects who receive any control Vi-containing vaccine (licensed unconjugated or conjugated) after the primary series and following booster doses.
Following the primary series (which may consist of one or several doses), serum samples may be collected at approximately day 14 and 28, at 6 months and then at 1 year and 3 years as a minimum.

Following a booster dose, a rapid rise in anti-Vi is expected if there has been efficient priming of the immune system. Therefore it is suggested that sera should be obtained at approximately 6 days, 10 days and 28 days after the booster dose, and then at preplanned intervals.

To reduce the number of samples taken from each participant, groups could be further randomized to provide samples at different time points. It is suggested that all subjects should at least provide samples before vaccination and on day 28 post-vaccination. Longer-term assessments of postprimary immunization and postbooster levels should be planned at least for subgroups of vaccinated subjects.

C.2.3.2 Immune memory
Due to concerns that vaccination with unconjugated Vi polysaccharide can lead to hyporesponsiveness to sequential doses, and may potentially blunt the immune response to a conjugated Vi vaccine, unconjugated Vi polysaccharide should not be administered to subjects primed with a candidate Vi conjugate vaccine in order to demonstrate that the initial dose(s) of the conjugate elicited a T cell-dependent immune response.

Whether a T cell-dependent initial immune response has been elicited by the initial dose or doses can be assessed by administering a further dose of the Vi conjugate vaccine after an interval of approximately 6–12 months. The immune response observed (ideally by measuring not only anti-Vi IgG but also functional antibody, antibody avidity and cell-mediated immunity using ELISPOT) following a single dose of Vi conjugate vaccine administered to subjects who completed a primary series of the same vaccine can be compared with the response to a first dose administered to previously unvaccinated subjects of the same age. The immune response to a single dose of the Vi conjugate vaccine in primed subjects should be superior to that in subjects who are Vi naive (see section C.3.4 for additional information regarding the administration of booster doses, including the administration of Vi conjugate vaccine to subjects who previously received conjugated or unconjugated Vi vaccines).

C.2.4 Analyses of immune responses
Although elicitation of anti-Vi IgG by vaccination has been shown to correlate with protection, the minimum concentration of anti-Vi IgG required for protection against typhoid remains uncertain (61, 65, 67, 68, 121).

The assessment of anti-Vi IgG concentrations should take into account all of the following factors:
- Proportions of vaccinees who achieve concentrations above one or more predefined threshold concentrations – analyses of protective efficacy observed over time in a prospective, randomized and placebo-controlled study with one Vi conjugate vaccine in children who were first vaccinated when aged 2–5 years have suggested a benchmark (or threshold) value that could be applied to the interpretation of anti-Vi IgG concentrations (65, 67, 68, 83, 121). Based on the assay used in these studies to assess stored serum samples, a threshold value of 4.3 µg/ml anti-Vi antibody measured by ELISA (83) appears to be associated with a high level of sustained protection lasting approximately 4 years after vaccination. If the antibody decay curve for a candidate conjugate vaccine resembles that of the vaccine that was used in the study, then the antibody concentrations at earlier time points after vaccination should considerably exceed this threshold value. Until an international standard becomes available, sponsors who wish to apply this threshold value to the results of their own assays need to perform a calibration against the assay used in the study of efficacy mentioned above (83).

- Seroconversion rates – seroconversion may be defined as a change from seronegative before vaccination to seropositive after vaccination (e.g. based on the assay cut-off or based on achieving a defined threshold value), or as at least a four-fold increase from pre-vaccination concentrations to post-vaccination concentrations in subjects who were seropositive at baseline.

- Reverse cumulative distributions (RCDs).

- Geometric mean concentrations (GMCs).

- When selecting the most appropriate immune-response parameter to use for the primary assessment of immunogenicity in any one study, researchers should take into account the population selected for investigation, the anticipated pre-existing antibody concentrations that may reflect prior vaccinations or natural exposure, and whether the assessment relates to postprimary series immunizations or postboosting. Regardless of which parameter is selected for the predefined primary analysis (see section C.3), between-group comparisons based on other parameters should be presented.

C.3 Clinical study designs

C.3.1 Studies that compare conjugated Vi vaccines with unconjugated Vi vaccines

Studies that compare candidate Vi conjugate vaccines with licensed unconjugated Vi vaccines can only be conducted in subjects who are aged at least 2 years. Data
should be generated across the entire age range for which a claim for use will be sought. Studies should stratify subjects by appropriate age subgroups, or separate studies should be conducted in different age groups.

It is recommended that these studies are randomized and double blind. If the sponsor proposes to administer more than one dose of Vi conjugate in any age subgroup there will be a need to consider matching of the schedule in the unconjugated Vi vaccine control group. Sponsors should identify suitable non-typhoid vaccines that could be administered to the control group in order to avoid or at least to minimize the need for placebo injections. The selection of the unconjugated Vi control vaccine for each study should take into account the available evidence on safety and immunogenicity and should be discussed with the relevant NRA.

The primary comparison of immune responses could be based on:

- percentages that achieve anti-Vi IgG levels above predefined threshold values (e.g. as suggested in section C.2.4);
- seroconversion rates.

The immune responses should be measured in samples collected at day 28 after the initial vaccination series has been completed (i.e. after a single dose or after the last assigned dose of the primary series), or in samples collected at an alternative time point if this is justified by data on the antibody kinetic.

The primary analysis should demonstrate that the immune response to the Vi conjugate vaccine is at least non-inferior when compared with the immune response to the control vaccine. The predefined margin of non-inferiority should be carefully justified. Protocols may also plan for sequential analyses to assess whether there is superiority in immune responses to the Vi conjugate vaccine if the predefined criterion for concluding non-inferiority has been met.

**C.3.2 Studies that compare vaccinated groups with unvaccinated groups**

These studies should employ random allocation to the Vi conjugate candidate vaccine (i.e. the vaccinated group) or to a licensed non-typhoid vaccine from which study subjects may derive some benefit (i.e. the unvaccinated group).

This study design is most likely to be used for subjects who are younger than 2 years. There are no Vi-containing vaccines known to be efficacious in this age group, which means that data on the immune response cannot provide a direct bridge to vaccine efficacy. Therefore, other options need to be considered for interpreting the anti-Vi IgG immune response to a candidate Vi conjugate vaccine.

The immune responses in the group receiving the candidate Vi conjugate vaccine should be superior to those in the unvaccinated group. In addition, the immune responses observed after the last assigned dose has been administered may be compared with:
the immune response to unconjugated Vi vaccine in one or more older age groups; or
- the immune response to the same candidate Vi conjugate vaccine in one or more older age groups; or
- both of these responses.

The comparative data could be derived from subjects (e.g. children aged 2–5 years) enrolled in a randomized study of candidate Vi conjugate vaccine versus unconjugated Vi vaccine that has successfully demonstrated non-inferiority as described in section C.3.1.

The primary analysis should be based on demonstrating that the immune response to the candidate Vi conjugate vaccine is at least non-inferior when compared with the immune response to the control vaccine in another age group. However, comparing immune responses among age groups (and among regimens) is not straightforward. For example, seroconversion rates may be impacted by pre-existing antibodies, and final GMCs may vary by age. Therefore, it may be appropriate to place more weight on comparing the proportions of subjects that achieve post-vaccination anti-Vi IgG concentrations that rise above a threshold value (as discussed in section C.2.4).

C.3.3 Studies that compare conjugated Vi vaccines

The availability of licensed Vi conjugate vaccines will have implications for the design of clinical studies in all age groups. Some of the issues that will need to be taken into account include:

- whether the protective efficacy of any licensed Vi conjugate vaccine has been documented in a pre-licensure study of protective efficacy or by post-approval data on effectiveness, or both – if so, then conducting comparative immunogenicity studies against such a vaccine would allow for direct bridging between anti-Vi IgG concentrations and protection;
- whether data on efficacy or effectiveness point to a specific anti-Vi antibody concentration that strongly correlates with efficacy;
- whether and where Vi conjugate vaccines have been introduced into routine immunization programmes and in which age groups;
- investigators’ and subjects’ willingness to take part in studies that use a control group that receives an unconjugated Vi vaccine or an unvaccinated control group.

As more Vi conjugate vaccines become licensed, it is expected that there will be a transition towards conducting comparative studies between candidate and control Vi conjugate vaccines. The selection of the most appropriate licensed
Vi conjugate vaccine for a comparative study must be agreed between the sponsor and the NRA. However, the optimal candidate would be a Vi conjugate vaccine for which protective efficacy has been demonstrated; these data may come from post-approval studies of effectiveness. If no licensed conjugates have documented efficacy, then the extent of the data on comparative immunogenicity for each age group of interest for licensed Vi conjugate vaccines should be taken into account.

The aim of these studies is to demonstrate the non-inferiority of the immune response to the candidate vaccine when compared with the licensed Vi conjugate vaccine. If efficacy data have supported derivation of an anti-Vi antibody concentration that strongly correlates with protection, then the proportions of subjects that achieve at least this concentration after vaccination should be compared.

C.3.4 Antibody persistence and booster doses

Longer-term assessment of antibody persistence is considered essential. At the time when a Vi conjugate vaccine is initially approved there should be adequate documentation of anti-Vi concentrations for at least 1 year after the initial dose has been administered. The collection of further data on antibody persistence should be planned but, subject to agreement with the NRA, may be reported at intervals after the initial approval.

In studies that compare conjugated Vi vaccines with unconjugated Vi vaccines, data on antibody persistence should be analysed among the randomized groups. Using antibody decay curves observed following administration of unconjugated Vi vaccines, data for up to 1 year can indicate whether there is any difference between vaccines in the initial rate of decrease of anti-Vi antibody. While there is no established immunological correlate of protection, antibody persistence data may be viewed in terms of the percentages of vaccinees that have anti-Vi IgG concentrations above a predefined threshold for a specified period of time.

Determining the need for and the appropriate timing of a booster dose of Vi conjugate vaccine is not straightforward, and needs and timing may differ among age groups and populations – there may, for example, be a considerable natural boosting effect in highly endemic regions. Bacteraemia can be detected shortly after oral inoculation with S. Typhi, and several days before the onset of clinical symptoms. This suggests that it may not be appropriate to rely on immune memory responses to achieve a sufficiently rapid rise in anti-Vi antibody to protect individual subjects. In addition, data on one Vi conjugate vaccine suggest that protection against typhoid depends on maintaining a certain concentration of anti-Vi antibody (65, 67, 68, 83, 121).

Extensive post-approval data on antibody persistence and vaccine effectiveness are needed to support decisions on boosting. Nevertheless, in order to facilitate decisions regarding the introduction of booster doses,
it is recommended that studies in all age groups should plan to document at predetermined intervals immune responses to booster doses. Subjects may be sub-randomized to different schedules for booster doses after the initial dose or doses. As mentioned in section C.2.3, by including an unvaccinated control group, these data can also be used to demonstrate that the initial dose or doses elicited a T cell-dependent immune response.

In studies that compare a candidate Vi conjugate vaccine with an unconjugated Vi vaccine, it is important to analyse immune responses to a sequential (booster) dose of the Vi conjugate vaccine in different groups. These data can be used to determine whether prior exposure to unconjugated Vi polysaccharide may blunt the immune response to a conjugate vaccine as a result of inducing hyporesponsiveness. The data may indicate whether more than one dose of the conjugate vaccine is needed in these subjects, which would be important information for planning the introduction of Vi conjugate vaccines into regions where there has been extensive use of unconjugated Vi vaccines in the past.

The assessment of immune responses to a booster dose should be based on antibody concentrations found immediately before and after the booster dose. The rate of change in immune parameters after the booster dose, as well as the magnitude of the response observed, should be compared among groups primed with the same Vi conjugate vaccine, unprimed subjects and subjects that previously received unconjugated Vi vaccine. If the Vi conjugate vaccine is found to have efficiently primed the immune system, then the onset of the response after the booster dose should be faster, and the antibody concentrations achieved should be higher, than in the other groups (see section C.2.3).

C.3.5 Immune responses to, and effects of, the carrier protein
Proteins such as CRM197, DT, TT and rEPA have been used in the production of various Vi conjugate vaccines. Based on experience with other types of conjugate vaccines that use CRM197, DT or TT as the carrier protein, there is some potential that the immune response to the conjugated antigen may be reduced in subjects who have high levels of tetanus or diphtheria antitoxin before vaccination. This phenomenon should be explored during the development of Vi conjugate vaccines; this may be accomplished by analysing post-vaccination responses and comparing these with pre-vaccination antibody concentrations. The potential clinical significance of any effect requires careful consideration.

C.3.6 Co-administration with other vaccines
Concomitant administration of some types of conjugates with other vaccines already in routine use, including other conjugated vaccines, may give rise to detectable immune interference – which may be a depression or an enhancement
of antibody levels – although the magnitude of the effect may not necessarily be of clinical significance. The possible effects of co-administration on immune responses cannot be predicted simply by considering the vaccine content. Therefore, clinical studies are needed in which candidate Vi conjugate vaccines are co-administered with other vaccines that are representative of the types that for convenience and reasons related to a vaccine programme are likely to be given at the same time. These studies could examine co-administration with vaccines routinely used in infants and toddlers in endemic areas or co-administration with vaccines commonly used by travellers resident in non-endemic areas.

Co-administration studies may be conducted before or after initial licensure, or both, depending on the importance of being able to recommend co-administration with specific types of vaccines to facilitate use within existing vaccination programmes targeting specific age groups.

In co-administration studies the immune response to the Vi conjugate and to all other co-administered antigens should be evaluated. The approach to these studies is based primarily on demonstrating the non-inferiority of immune responses to antigens when vaccines are co-administered compared with each vaccine given alone, with careful justification of predefined non-inferiority margins.

C.4 Pre-licensure assessment of safety

There is no evidence that points to anticipation of specific safety issues for Vi conjugate vaccines. At present it is possible only to recommend that the assessment of safety in pre-licensure studies should follow the usual approaches to ensure comprehensive monitoring and data collection.

C.5 Postmarketing studies and surveillance

The information in the application dossier is likely to be restricted to studies of safety and immunogenicity that have been conducted in certain geographical areas and in populations with particular demographic features. In addition, the total population evaluated for safety in pre-licensure clinical studies may be limited such that only those adverse events that occur at a frequency of at least 1 per 1000 persons vaccinated can be described with any degree of confidence \(^{(120)}\). Therefore, it is considered critically important that well developed plans are put in place prior to licensure to ensure that vaccine safety and effectiveness are assessed during routine use in the post-approval period. In particular:

- Studies of vaccine effectiveness and impact should include a careful evaluation of any herd immunity effect of Vi conjugate vaccines. It may not be possible to collect vaccine-specific effectiveness data if more than one Vi conjugate is introduced concurrently in the same region, but the overall effectiveness of a programme that includes
Specific vaccines is still informative regardless of whether vaccines are delivered routinely or as an outbreak intervention.

- Further attempts should be made to identify an immunological correlate of protection. This requires that factors be considered in addition to the usual issues surrounding the approach selected to assess effectiveness.

- If the pre-licensure safety database is limited in size, or if any particular safety issues are observed during clinical studies or after approval, a dedicated post-authorization safety study may be necessary in addition to routine passive surveillance.

Sound and comprehensive data on safety and effectiveness cannot be collected by the sponsors alone. Therefore, collaborations should be planned between sponsors and public health bodies to ensure that adequate and reliable data are collected in areas where there is routine and widespread use of a Vi conjugate vaccine. Protocols should be developed before the initial approval, and should be included in the application dossier. These protocols can be refined once it is known where and how a vaccine will actually be used.

Other issues to be addressed after initial licensure include:

- Assessing longer-term antibody concentrations in selected cohorts, including antibody concentrations after booster doses are administered (see section C.3.4).

- Conducting safety and immunogenicity studies in populations that were not included in pre-licensure studies and in which there are good reasons to expect that immune responses may differ (e.g. in immunosuppressed people, or age groups not previously studied). Additional safety and immunogenicity studies may also be considered if there is a good scientific rationale for anticipating that the immune response to the Vi conjugate vaccine in the pre-licensure study population (e.g. residents in endemic areas) may not predict that in populations that have not been studied (e.g. residents in non-endemic areas who are travelling to endemic areas).

- Assessing the possibility that widespread use of a vaccine and high immunization coverage in a population where typhoid fever is endemic may lead to the emergence of otherwise rare Vi-negative variants of S. Typhi (124–127); these variants exist and can cause typhoid fever, albeit they have lower attack rates (128, 129).

All data collected should be regularly submitted to the responsible regulatory authorities so that any implications for the marketing authorization can be assessed, and appropriate actions can be taken.
Part D. Guidelines for NRAs

D.1 General guidelines

The general recommendations for NRAs and national control laboratories given in the Guidelines for national authorities on quality assurance for biological products (130) and the Guidelines for independent lot release of vaccines by regulatory authorities (131) should be followed.

These Guidelines specify that no new biological substance should be released until consistency in manufacturing and quality has been demonstrated by regularly releasing consistent batches.

The detailed procedures for production and quality control, and any significant changes in these that may affect the quality, safety or efficacy of a Vi polysaccharide conjugate typhoid vaccine, should be discussed with and approved by the NRA. The NRA may obtain the product-specific working reference from the manufacturer and use this for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in ensuring the quality of vaccines. In particular, the NRA should carefully monitor production records and the results of quality-control tests for clinical lots, as well as results for a series of consecutive lots of the final bulk and final product.

D.2 Official release and certification

A vaccine lot should be released only if it fulfils the national requirements and Part A of these Guidelines.

A model protocol for the manufacturing and control of typhoid conjugate vaccines is shown in Appendix 1; this protocol should be signed by the responsible official of the manufacturing establishment, and should be prepared and submitted to the NRA in support of a request to release the vaccine for use.

A certificate signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine meets all national requirements as well as Part A of these Guidelines. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory determination of critical quality parameters (such as the ratio of free Vi polysaccharide to bound Vi polysaccharide) as well as the expiry date assigned on the basis of the shelf-life of the vaccine should be stated. A model NRA Lot-release Certificate is given in Appendix 2. A copy of the model protocol should be attached to the lot-release certificate – the purpose of which is to facilitate the exchange of typhoid conjugate vaccines between countries.
Authors and acknowledgments

The first draft of these Guidelines was prepared by Dr J. Mathew, Post Graduate Institute of Medical Education and Research, India and Dr M. Levine, University of Maryland School of Medicine, USA (General considerations); Dr S. Rijpkema, National Institute for Biological Standards and Control, England (Part A); Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA (Part B); Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England (Part C); Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland.

Consolidation of each part of the Guidelines then took place following three expert consultations: (a) joint Korea Food and Drug Administration (KFDA) and WHO working group held in Jeju, Republic of Korea, 5–7 September 2012; (b) WHO clinical working group held in London, 7–8 January 2013, for which financial and technical support was provided by Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; and (c) WHO informal consultation held in Geneva, 29–30 April 2013.

Acknowledgments are due to the following experts who helped to improve earlier versions by providing written comments and proposed changes: Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr I. Feavers, National Institute for Biological Standards and Control, England; Dr M. Levine, University of Maryland School of Medicine, USA; Dr A. Pollard, Oxford University, England.

Acknowledgments are extended to the following individuals who participated in discussions at the joint KFDA/WHO working group: Dr C. Ahn, National Institute of Food and Drug Safety Evaluation, Republic of Korea; Dr M. Bonnet (IFPMA representative), Sanofi Pasteur, France; Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr R. Carbis, International Vaccine Institute, Republic of Korea; Dr D. Cardoso-Gonzalez (DCVMN representative), Finlay Institute, Cuba; Dr D. Garcia, National Drug and Health Products Safety Agency, France; Dr H. Izumiya, National Institute of Infectious Diseases, Japan; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr C. Jones (Rapporteur), National Institute for Biological Standards and Control, England; Dr B.G. Kim, Korea Food and Drug Administration, Republic of Korea; Dr C.K. Lee (Chair), Korea Food and Drug Administration, Republic of Korea; Dr T. King Jr, Food and Drug Administration, Philippines; Dr I. Knezevic, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Ms D. Kusmitiaty, National Agency of Drug and Food Control, Indonesia; Dr M. Levine, University of Maryland School of Medicine, USA; Dr L. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr J. Mathew, Post Graduate Institute
of Medical Education and Research, India; Dr G. Meller (Observer), Bill & Melinda Gates Foundation, USA; Dr M. Morita, National Institute of Infectious Diseases, Japan; Dr P. Namgyal, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Ms N. Nurainy (DCVMN representative), Biofarma, Indonesia; Dr H.J. Oh, Korea Food and Drug Administration, Republic of Korea; Dr M. Paste (IFPMA representative), GlaxoSmithKline Biologicals, Belgium; Dr A. Ramkishan, Ministry of Health and Family Welfare, India; Dr S. Rijpkema, National Institute for Biological Standards and Control, England; Dr S. Sahastrabuddhe, International Vaccine Institute, Republic of Korea; Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr S. Szu, National Institutes of Health, USA; Ms J. Tresnabudi (Observer), Biofarma, Indonesia; Dr M. Zeng, National Institutes of Food and Drug Control, China. The following KFDA senior managers and staff members are also acknowledged: Ms Y. Choi, Dr S.T. Chung, Ms S.Y. Eum, Dr S.H. Hong, Dr S.J. Kang, Dr S.Y. Kang, Dr K.H. Lee, Dr K.T. Nam, Dr I.S. Shin and Dr Y. Sohn.

Acknowledgments are due to the following experts who attended the WHO clinical working group and who reviewed clinical data and agreed upon the key points to be considered in the clinical guidelines: Dr I. Feavers, National Institute for Biological Standards and Control, England; Dr E. Griffiths (Chair), England; Dr I. Knezevic, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr M. Levine, University of Maryland School of Medicine, USA; Dr C. Nelson (Rapporteur), Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr A. Pollard, Oxford University, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency; Dr S. Rijpkema, National Institute for Biological Standards and Control, England.

The second draft of these Guidelines was prepared by the following individuals taking into consideration: (a) written comments from a first public consultation from March to April 2013 following the uploading of the first draft document onto the WHO Biologicals website; and (b) discussion during the 2013 WHO informal consultation: Dr J. Mathew, Post Graduate Institute of Medical Education and Research, India (General considerations); Dr S. Rijpkema, National Institute for Biological Standards and Control, England (Part A); Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA (Part B); Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England (Part C); Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland.

Acknowledgments are due to the following experts who provided written comments during the first public consultation: Dr M. Bonnet, Sanofi Pasteur, France; Dr P. Chagnaud, National Drug and Health Products Safety Agency,
France; Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr S. Dutta, National Institute of Cholera and Enteric Diseases, India; Ms M.J. Escoto-López, Center for State Control on the Quality of Drugs, Cuba; Dr E. Griffiths, England; Dr A. Goel, Biological E, India; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr O. Le Doledec, National Drug and Health Products Safety Agency, France; Professor M. Levine, University of Maryland School of Medicine, USA; Dr L. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr A. Merkle, Paul-Ehrlich-Institut, Germany; Dr K. Meunier, National Drug and Health Products Safety Agency, France; Dr T. Mongeau, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr S. Morgeaux, National Drug and Health Products Safety Agency, France; Dr T. Morris, United States Pharmacopoeial Convention, USA; Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr H.J. Oh, Ministry of Food and Drug Safety, Republic of Korea; Dr S. Park, Ministry of Food and Drug Safety, Republic of Korea; Dr M. Paste, GlaxoSmithKline Biologicals, Belgium; Dr B. Patnaik, Bharat Biotech International, India; Dr S. Rijpkema, National Institute for Biological Standards and Control, England; Dr J. Robbins, USA; Dr R. Schneerson, USA; Dr S. Sontakke, Health Canada, Canada; Dr S. Sus, Eunice Kennedy Shriver National Institute of Child Health and Human Development, USA; Dr W. Van Molle, Scientific Institute of Public Health, Belgium; Dr A. Worobec, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Ms D. Kusmiaty, National Agency of Drug and Food Control, Indonesia; Dr G. Xie, China National Biotec Group, China; Dr M. Zeng, National Institutes of Food and Drug Control, China; Japan Paediatric Society (anonymous contribution); State Food and Drug Administration and Center for Drug Evaluation of China (anonymous contribution).

Acknowledgments are due to the following experts who participated in the 2013 WHO informal consultation: Dr A. Bentsi-Enchill, Department of Immunization, Vaccines and Biologicals, World Health Organization, Switzerland; Dr M. Bonnet (IFPMA representative), Sanofi Pasteur, France; Dr J. Cipollo, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr J. Cirunay, Food and Drug Administration, Philippines; Dr R. Carbis, International Vaccine Institute, Republic of Korea; Dr D. Cardoso-Gonzalez (DCVMN representative), Finlay Institute, Cuba; Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Ms M.J. Escoto-López, Center for State Control on the Quality of Drugs, Cuba; Dr I. Feavers, National Institute for Biological Standards and Control, England; Dr D. Garcia, National Drug and Health Products Safety Agency, France; Mr K. Gopinathan, Bharat Biotech International, India; Dr E. Griffiths (Chair), England; Ms W. Jaroenkunathum, Ministry of Public Health, Thailand; Dr C. Jones, National Institute for Biological Standards and Control, England; Dr C.K. Lee (Co-Chair), Korea Food and Drug
Administration, Republic of Korea; Dr I. Knezevic, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr H. Langar, WHO Regional Office for the Eastern Mediterranean, World Health Organization, Egypt; Dr M. Levine (Rapporteur), University of Maryland School of Medicine, USA; Dr L.B. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr J. Mathew, Post Graduate Institute of Medical Education and Research, India; Dr E. Mohamed (DCVMN representative), The Biovac Institute, South Africa; Dr M. Morita, National Institute of Infectious Diseases, Japan; Dr C. Nelson, Coalition Against Typhoid Secretariat, Sabin Vaccine Institute, USA; Dr V.C. Nguyen, National Institute of Hygiene and Epidemiology, Viet Nam; Dr S. Nishioka, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr H.J. Oh, Korea Food and Drug Administration, Republic of Korea; Dr M. Paste (IFPMA representative), GlaxoSmithKline Biologicals, Belgium; Dr A. Podda, Novartis Vaccines Institute for Global Health, Italy; Dr A. Pollard, Oxford University, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, England; Dr A. Ramkishan, Ministry of Health and Family Welfare, India; Dr S. Rijpkema, National Institute for Biological Standards and Control, England; Dr J. Robbins, USA (via teleconference); Dr S. Sahastrabuddhe, International Vaccine Institute, Republic of Korea; Dr J. Shin, Department of Essential Medicines and Health Products, World Health Organization, Switzerland; Dr S. Szu, National Institutes of Health, USA; Ms J. Tresnabudi, Biofarma, Indonesia; Ms G. Trisnasari (DCVMN representative), Biofarma, Indonesia; Ms A. Visala, Food and Drugs Administration, India; Dr M. Zeng, National Institutes of Food and Drug Control, China.

A further improved draft was prepared following written submissions on the second draft, and the contributions of the following experts are acknowledged: Ms J. Dahlan, National Agency of Drug and Food Control, Indonesia; Mr K. Gopinathan, Bharat Biotech International, India; Ms W. Jaroenkonatham, Ministry of Public Health, Thailand; Dr M. Levine, University of Maryland School of Medicine, USA; Dr L. Martin, Novartis Vaccines Institute for Global Health, Italy; Dr S. Szu, National Institutes of Health, USA; Ms G. Trisnasari, Biofarma, Indonesia.

During a second round of public consultation on document WHO/BS/2013.2215 organized through the WHO Biologicals website from July to September 2013 further comments were provided by the following experts: Dr F. Cano, Dr D. Garcia, Dr S. Morgeaux and Dr M. Surgot, National Drug and Health Products Safety Agency, France; Dr J. Cipollo and Dr T. Mongeau, United States Food and Drug Administration Center for Biologics Evaluation and Research, USA; Dr H.J. Oh, Ministry of Food and Drug Safety, Republic of Korea; Dr S. Rijpkema, National Institute for Biological Standards and Control, England.
References


68. Thiem VD et al. The Vi conjugate typhoid vaccine is safe, elicits protective levels of IgG anti-Vi, and is compatible with routine infant vaccines. *Clinical and Vaccine Immunology*, 2011, 18:730–735.


Appendix 1

Model protocol for the manufacturing and control of typhoid conjugate vaccines

The following protocol is intended for guidance and indicates the minimum information that should be provided by the manufacturer to an NRA. Information and tests may be added or omitted as required by an NRA.

It is thus possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that accompanies the vaccine container. If the protocol is being submitted in support of a request to permit importation, it must also be accompanied by a lot-release certificate from the NRA of the country where the vaccine was produced, stating that the product meets national requirements as well as the recommendations in Part A of this document.

Summary information on the final lots

International name of product: ________________________________
Commercial name: ________________________________
Product licence (marketing authorization) number: ________________________________
Country: ________________________________
Name and address of manufacturer: ________________________________
Final packing lot number: ________________________________
Type of containers: ________________________________
Number of containers in this packing lot: ________________________________
Final container lot number: ________________________________
Number of filled containers in the final lot: ________________________________
Date of manufacture: ________________________________
Nature of final product: ________________________________
Preservative used and nominal concentration: ________________________________
Volume of each recommended single human dose: ________________________________
Number of doses per final container: ________________________________
Summary of composition: ________________________________

(Include a summary of the qualitative and quantitative composition of the vaccine per single human dose; include the conjugate, any adjuvant used and other excipients)
Shelf-life approved (months): .................................................................
Expiry date: ...........................................................................................
Storage conditions: ................................................................................

The following sections are intended for reporting the results of the tests performed during the production of the vaccine, so that the complete document will provide evidence of consistency in production; thus, if any test had to be repeated, this information must be indicated. Any abnormal results should be recorded on a separate sheet.

**Detailed information on manufacture and control**

**SUMMARY OF STARTING MATERIALS**

It is possible that a number of bulk lots may be used to produce a single final lot. A summary of the bulk polysaccharide, activated saccharide, bulk carrier protein and bulk conjugate lots that contributed to the final lot should be provided.

**CONTROL OF TYPHOID Vi POLYSACCHARIDE**

Bacterial strain

Identity of *Salmonella* Typhi Ty2 or

*Citrobacter freundii*: ________________________________________________

Origin and short history: ___________________________________________

Authority that approved the strain: _________________________________

Date approved: _________________________________________________

Bacterial culture media for seed-lot preparation and Vi production

Free from ingredients that form precipitate when CTAB is added: ___________

Free from toxic or allergenic substances: ______________________________

Any components of animal origin (list): _______________________________

Certified as TSE-free: _____________________________________________

Master-seed lot

Lot number: ______________________________________________________

Date master-seed lot established: ____________________________________

Working-seed lot

Lot number: ______________________________________________________

Date working-seed lot established: _________________________________

Type of control tests used on working-seed lot: ________________________

Date seed lot reconstituted: _________________________________________
Control of single harvests

For each single harvest, indicate the medium used; the dates of inoculation; the temperature of incubation; the dates of harvests and harvest volumes; the results of tests for bacterial growth rate, pH, purity and identity; the method and date of inactivation; the method of purification; and the yield of purified polysaccharide.

Control of purified typhoid Vi polysaccharide

<table>
<thead>
<tr>
<th>Lot number:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of manufacture:</td>
<td></td>
</tr>
<tr>
<td>Volume:</td>
<td></td>
</tr>
</tbody>
</table>

**Identity**

<table>
<thead>
<tr>
<th>Date tested:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
</tbody>
</table>

**Molecular size or mass distribution**

<table>
<thead>
<tr>
<th>Date tested:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
</tbody>
</table>

**Polysaccharide content**

<table>
<thead>
<tr>
<th>Date tested:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Method used:</td>
<td></td>
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<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
</tbody>
</table>

**O-acetyl content**

<table>
<thead>
<tr>
<th>Date tested:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
</tbody>
</table>

**Moisture content**

<table>
<thead>
<tr>
<th>Date tested:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
</tbody>
</table>
Protein impurity
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Nucleic acid impurity
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Phenol content
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Endotoxin content
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Residues of process-related contaminants
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Control of modified polysaccharide
Lot number: ____________________________
Method of chemical modification: __________________________

Extent of activation for conjugation
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________
Molecular size or mass distribution

Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ________________________________

CONTROL OF CARRIER PROTEIN

Microorganisms used
Identity of strain used to produce carrier protein: ________________________________
Origin and short history: ________________________________
Authority that approved the strain: ________________________________
Date approved: ________________________________

Bacterial culture media for seed-lot preparation
and carrier-protein production
Free from ingredients that form precipitate when CTAB
is added: ________________________________
Free from toxic or allergenic substances: ________________________________
Any components of animal origin (list): ________________________________
Certified as TSE-free: ________________________________

Master-seed lot
Lot number: ________________________________
Date master-seed lot established: ________________________________

Working-seed lot
Lot number: ________________________________
Date established: ________________________________
Type of control tests used on working-seed lot: ________________________________
Date seed lot reconstituted: ________________________________

Control of carrier-protein production
List the lot numbers of harvests: indicate the medium used; the dates of inoculation;
the temperature of incubation; the dates of harvests and harvest volumes; the results
of tests for bacterial growth rate, pH, purity and identity; the method and date of
inactivation; the method of purification; and the yield of purified carrier protein.
Provide evidence that the carrier protein is nontoxic.
Purified carrier protein
Lot number: ____________________________
Date produced: _________________________

Identity
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: _________________________________

Protein impurity
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: _________________________________

Nucleic acid impurity
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: _________________________________

Modified carrier protein
Lot number: ____________________________
Date produced: _________________________
Method of modification: __________________

Extent of activation
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: _________________________________

CONTROL OF PURIFIED BULK CONJUGATE

Production details of bulk conjugate
List the lot numbers of the saccharide and carrier protein used to manufacture the conjugate vaccines, the production procedure used, the date of manufacture and the yield.
Tests on purified bulk conjugate

Identity
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Endotoxin content
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

O-acetyl content
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Residual reagents
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Vi polysaccharide content
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Conjugated and unbound (free) polysaccharide
Date tested: ____________________________
Method used: __________________________
Specification: __________________________
Result: ________________________________

Protein content
Date tested: ____________________________
Method used: __________________________
<table>
<thead>
<tr>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conjugation markers</strong></td>
<td></td>
</tr>
<tr>
<td>Date tested:</td>
<td></td>
</tr>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
<tr>
<td><strong>Absence of reactive functional groups (capping markers)</strong></td>
<td></td>
</tr>
<tr>
<td>Date tested:</td>
<td></td>
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<tr>
<td>Method used:</td>
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<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
<tr>
<td><strong>Ratio of polysaccharide to protein</strong></td>
<td></td>
</tr>
<tr>
<td>Date tested:</td>
<td></td>
</tr>
<tr>
<td>Method used:</td>
<td></td>
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<tr>
<td>Specification:</td>
<td></td>
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<tr>
<td>Result:</td>
<td></td>
</tr>
<tr>
<td><strong>Molecular size or mass distribution</strong></td>
<td></td>
</tr>
<tr>
<td>Date tested:</td>
<td></td>
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<tr>
<td>Method used:</td>
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<tr>
<td>Specification:</td>
<td></td>
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<tr>
<td>Result:</td>
<td></td>
</tr>
<tr>
<td><strong>Bacterial and mycotic bioburden</strong></td>
<td></td>
</tr>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Media:</td>
<td></td>
</tr>
<tr>
<td>Volume tested:</td>
<td></td>
</tr>
<tr>
<td>Date of inoculation:</td>
<td></td>
</tr>
<tr>
<td>Date of end of test:</td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
<tr>
<td><strong>Specific toxicity of carrier protein (where appropriate)</strong></td>
<td></td>
</tr>
<tr>
<td>Method used:</td>
<td></td>
</tr>
<tr>
<td>Strain and type of animals:</td>
<td></td>
</tr>
<tr>
<td>Number of animals:</td>
<td></td>
</tr>
<tr>
<td>Route of injection:</td>
<td></td>
</tr>
</tbody>
</table>
Volume of injection: ________________________________
Quantity of protein injected: ________________________________
Date of start of test: ________________________________
Date of end of test: ________________________________
Specification: ________________________________
Result: ________________________________

\[ \text{pH} \]
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ________________________________

\[ \text{Appearance} \]
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ________________________________

Depending on the conjugation chemistry used to produce the vaccine, tests should also be included to demonstrate that amounts of residual reagents and reaction by-products are below a specified level.

**CONTROL OF FINAL BULK**
Lot number: ________________________________
Date prepared: ________________________________

**Preservative (if used)**
Name and nature: ________________________________
Lot number: ________________________________
Final concentration in the final bulk: ________________________________

**Stabilizer (if used)**
Name and nature: ________________________________
Lot number: ________________________________
Final concentration in the final bulk: ________________________________

**Adjuvant (if used)**
Name and nature: ________________________________
Lot number: ________________________________
Final concentration in the final bulk: ________________________________
Tests on final bulk

Bacterial and mycotic sterility

Method used: ________________________________
Media: ______________________________________
Volume tested: _______________________________
Date of inoculation: ___________________________
Date of end of test: ___________________________
Specification: _________________________________
Result: ______________________________________

FILLING AND CONTAINERS

Lot number: _________________________________
Date of sterile filtration: _______________________
Date of filling: _______________________________
Volume of final bulk: __________________________
Volume per container: __________________________
Number of containers filled (gross): ______________
Date of lyophilization (if applicable): ____________
Number of containers rejected during inspection: ___________
Number of containers sampled: ___________________
Total number of containers (net): _________________
Maximum duration approved for storage: _________________
Storage temperature and duration: _________________

CONTROL TESTS ON FINAL LOT

Inspection of final containers

Date tested: _________________________________
Method used: _________________________________
Specification: _________________________________
Results: ______________________________________
Appearance before reconstitution:1 _________________________________
Appearance after reconstitution:1 _________________________________
Diluent used: ____________________________________
Lot number of diluent used: _________________________

1 This applies only to lyophilized vaccines.
**Tests on final lot**

**Identity**
- Date tested: ____________________________
- Method used: __________________________
- Specification: __________________________
- Result: ________________________________

**Sterility**
- Method used: __________________________
- Media: ________________________________
- Number of containers tested: __________
- Date of inoculation: ____________________
- Date of end of test: _____________________
- Specification: __________________________
- Result: ________________________________

**Polysaccharide content**
- Date tested: __________________________
- Method used: __________________________
- Specification: __________________________
- Result: ________________________________

**Unbound (free) polysaccharide**
- Date tested: __________________________
- Method used: __________________________
- Specification: __________________________
- Result: ________________________________

**O-acetyl content**
- Date tested: __________________________
- Method used: __________________________
- Specification: __________________________
- Result: ________________________________

**Molecular size or mass distribution**
- Date tested: __________________________
- Method used: __________________________
- Specification: __________________________
- Result: ________________________________
Endotoxin or pyrogen content
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ______________________________________

Adjuvant content and degree of adsorption (if applicable)
Date tested: ________________________________
Nature and concentration of adjuvant per single human dose: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ______________________________________

Preservative content (if applicable)
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ______________________________________

General safety
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ______________________________________

pH
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ______________________________________

Moisture content
Date tested: ________________________________
Method used: ________________________________
Specification: ________________________________
Result: ______________________________________

---

2 This applies only to lyophilized vaccines.
Osmolality
Date tested: 
Method used: 
Specification: 
Result: 

Control of diluent (if applicable)
Name and composition of diluent: 
Lot number: 
Date of filling: 
Type of diluent container: 
Appearance: 
Filling volume per container: 
Maximum duration approved for storage: 
Storage temperature and duration: 
Other specifications: 

CONTROL OF ADJUVANT

Summary of production details for the adjuvant
When an adjuvant suspension is provided to reconstitute a lyophilized vaccine, a summary of the production and control processes should be provided. The information provided and the tests performed depend on the adjuvant used.

Summary information for the adjuvant
Name and address of manufacturer: 
Nature of the adjuvant: 
Lot number: 
Date of manufacture: 
Expiry date: 

Tests on the adjuvant
Adjuvant content
Date tested: 
Method used: 
Specification: 
Result: 

---

3 This section is required only when an adjuvant is provided separately to reconstitute a lyophilized vaccine.
Annex 3

Appearance
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: ____________________________

Purity or impurity
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: ____________________________

pH
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: ____________________________

Pyrogenicity
Date tested: ____________________________
Method used: ____________________________
Specification: ____________________________
Result: ____________________________

Sterility
Method used: ____________________________
Media: ____________________________
Number of containers used: ____________________________
Date of inoculation: ____________________________
Date of end of test: ____________________________
Specification: ____________________________
Result: ____________________________

*A pyrogen test of the adjuvant is not needed if a pyrogen test was performed on the adjuvanted reconstituted vaccine.*
CERTIFICATION BY THE MANUFACTURER

Name of head of quality control (typed) ________________________________

Certification by the person from the control laboratory of the manufacturing company taking overall responsibility for the production and quality control of the vaccine

I certify that lot no. ______________________ of typhoid conjugate vaccine, whose number appears on the label of the final containers, meets all national requirements and/or satisfies Part A\(^5\) of WHO Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines (2014)\(^6\).

Signature __________________________________________________________
Name (typed) ______________________________________________________
Date ______________________________________________________________

10. Certification by the NRA

If the vaccine is to be exported, attach a Lot-release Certificate from the NRA (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

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\(^5\) With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

Appendix 2

Model NRA Lot-release Certificate for typhoid conjugate vaccines

Certificate no. __________________________

The following lot(s) of typhoid conjugate vaccine produced by _______________ _______________1 in _________________,2 whose numbers appear on the labels of the final containers, meet all national requirements3 and Part A4 of the WHO Guidelines on the quality, safety and efficacy of typhoid conjugate vaccines (2014)5 and complies with WHO Good manufacturing practices: main principles for pharmaceutical products;6 Good manufacturing practices for biological products;7 and Guidelines for independent lot release of vaccines by regulatory authorities.8

The release decision is based on _____________________________9

Final lot number: _____________________________
Number of human doses released in this final lot: _____________________________
Expiry date: _____________________________

The Director of the NRA (or other appropriate authority)

Name (typed) _____________________________
Signature _____________________________
Date _____________________________

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1 Name of manufacturer.
2 Country of origin.
3 If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.
4 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
9 Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.