Towards a sustainable, quality and affordable *Haemophilus influenzae* type b vaccine for every child in the world

The right to live

Ahd HAMIDI
Towards a sustainable, quality and affordable *Haemophilus influenzae* type b vaccine for every child in the world

The right to live

Proefschrift

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'Health is a crown that the healthy wear but only the ill perceive'

Arab proverb
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Acronyms, abbreviations, and definitions

ADH: adipic acid dihydrazide
AEC: anion exchange chromatography
B-cell: B-cell or B lymphocyte is a type of lymphocyte (white blood cell) that plays a role in antibody-mediated immunity
BCR: B-cell receptor
BE: Biological E. (vaccine manufacturer in Hyderabad, India)
BF: Bio Farma (vaccine manufacturer in Bandung, Indonesia)
BSA: bovine serum albumin
CAPEX: capital expenditure
COGs: cost of goods
CNBr: cyanogen bromide
DCVMN: Developing Countries Vaccine Manufacturers Network
DoE: design of experiment
DSC: differential scanning calorimetry
DT(w)P: Diphtheria, Tetanus, and (whole cell) Pertussis
EDC: N-ethyl-Ni-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDAC: 1-ethyl-3(3-dimethylaminopropyl)carbodiimide
ELISA: enzyme-linked immune sorbent assay
EP: European Pharmacopoeia
GAVI: GAVI Alliance (formerly The Global Alliance for Vaccines and Immunization)
Glovax: pharmaceutical company in Seoul (Korea)
GMP: Good Manufacturing Practice
GPC: gel permeation chromatography
HbO-HA: PRP oligosaccharide conjugated to human albumin carrier protein
HepB: Hepatitis B
Hib: \textit{Haemophilus influenzae} type b
1H-NMR: proton nuclear magnetic resonance
HPSEC: high performance size exclusion chromatography
HTS: high throughput screening
ICH: international conference on harmonization
IgG: immunoglobulin G
Intravacc: Institute for Translational Vaccinology
IPC: in process control
IPV: inactivated polio vaccine
kDa: Kilodalton
LAL: limulus amoebocyte lysate
LCB: Laboratory for Control of Biological Products at the Netherlands Vaccine Institute
MDG: Millennium Development Goals
$M_r$: relative molecular mass
$M_w$: weight-average molecular mass
$M_n$: number-average molecular mass
NAD: nicotinamide adenine dinucleotide
NIBSC: National Institute for Biological Standards and Control (UK)
NMR: nuclear magnetic resonance
NIH: National Institutes of Health (US)
NRA: national regulatory authority
NVI: Netherlands Vaccine Institute
OD: optical density
OECD: Organization for Economic Cooperation and Development
OFAT: one factor at a time
OPEX: operating expenditure
PAT: process analytical technology
PBS: phosphate buffered saline
PRP: polyribosylribitol phosphate (Hib capsular polysaccharide)
PRP-AH: ADH-modified PRP
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<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>PRP-D:</td>
<td>PRP conjugated to diphtheria toxoid</td>
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<td>PRP-HbOC:</td>
<td>PRP conjugated to the diphtheria toxin mutant (CRM197)</td>
</tr>
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<td>PRP-OMP:</td>
<td>PRP conjugated to the outer membrane protein of <em>Neisseria meningitidis</em> group</td>
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<td>PRP-T:</td>
<td>PRP conjugated to tetanus toxoid</td>
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<td>QbD:</td>
<td>quality by design</td>
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<td>QC:</td>
<td>quality control</td>
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<td>RI:</td>
<td>refractive index</td>
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<td>RIVM:</td>
<td>Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment, the Netherlands)</td>
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<td>R&amp;D:</td>
<td>research and development</td>
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<td>SEC:</td>
<td>size exclusion chromatography</td>
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<td>SIBP:</td>
<td>Shanghai Institute of Biological Products (a subsidiary of China National Biotech Group, National Pharmaceutical Group Corporation in Beijing, China)</td>
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<td>SII:</td>
<td>Serum Institute of India (vaccine manufacturer in Pune, India)</td>
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<td>SGPDP:</td>
<td>stage-gated product development process</td>
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<td>SPF:</td>
<td>specific pathogen free</td>
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<td>T-cell:</td>
<td>T-cell or T lymphocyte is a type of lymphocyte (white blood cell) that plays a role in antibody independent or cellular immunity</td>
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<td>TMB:</td>
<td>tetramethyl benzidine</td>
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<td>ToBI:</td>
<td>toxin binding inhibition test</td>
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<tr>
<td>$t_R$:</td>
<td>retention time</td>
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<td>TTd:</td>
<td>tetanus toxoid</td>
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<tr>
<td>UV:</td>
<td>ultraviolet</td>
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<td>WFI:</td>
<td>water for injection</td>
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<td>WHO:</td>
<td>World Health Organization</td>
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Chapter 1

Introduction
About *Haemophilus influenzae*

*Haemophilus influenzae* is a non-motile, small gram-negative coccobacillary facultative anaerobic bacterium (figure 1) that was found by Richard Pfeiffer in patients during an influenza outbreak in 1892 [1]. *Haemophilus influenzae* was therefore thought to cause influenza until 1933 when it became clear that influenza is caused by a virus and not by *Haemophilus influenzae* bacterium.

![Figure 1: Gram stain of *Haemophilus influenzae* type b bacteria.](image)

*Haemophilus influenzae* strains are divided into two groups: unencapsulated (without a capsular polysaccharide) and encapsulated. Unencapsulated strains do not react with typing antisera and are referred to as nontypeable while encapsulated strains are reactive. Virulence properties of capsulated *Haemophilus influenzae* are most probably related to the capsular polysaccharide structure, therefore unencapsulated strains are considered to be less virulent. This chapter will focus on encapsulated strains.
Six serotypes of encapsulated *Haemophilus influenzae* (a through f) were identified by Pittman [2], based on their antigenically distinct polysaccharide capsule, and the structures thereof [3]. *Haemophilus influenzae* requires special growth factors, hemin (factor X) and nicotinamide adenine dinucleotide (NAD, factor V). The differences in growth factors requirements of *Haemophilus* serotypes allow for their differentiation.

**Haemophilus influenzae** disease

Mucosal contact with *Haemophilus influenzae* is the initial step in the pathogenesis. Disease caused by *Haemophilus influenzae* (figure 2 [4]) usually begins in the upper respiratory tract and can be manifested as an invasive infection (e.g. pneumonia, meningitis and epiglottitis) or as a less serious infection (e.g. otitis media and sinusitis) [5].

*Figure 2: Tissues infected by Haemophilus influenzae strains [4].*

*Haemophilus influenzae* infection is transmitted by droplets from infected (but not necessarily symptomatic) humans. In general the *Haemophilus* bacteria enters the body directly through the nasopharynx and the blood capillaries. The polysaccharide capsule allows these organisms to resist phagocytosis and complement-mediated lysis in the host. The exact mode of invasion to the bloodstream is not totally known.
Most of *Haemophilus* infections caused worldwide are due to *Haemophilus influenzae* type b (Hib). The clinical manifestation of Hib infections can vary per country. Worldwide tracking of different clinical manifestations of *Haemophilus influenzae* type b depends strongly on the frequency and likelihood that blood cultures are collected from the patient and on the laboratory capacity of the country for successfully isolating the causative organism. In general the most common manifestation of Hib infection is pneumonia.

All infections caused by Hib are often referred to as “Hib-disease”. Hib-disease mostly affects young children, below 2 years, elderly and immunocompromised people. *Haemophilus influenzae* type b can be prevented by vaccination.

***Haemophilus influenzae* type b prevention**

*Haemophilus influenzae* type b (Hib) infection can be prevented by active immunization meaning vaccination. In the 1970s the first Hib vaccine, a purified Hib capsular polysaccharide vaccine (polyribosylribitol phosphate, PRP), was developed. Hib vaccine induces antibodies to PRP which is the most important antigen in acquiring protective immunity. The human immune response to PRP is T-cell independent meaning humoral antibody response is simulated in the absence of T-cells. B-cells provide the primary response without a contribution from T-helper cells. The B-cell receptor (BCR), a protein on the outer surface of the B-cell, can bind to the polysaccharide. Because of the inability to induce T-cell dependent memory, polysaccharide vaccines are poorly immunogenic in children under the age of 18 months. In 1985, the polysaccharide vaccine was licensed for use in children of 18 month or older as it was ineffective in infants at 3-17 months of age [6].

Conjugation (covalent linkage) of bacterial polysaccharides to proteins can induce protective immunity by enhancing the immunogenicity to the polysaccharide. Conjugation of PRP to a T-cell dependent protein antigens has resulted in a new generation of Hib vaccines starting from 1987, the protein antigen attached to the Hib polysaccharide allow T-cells to help polysaccharide-specific B cells (figure 3).
Currently three different Hib conjugate vaccines based on the native Hib polysaccharide (isolated from Hib bacteria) are registered for use in young infants starting from the age of 2-3 month: PRP-OMP (PRP conjugated to the outer membrane protein of Neisseria meningitidis group b), PRP-HbOC (PRP conjugated to diphtheria toxin mutant, CRM197) and PRP-T (PRP conjugated to tetanus toxoid). A forth conjugate vaccine, PRP-D (PRP conjugated to diphtheria toxoid) is selectively used for vaccination of unvaccinated children aged 15-59 months or as a booster vaccine after the primary infant vaccination is completed. This is due to the limited immunogenicity of this vaccine and the variable protective efficacy in infants younger than 12-15 months. The four Hib conjugate vaccines differ by protein carrier, method of conjugation and polysaccharide size [7]. All these Hib conjugate vaccines have been subject to clinical trials [8]. A serological correlate of protection was demonstrated in these trials [9]. A polysaccharide antibody concentration of >0.15 µg/ml is considered to be a serological marker for short-term protection and an antibody concentration of ≥1.0 µg/ml as a marker for long-term
protection (1 month after the completion of primary immunization). In 2004, a fifth Hib conjugate vaccine composed of a fully synthetic PRP, conjugated to tetanus toxoid, was introduced [10]. Hib conjugate vaccines are available both as standalone vaccines as well as in combination with DTP (diphtheria, tetanus and pertussis), DTP-Hepatitis B and/or IPV (Inactivated Polio Vaccine).

Use of *Haemophilus influenzae* type b vaccines

Hib conjugate vaccines were widely introduced in high-income countries shortly after licensure. Because of the lack of information on Hib-disease burden, high cost, and limited vaccine supply many low- and middle-income countries didn’t introduce Hib vaccines in their national immunization programs for many years. In 1997, more than one decade after Hib vaccines had been widely introduced in high-income countries only one low-income country (Gambia) had included Hib vaccine in its national immunization program. The vaccine was donated by a manufacturer [11]. Consequently, in 2000 Hib still caused about 400,000 deaths among children under 5 years of age and on an average about eight million of serious illnesses worldwide [12]. A large number of survivors of Hib-disease are left with permanent disabilities such as mental retardation or deafness.

In 2000, a new international initiative, the Global Alliance for Vaccines and Immunization (GAVI) was launched, aiming at delivering underused vaccines, including Hib, to the poorest children in the world. In 2004, only few low- and middle-income countries had introduced Hib vaccines. It’s only after 2005 [13], when certain favorable conditions were created, that a significant number of low- and middle-income countries introduced Hib vaccines. In figure 4 the progress made between 1997 and 2013 regarding the introduction of Hib vaccine in national immunization programs is shown.
The main favorable condition for the introduction of Hib vaccine in low- and middle-income countries was the entrance of new manufactures into the market, decreasing concerns about limited supply of the vaccines and increasing the chances of a price reduction. The Hib vaccine market was dominated for over 20 years by vaccine manufacturers located in high-income countries. However, since 2004 emerging vaccine manufacturers in low- and middle-income countries succeeded in licensing several Hib vaccines. A synthetic Hib vaccine, developed by Center for Genetic Engineering and Biotechnology in Cuba, was introduced in Cuba’s national immunization program in 2004. In 2008, Serum Institute of India (SII, India) got its first Hib vaccine prequalified and Biological E limited (BE, India) in 2011. Another favorable condition for the introduction of Hib vaccine was the WHO recommendation in 2006 for global use of this vaccine, based on multiple burden assessment studies. In addition, GAVI played a very critical role in creating the
proper market conditions leading to a high demand of this vaccine. The weighted average price GAVI paid for a pentavalent vaccine including a Hib component (DTP-HepB-Hib) has been stable for years at about US$ 3.56 a dose (figure 5). In 2011, SII offered to sell liquid pentavalent vaccine including Hib at a price of US$ 1.75 a dose and in 2013 BE decided to reduce the price of this vaccine to US$ 1.19 a dose [15-19]. Hib is the most expensive of all five antigens.

![Figure 5: GAVI price for pentavalent vaccine including Hib [15-17], number of manufacturers and price decline achieved.](image)

Hib vaccination has resulted in a decrease of Hib-disease incidence in the countries where the vaccine has been introduced in the national immunization programs [20-25]. For example in Gambia it was seen that during 2008-2010 (10-12 years after the introduction of the vaccine), the annual incidences of Hib meningitis and all invasive Hib-disease in children below 5 years was less than 5 cases per 100,000 children.
Thesis outline

The first *Haemophilus influenzae* type b (Hib) conjugate vaccine was licensed in 1987. Introduction of this vaccine in high-income countries was fast. By 1998, 50% of these countries had introduced the vaccine into their national immunization programs while only one low-income country (Gambia) had used this vaccine in its national immunization program. The introduction of Hib conjugate vaccines led to a dramatic decrease of the incidence of Hib-disease. The introduction of Hib vaccine into national immunization programs of low-and middle-income countries was mainly hampered by lack of information, high price and shortage of the vaccine. Furthermore, most of the vaccine manufacturers in these countries had no access to conjugation technology. The aims of this thesis were: 1) to develop a Hib conjugate vaccine production process, without infringing on any existing patents or intellectual properties making sure that the process is transferable and can lead to the production of an affordable Hib vaccine, 2) to develop quality control tests, 3) to transfer the Hib production process, related quality control tests and materials to a number of emerging manufacturers and make sure that the process is successfully implemented, 4) to generate enough (pre)clinical data and share the data with all partners and 5) to identify possibilities for process optimization by using a mathematical modeling tool.

The National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM, in Bilthoven, The Netherlands) had extensive experience with several conjugate vaccines [26-29] and with technology transfer in general. In line with this tradition, RIVM\(^1\) decided to develop a Hib conjugate vaccine and transfer the technology to a number of emerging vaccine manufacturers in low- and middle-income countries (chapter 2), to enable them to ensure a sustainable supply of a quality and affordable Hib conjugate vaccine to their national immunization programs and to UNICEF/ GAVI. In this way, the Hib project would contribute to the United Nations Millennium Development Goals (MDGs) and to GAVI’s goal in reducing

\(^1\) RIVM delegated essential vaccine tasks to the Netherlands Vaccine Institute (NVI) between 2003 and 2010. In 2013, a large part of the Research and Development Unit of RIVM and NVI including all technology transfer activities and the Animal Research Centre were housed in Intravacc (Institute for Translational Vaccinology).
child mortality by increasing access to underused vaccines. In chapter 2 an overall overview of the Hib-project is given including the main timelines and deliverables. In 2001, three years after the Hib project had started at RIVM, a process for the production of a Hib conjugate vaccine (including related quality control tests) was developed and technology transfer was started. The process developed was based on RIVM’s (later NVI and now Intravacc) prior knowledge (chapter 3). The targeted and integrated development approach followed for this new Hib conjugate vaccine was regulatory and partner driven, defining the target product profile and quality attributes at the start of the project in close collaboration with the partner(s) and/or national regulatory authorities. The development approach followed is explained in chapter 3. Further, in chapter 3 a more advanced approach (using mathematical modeling) is described. Recently, the Hib process was used to test the suitability of this approach for vaccine development. Therefore Intravacc’s process for the production of Hib conjugate vaccine was simulated using SuperPro Designer, the Hib model developed using SuperPro was very predictive. This model was used to identify potential process improvements which may lead to further reduction in the price of the vaccine. Further, quality control tests were developed by RIVM and transferred to the technology transfer partners. Besides, physicochemical tests needed for the product release additional immunological methods were developed in order to facilitate the process development (chapter 4). Three of the ELISA’s (enzyme-linked immunosorbent assays) that have been very valuable during the process development, are discussed in chapter 4. These are the PRP-ELISA, the antigenicity ELISA and the anti-PRP IgG ELISA (developed as part of the mice immunogenicity test). These ELISA methods were used during process development, process implementation, scaling up and for lot release purposes after proper qualification and validation. As part of the Hib project the technology transfer partners received materials (including the seed lot and reference samples), all relevant documentation on the process and quality control tests, and all (pre)clinical data generated bij RIVM. RIVM produced a (pre)clinical lot together with the first partner (Bio Farma, Indonesia) and tested this lot in a preclinical study as discussed in chapter 5. The partners were
able to use the preclinical data to support their clinical dossier. The (pre)clinical lot was later tested in a phase 1 clinical study by Bio Farma in close collaboration with RIVM.

Ultimately the Hib knowhow was successfully transferred to four manufacturers: Bio Farma (Indonesia), Biological E. limited (BE, India), Serum Institute of India (SII, India) and Shanghai Institute of Biological Products (SIBP, China). Technology transfer to SIBP was facilitated by Glovax (Korea). Two of these manufacturers, SII and BE, are now playing a very important role by producing large volumes of affordable Hib conjugate vaccines both standalone and in combination with other antigens. Although the price of the pentavalent vaccine (DTP-HepB-Hib) was stable for many years, this price dropped with 66% thanks to two of Intravacc’s Hib-partners (Serum Institute of India and Biological E.). In 2013, a pentavalent vaccine was made available by BE to GAVI for US$ 1.19 per dose compared to US$ 3.56 per dose. In chapter 6 all lessons learned during the Hib project are summarized and the project approach used is explained.

Before starting with the Hib project the expected cost of goods was calculated as part of the project feasibility study (chapter 7) using Excel. Recently, the cost of goods was recalculated using mathematical modeling tools (SuperPro Designer). COGs calculated using both methods was in the same range and was found to be realistic taking the present sales price into account. The advantages of using a mathematical model are discussed in chapter 7. Having a mathematical model available allows the performance of a sensitivity analysis to determine the impact of certain changes on for example COGs.

In chapter 8 a general discussion is given. The approach followed during the process development and technology transfer was very successful and can be useful for other technology transfer projects. The Hib project did contribute in assuring the availability of affordable quality Hib conjugate vaccines and thereby in increasing the introduction of Hib vaccines into national immunization programs of low- and middle-income countries. Consequently the Hib project contributed to the United Nations Millennium Development Goals (MDGs) in reducing child mortality. Further, the use of a rational design approach (for example mathematical modeling tools) can give better insight in the possibilities to achieve cost price reduction, process understanding and/or process
optimization. It’s advisable to use this rational approach during vaccine development. Modeling tools can also be very useful during the manufacturing phase. These tools can for example be used to reduce batch cycle times and to debottleneck an existing facility.
Development and technology transfer of Haemophilus influenzae type b conjugate vaccines for developing countries

Michel Beurrett
Ahd Hamidi
Hans Kreeftenberg

Vaccine 2012; 30: 4897-4906
Abstract

This paper describes the development of a *Haemophilus influenzae* type b (Hib) conjugate vaccine at the National Institute for Public Health and the Environment/Netherlands Vaccine Institute (RIVM/NVI, Bilthoven, The Netherlands), and the subsequent transfer of its production process to manufacturers in developing countries. In 1998, at the outset of the project, the majority of the world’s children were not immunized against Hib because of the high price and limited supply of the conjugate vaccines, due partly to the fact that local manufacturers in developing countries did not master the Hib conjugate production technology.

To address this problem, the RIVM/NVI has developed a robust Hib conjugate vaccine production process based on a proven model, and transferred this technology to several partners in India, Indonesia, Korea and China. As a result, emerging manufacturers in developing countries acquired modern technologies previously unavailable to them. This has in turn facilitated their approach to producing other conjugate vaccines.

As an additional spin-off from the project, a World Health Organization (WHO) Hib quality control (QC) course was designed and conducted at the RIVM/NVI, resulting in an increased regulatory capacity for conjugate vaccines in developing countries at the National Regulatory Authority (NRA) level. For the local populations, this has translated into an increased and sustainable supply of affordable Hib conjugate-containing combination vaccines. During the course of this project, developing countries have demonstrated their ability to produce large quantities of high-quality modern vaccines after a successful transfer of the technology.
Introduction

The National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM, Bilthoven, The Netherlands), which, in the period 2003–2010, delegated essential vaccine missions to the Netherlands Vaccine Institute (Nederlands Vaccine Instituut, NVI), has a long history in the development and transfer of vaccine technology to vaccine manufacturers in developing and developed countries [30]. The transfer of fermentor-based DTwP (Diphtheria-Tetanus-[whole cell] Pertussis) and BCG technology, as well as the Vero cell-based micro-carrier technology for inactivated polio vaccine (IPV) [31], are worth mentioning. Usually, the transfer of technology occurred in bilateral collaborations and during courses organized by the RIVM/NVI, under the auspices of the World Health Organization (WHO) Global Learning Opportunities for Vaccine Quality (GLO/VQ), or in collaboration with the Developing Countries Vaccine Manufacturers Network (DCVMN).

Since 1997, the WHO has recommended inclusion of the *Haemophilus influenzae* type b (Hib) conjugate vaccine in national immunization programs, wherever resources are available and disease burden data show that this is a priority [32-36]. Despite this recommendation, the majority of children in developing countries had no access to the Hib conjugate vaccine at that time because of insufficient data on disease burden [37] and vaccine impact, lack of awareness, and focus on other priorities [38]. In addition, there was also only one supplier of Hib conjugate vaccine to the United Nations International Children’s Emergency Fund (UNICEF) in those days [39], and for most countries, the price of a three-dose regimen of Hib conjugate vaccine far exceeded the combined price of all other vaccines in the routine infant immunization schedule defined by the WHO Expanded Program on Immunization (EPI). Due to the lack of competition, the price of the vaccine remained almost the same over many years [39].

Vaccine manufacturers in developing countries did not have access to Hib conjugate technology due to patents and proprietary know-how [40,41], and in the absence of reliable market forecast, were hesitant to invest in the development of such vaccines. Furthermore, vaccine manufacturers in industrialized countries began phasing out the
production of their traditional, less expensive, DTwP vaccines. Up to this point, these were made available for low prices to UNICEF for use in developing countries.

To address this problem and pursue its historical role as a vaccine technology transfer center, the RIVM/NVI launched a project in 1998 to develop an up-scalable, commercially viable, Hib conjugate vaccine production process, without infringing on any existing patents, and by using technology easily transferable to vaccine manufacturers in developing countries. Additional support for the development of combination vaccines that include a Hib conjugate component meant that these manufacturers, by gaining access to Hib conjugate vaccine technology, could ensure a sustainable supply of affordable and quality vaccines in the future. With this project, the RIVM/NVI aimed to contribute to the United Nations Millennium Development Goals (MDG), particularly MDG 4 (“Reduce by two-thirds, between 1990 and 2015, the under-five mortality rate”), and support the GAVI Alliance, the DCVMN [42] and the WHO [35,43] in their objectives to introduce Hib conjugate vaccines worldwide.

The RIVM/NVI Hib vaccine project and partnerships

At the inception of the project, four different Hib conjugate vaccines with an excellent safety record had been used since the 1980s in industrialized countries [44, 45]. A diphtheria toxoid conjugate (PRP-D) was the first one introduced [46], soon followed by a mutant-diphtheria-toxin conjugate (PRP-CRM) [47], a meningococcal outer-membrane protein conjugate (PRP-OMP) [48], and finally a tetanus toxoid (TTd) conjugate (PRP-T) [49]. Later on, a new type of conjugate vaccine in which the Hib saccharide antigen is prepared by chemical synthesis has been introduced [50]. Further improvement of existing conjugation methods also ensures that Hib vaccines are optimized through more rigorous methodology [51, 52].

The RIVM/NVI opted for a lyophilized PRP-T vaccine, based on the conjugation method originally described by John Robbins and collaborators (National Institutes of Health, Bethesda, USA) [53-55]. This unpatented technology had a proven track record, as similar
vaccines had already been used for many years in national immunization programs. Availability of TTd in developing countries through local production was an additional reason to favor a PRP-T vaccine. Since lot-release specifications are product-specific and were already established for existing technologies like PRP-T [56, 57], lengthy validation of lot-release criteria in extensive clinical trials could be avoided. Also, this technology bypassed ethical issues to justify clinical evaluation of a Hib conjugate vaccine based on a new and unknown conjugation technology in the presence of highly safe and effective licensed Hib vaccines.

The ultimate objective of this project was to make a tetra- or pentavalent combination vaccine that could be administered in one injection, by reconstituting the lyophilized Hib conjugate with existing vaccines, such as DTwP or DTwP-HepB (DTwP with hepatitis B). This approach secured optimal stability of the Hib component and simplified quality control (QC) testing, and would allow a dose reduction study by reconstituting the Hib component in a larger volume of DTwP-HepB vaccine. In anticipation of inferior stability, a liquid monovalent Hib vaccine was considered incompatible with this goal. It is worth noting that official tenders from international organizations, as well as local markets, initially called for a lyophilized-liquid presentation, and then evolved toward a fully liquid presentation. In time, our partners decided to make this necessary transition, while continuing their efforts with the initial presentation. There was also a discussion over the possibility of cutting cost by lowering the dose, while at the same time improving the yield during production. Our partners have mostly investigated the second idea, and decided to keep a well-established dosage (10 µg) [56, 57], although other manufacturers have demonstrated that a lower dose (e.g. 2.5 µg) could afford a protective immune response [58-60].

Bio Farma (BF, Bandung, Indonesia), a state-owned vaccine manufacturer, was the first potential partner interested in the RIVM/NVI Hib project. Soon after preliminary investigations, the RIVM/NVI and BF discussed a project plan, including possible technical or regulatory complications. These involved strain choice, the need to avoid using media components of animal origin to reduce Bovine Spongiform Encephalopathy (BSE)-related regulatory problems, the presentation form, and discussion of regulatory aspects with the Indonesian National Regulatory Authority (NRA).
An effective communication system was set up between the two project teams at BF and the RIVM/NVI for managerial purposes and to avoid unnecessary delays. Project-planning tools were implemented to coordinate the various activities and to monitor the critical paths. In addition, a training program was organized at the RIVM/NVI for BF technical staff, as well as at BF’s facilities in Indonesia.

By mid-2001, a pilot scale process that was commercially viable and readily transferable to manufacturers in developing countries had been established. During that period, all essential release and in-process QC tests relevant to the process had also been developed. Meanwhile, the project attracted more partners from both the public and private sector. By the end of 2001, the RIVM/NVI had signed cooperation agreements with BF and Biological E. (BE, Hyderabad, India) and, in 2002, with Serum Institute of India (SII, Pune, India). In 2004, these were formalized into final license agreements (see Table 1). In 2006, a license agreement was signed with Glovax (Seoul, Korea), in partnership with the Shanghai Institute for Biological Products (SIBP, Shanghai, China) for production of the vaccine. With each partner, technology transfer and license rights agreements were arranged to cover cost incurred by the RIVM/NVI during the development and transfer of the Hib technology.

Early 2002, the partners received further training at the RIVM/NVI in all aspects of the developed technology, and they invested in facilities and equipment to produce Hib conjugate vaccines. Moreover, all documentation on the production process and QC tests was made available to the partners. In the meantime, the RIVM/NVI continued to produce experimental conjugate batches, and BF personnel spent several months at the RIVM/NVI to help in this endeavor. In 2004, the technology transfer of the Hib process to BF was successfully completed, and the RIVM/NVI Hib production process and related QC testing were implemented at their manufacturing facility in Bandung. In December 2005, both BE and SII in India had all the process steps and related QC testing running, and in 2007 SIBP had also successfully implemented the process in China (see Table 1).
**Table 1.** Chronology of the Hib vaccine technology transfer project.

<table>
<thead>
<tr>
<th>Date</th>
<th>Milestone</th>
<th>Partner</th>
<th>Financing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>Start Hib project at RIVM: project plan and set-up of R&amp;D team</td>
<td>RIVM</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>BF R&amp;D team visit to RIVM: basic process and QC training</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seedlot production under GMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRP production under GMP (commercial scale; process consistency)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>Conjugation process established (pilot-scale)</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cooperation agreement BF-RIVM</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tripartite cooperation agreement BE-BF-RIVM</td>
<td>BE &amp; BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Training BF and BE on-site (PRP production)</td>
<td>BE &amp; BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Production of clinical lots (PRP and TTd production at BF; conjugation at RIVM)</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cooperation agreement SII-RIVM</td>
<td>SII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basic process and QC training</td>
<td>SII &amp; BE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transfer of Hib project from RIVM to NVI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>Publication of seedlot report, brochure for clinical investigators, residual reagents report, and requirements for Hib vaccine</td>
<td>TNO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toxicology study and report</td>
<td>BF, SII &amp; BE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Follow-up on-site training</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phase 1 clinical trial in adults (monovalent Hib, lyophilized)</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>Technology transfer completed</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phase 1 clinical trial in adults (monovalent Hib, lyophilized)</td>
<td>SII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phase 1 &amp; 2 clinical trials in infants (pentavalent DTwP-HepB-Hib, lyophilized)</td>
<td>BF</td>
<td></td>
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<tr>
<td></td>
<td>PCT patent application filing</td>
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<tr>
<td></td>
<td>License agreements (technical services, licensing fees and royalties)</td>
<td>BF, SII &amp; BE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>License agreement (technical services, licensing fees and royalties)</td>
<td>SII &amp; BE</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Technology transfer completed</td>
<td>SII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>License agreement (technical services, licensing fees and royalties)</td>
<td>Glovax</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basic process and QC training</td>
<td>Glovax &amp; SIBP</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Follow-up on-site training; technology transfer completed</td>
<td>SII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indian market license for monovalent Hib-lyo.</td>
<td>DCVMN &amp; NRAs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hib QC course</td>
<td>WHO</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>Hib QC course (twice)</td>
<td>SII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WHO prequalification for monovalent Hib-lyo.</td>
<td>DCVMN &amp; NRAs</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>Patent publication: USA [32], India</td>
<td>SII</td>
<td></td>
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<tr>
<td></td>
<td>Indian market license for pentavalent DTwP-HepB-Hib fully liquid</td>
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<tr>
<td>2010</td>
<td>Contract termination</td>
<td>BF</td>
<td></td>
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<tr>
<td></td>
<td>Patent publication: Europe [33], Indonesia, Japan</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>WHO prequalification for Hib combination vaccines (DTwP-Hib-lyo., DTwP-HepB-Hib-lyo. &amp; DTwP-HepB-Hib fully liquid)</td>
<td>SII</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Transfer of Hib project from NVI to RIVM</td>
<td>SII &amp; GAVI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Price reduction agreement</td>
<td>BE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WHO prequalification for DTwP-HepB-Hib-lyo.</td>
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</tbody>
</table>

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* RIVM/NVI provided an initial seed capital of ~€ 0.7 M.
* A general memorandum of understanding (MoU) was signed between RIVM and BF in 1993.
* During the period 1998–2011, the combined financial contribution of the four partners amounted to ~€ 3.5 M. This has covered a large part of all personnel, supplies and investment costs incurred by RIVM/NVI.
* Subcontractor.
* Glovax signed a separate agreement with SIBP for the manufacturing of Hib vaccine.
Process and analytical techniques

A suitable Hib strain was selected (A760705; Bacterial Meningitis Reference Laboratory, Academic Medical Center, Amsterdam), isolated in 1976 in the Netherlands from a two-year-old patient. This strain was fully characterized and had a traceable history. A seedlot system was established, and several ampoules of the master seedlot were then sent to each partner. The RIVM/NVI then set up Hib cultivation under Good Manufacturing Practice (GMP) conditions at a working volume of 350 L.

The process developed for the purification of the Hib polysaccharide (polyribosylribitol phosphate, PRP) was relatively simple and resulted in a high product yield. Extraction of PRP from the concentrated culture supernatant gave on average 60 g of pure PRP. Three consecutive PRP lots, prepared under GMP conditions from the same 350 L culture supernatant, consistently satisfied all requirements from the European Pharmacopeia (EP) and the WHO [56, 57], in particular with protein (0.33%) and nucleic acid (0.75%) contaminants well below the set specification (<1%). Purified PRP was unambiguously identified by proton nuclear magnetic resonance (1H NMR), by comparison with published reference spectra [63, 64]. The entire PRP purification process was eventually patented by the RIVM/NVI in order to protect its partners [61, 62].

The production process was divided into several steps to provide a smooth operation with acceptable yields. In this respect, we investigated storage conditions of the concentrated Hib culture supernatant and of the purified PRP. The choice of storage conditions (4 °C in a neutral buffer) for concentrated and purified Hib conjugate bulk allowed for maximum flexibility in timing of the conjugation and lyophilization steps.

In the selected conjugation method [53-55], the PRP is first modified with cyanogen bromide in alkaline conditions, and afterward immediately modified with an adipic acid dihydrazide (ADH) linker. Carboxylic groups on the protein carrier are activated in-situ with N-ethyl-Ni-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), which allows covalent coupling with the hydrazide groups present on the ADH-modified PRP (PRP-AH) to occur.
Early on, we observed that long exposure of PRP to alkaline conditions during the modification reaction produced inconsistent results, due to the hydrolysis of the polysaccharide. The process was thus amended to allow for a pre-treatment in alkaline buffer before adding the activation and modification reagents. In these conditions, the depolymerization of PRP reached a stable point, which afforded a PRP-AH product of consistent relative molecular mass (Mr 200-250 kDa) [51]. Finally, a size exclusion chromatography (SEC) purification step was used to separate the conjugate from non-conjugated PRP and TTd, as described in the original method [53].

To lower the hurdles intrinsically linked to the adoption of novel technology, a conscious effort was made to select simple and relatively inexpensive test methods and production processes. The use of expensive and technically challenging techniques (e.g. NMR) was limited to the validation of a few key process steps at the RIVM/NVI, in particular to demonstrate the absence of residual reagents and contaminants. Figure 6 gives an overview of the analytical techniques used for the monitoring of the consecutive process steps, as well as the analysis of the various intermediate and final products. A number of analytical methods were specifically developed for this project: among others, a PRP ELISA for the quantification of PRP in process samples (data not shown), and a set of different antigenicity ELISAs for the characterization of the conjugate (see figure 7). With the latter assay, different combinations of coating antibodies (either burro anti-Hib or horse anti-TTd) and of detection conjugates (peroxidase-labeled anti-Hib or anti-TTd) were used to ascertain the covalent nature of the Hib conjugates tested. Anti-Hib monoclonal antibodies were not available and the assay had to rely on the use of a unique non-renewable product (burro anti-Hib serum, gift of Dr. John Robbins, NIH, USA). As expected, a control made of a simple mixture of PRP and TTd did not react positively when coating and detection reagents were directed against different antigens. Based on the standard ELISA immunogenicity test for human sera [65], an assay for mouse sera was also developed (see figure 8). The response of individual mice to the PRP-T conjugate was widespread (the number of positive responders varied considerably),
as was also observed with commercially available PRP-T vaccines used as positive controls. As expected, unconjugated PRP gave a negative response.

**Figure 6.** Products, process steps and related analytical techniques. Various assays were routinely used to determine the composition of PRP, PRP-AH and PRP-T, and to verify that the intermediate and final products complied with EP and WHO recommendations for PRP-T specifications [27, 28]. Technical details for some of these analytical techniques are given in the next figures. 

- **Seedlot**
  - cultivation: molecular mass ($M_\text{r}$) - PRP - protein - nucleic acid
  - purification: $M_\text{r}$ - PRP - protein - nucleic acid

- **PRP**
  - modification: $M_\text{r}$ - modification monitoring
  - purification: $M_\text{r}$ - residual ADH

- **PRP-AH**
  - conjugation: $M_\text{r}$ - conjugation monitoring - residual TTD
  - purification: PRP - protein

- **PRP-T**
  - formulation: sterility
  - lyophilization: lyophilization monitoring

- **Vaccine**
  - $M_\text{r}$ - PRP - free PRP - glass transition - residual moisture - sterility

Legend:
- **a** HPSEC (see Figures 4 and 5).
- **b** PRP-containing samples hydrolyzed in alkali and PRP RU quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [36].
- **c** Coomassie colorimetric assay for contaminating protein (dye-binding method) [37].
- **d** UV scan test for contaminating nucleic acids.
- **e** Orcinol colorimetric assay for ribose (part of PRP RU) [38].
- **f** Phosphomolybdic acid colorimetric assay for organic phosphate (part of PRP RU) [39].
- **g** Folin reagent colorimetric assay for contaminating protein [40].
- **h** LAL assay for contaminating lipopolysaccharide (endotoxin).
- **i** H-NMR spectrum of purified PRP compared to published data [34, 35].
- **j** TNBS colorimetric assay for hydrazide groups (ADH) [41, 42].
- **k** BCA colorimetric assay for bound protein in PRP-T [43].
- **l** Sodium deoxycholate (DOC) precipitation of PRP-T, then assay for unconjugated PRP-AH in PRP-T [44].
- **m** Lyophilized vaccine buffer-exchanged (10 kDa filters) to remove sucrose.
- **n** Glass transition temperature ($T_c$) measured by DSC (lyophilized product).
- **o** Coulometric assay for residual moisture (lyophilized product).
**Figure 7.** Antigenicity ELISAs.  
Antigenicity was measured by ELISA with different combinations of antisera (burro anti-Hib, gift of Dr. John Robbins, NIH, USA, and horse anti-TTd, from RIVM/NVI) used alternatively as coating antigen or detection antigen. BAH: burro anti-Hib; HAT: horse anti-TTd, BAH~PO: BAH conjugated to horseradish peroxidase; HAT~PO: HAT conjugated to horseradish peroxidase. PRP+TTd (- - - light blue): control mixture of unconjugated PRP and TTd in the same ratio as expected in the final conjugate. Ref1 (— - - pink) and 2 (— - green): positive controls (commercially available PRP-T vaccines). The liquid vaccine bulk (clinical batch 5, — dark blue) was tested in duplicate.  
a. BAH coating and HAT~PO detection: only PRP-T conjugates can bind and be detected. PRP+TTd gives a negative response.  
b. BAH coating and BAH~PO detection: the antigenicity of conjugated PRP is similar to that of the original (unconjugated) PRP. PRP+TTd gives a positive response.

**Figure 8.** Mouse immunogenicity ELISA.  
Randomized groups of ten female NIH mice were immunized with 1 µg PRP, which corresponds to 1/10 of the human dose. Two doses were given subcutaneously at 2 week interval (at wk 0 and wk 2) and blood samples were taken 2 weeks after the last immunization (at wk 4). Immunogenicity of individual mouse sera was measured by ELISA [45] using HbO-HA (PRP oligosaccharide conjugated to human albumin; supplied by the National Institute for Biological Standards and Control, NIBSC, UK) as coating reagent. Detection of anti-Hib antibodies was done with biotinylated goat anti-mouse-IgG, followed by a streptavidin-peroxidase conjugate.  
a. Immunization with PRP (not conjugated): no positive responders.  
b. Immunization with PRP-T (experimental batch 51A): 5 out of 10 mice were identified as positive responders, as was also observed on average with commercially available PRP-T vaccines (data not shown).
A panel of physicochemical assays was also used throughout to monitor the process and to characterize products. Various standard colorimetric assays [67-74] were routinely used to determine the composition of PRP, PRP-AH and PRP-T, and to verify compliance with our specifications (PRP-AH: 1 mol ADH per 20-50 RU; PRP-T: PRP/protein ratio 0.3–0.6, w/w) and the EP and WHO recommendations for PRP-T [56, 57]. Raw and purified PRP was analyzed by high performance size exclusion chromatography (HPSEC), simultaneously allowing the determination of Mr, polymer heterogeneity, and the detection of contaminants, such as proteins and nucleic acids (see figure 9).

**Figure 9.** HPSEC analysis of PRP. HPSEC analysis was carried out on two OHpak SB-805 and SB-804 columns (300 ´ 8 mm, with the addition of an SB-G guard column, 50 ´ 6 mm; Shodex) mounted in series, with 10 mM phosphate-buffered saline (PBS, pH 7-2) as eluent, at 1 mL/min and 35°C. Elution was monitored with a RI and a multiple-wavelength UV detector. The Mr value of the polysaccharides was determined by reference to the retention times (t_R) of defined pullulan standards (M_w 5·8–853 kDa, M_w/M_n ~1·1). The elution limits of the column set were determined by using high Mr dextran (M_r 5–40 ´ 10^6 kDa) and deuterium oxide (D_2O). Detection of PRP was done by measurement of the RI signal (— black). Impurities (proteins and nucleic acids) were detected by UV (215 nm, — - - blue; 260 nm, — - pink; 280 nm, — - - brown) and RI (— black).

a. Raw PRP (experimental batch 38) at the start of the purification process: large amounts of proteins and nucleic acids are clearly visible on the UV profiles.

b. Pure PRP (experimental batch 38, t_R 14·35 min, M_r 664 kDa): low residual amounts of proteins and nucleic acids are still visible (t_R <12·5 min and >17 min) on the UV profiles. Low-mass impurities (t_R >17 min) were further removed during ultrafiltration of PRP-AH (see Figure 5).
This method proved to be more sensitive than the standard UV test, especially for the detection of high-Mr nucleic acids, which had to be almost completely eliminated during purification of PRP to prevent undesirable effects during conjugation. The same HPSEC conditions were used for the real-time monitoring of the modification and the conjugation steps (see figure 10). In both cases, the remaining amount of residual reagents (free ADH), or unconjugated antigen (free TTd), was calculated and used to determine the end point (≤5% residual compound) of these two reactions.

**Figure 10.** HPSEC monitoring of the conjugation process.

HPSEC was carried out as explained previously (see Figure 4). Samples were injected at regular intervals to monitor progress of the reactions.

a Detection of the ADH-modified PRP (PRP-AH; clinical batch 10) was done by UV (215 nm) and RI (not shown): the presence of hydrazide groups on PRP-AH (t_R ~15 min, Mr 215 kDa) made the polysaccharide visible by UV (modification reaction mixture, — - - blue; after one cycle of ultrafiltration, — - pink; purified PRP-AH, - - - brown), while the original PRP (data not shown) and the CNBr-activated PRP (— black) essentially showed no UV absorbance (except for their impurities). Free ADH (t_R ~22 min; ≤5% in the purified product, calculated as the ratio of the areas of the free ADH peak versus the PRP-AH peak), and low-mass impurities (t_R >17 min), were removed during ultrafiltration of PRP-AH.

b Detection of the conjugate (PRP-T; experimental batch 52) was done at various time points by UV (280 nm; t_0 — black; 30 min, — - - blue; 60 min, — - pink; 120 min, - - - brown; end at 280 min, . . . green) and RI (not shown): the gradual disappearance of uncoupled TTd (t_R ~17.5 min; ≤5% at the end of the conjugation, calculated as the ratio of the area of the free TTd peak versus the total area of all peaks) occurred simultaneously with the formation of conjugate (t_R 13–14 min, final Mr 1,411 kDa). Unconjugated PRP-AH (t_R ~15 min) was not visible as a distinct peak, even at its optimal wavelength (215 nm).

The first clinical lots of lyophilized Hib conjugate vaccine were jointly produced by BF and RIVM/NVI. They were utilized to perform different stability studies and to demonstrate manufacturing consistency of consecutive batches. Both intermediate and final lyophilized products
Hib vaccines for developing countries were shown to be very stable at 4 °C: up to 24 weeks, or 3 years, respectively. In particular, the amount of free PRP stayed well below the set specification (≤20% of total PRP) throughout the entire study.

Table 2. Stability of liquid bulk and lyophilized clinical lot.

<table>
<thead>
<tr>
<th></th>
<th>Liquid bulk (4°C)</th>
<th>Lyophilized (4°C)</th>
<th>Lyophilized (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;0&lt;/sub&gt;</td>
<td>24 wk</td>
<td>t&lt;sub&gt;0&lt;/sub&gt;</td>
<td>36 mo.</td>
</tr>
<tr>
<td>Molecular mass (M)&lt;sup&gt;r&lt;/sup&gt; (kDa)</td>
<td>1,456</td>
<td>1,291</td>
<td>1,315</td>
</tr>
<tr>
<td>Peak sharpness&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400</td>
<td>509</td>
<td>439</td>
</tr>
<tr>
<td>Free PRP&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>5·2</td>
<td>4·6</td>
<td>12·8</td>
</tr>
<tr>
<td>T&lt;sub&gt;G&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (°C)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>57</td>
</tr>
</tbody>
</table>

The vaccine liquid bulk (clinical batch 5) was stored at 4°C, and tested at various time points. Visual inspection indicated that the product remained clear. The product passed bio burden, immunogenicity, and toxicity tests (at t<sub>0</sub> and 24 weeks), as well as the antigenicity test (at t<sub>0</sub> and 4, 12 and 24 weeks). Specific toxicity and reversion to toxicity (tested at 4°C and 37°C) of TTd were measured by standard tests (EP). Antigenicity of the conjugate (see Figure 2), as well as PRP immunogenicity of mouse sera (see Figure 3), were measured as previously described. TTd immunogenicity of mouse sera was measured with the standard toxin-binding inhibition test (ToBI) test (EP). Endotoxin (2·98 IU/µg) was measured at t<sub>0</sub> by the Limulus amoebocyte lysate (LAL) test (EP), and was well below the set limit (<10 IU/µg PRP) [27].

The lyophilized clinical lot (batch FPH012) was prepared from a formulation (addition of Tris buffer, and sucrose as bulking agent) of the liquid vaccine bulk (clinical batch 5). The five-dose vaccine vials were stored at 4°C, and tested at various time points. Visual inspection indicated that the product remained clear. The product was shown to be sterile at t<sub>0</sub>. The product passed the immunogenicity test (at t<sub>0</sub> and 12 and 36 months), as well as the antigenicity test (at t<sub>0</sub> and 3, 9, 12 and 36 months). Antigenicity of the conjugate (see Figure 2), as well as immunogenicity of mouse sera (see Figure 3), were measured as previously described. The following tests were conducted only at t<sub>0</sub>. The total amount of PRP per dose (9·3 µg) was measured by the orcinol test with D(-)-ribose (Rib) as standard [38], after buffer exchange (10 kDa filters) to remove sucrose. The total amount of TTd per dose (25·5 µg) was measured by HPSEC (UV at 280 nm), by comparison with the peak of the original liquid bulk of known protein concentration. The residual moisture (1·27%) was measured by the standard coulometric titration test (EP). The pH value was 6·74 (measured at 25°C). A stress test was also conducted at 37°C for a maximum of 4 weeks.

n.a.: not applicable.

n.d.: not determined.

<sup>a</sup> M<sub>r</sub> was measured by HPSEC against a pullulan calibration curve (see Figure 4). The total plate count value (N) for the conjugate peak (RI data) was used to express peak sharpness: broadening indicates the apparition of free PRP due to the degradation of the conjugate.

<sup>b</sup> Free PRP (% of total PRP) was measured by the orcinol test [38] after DOC-HCl precipitation of the conjugate [44]. Free PRP of the lyophilized vaccine was measured after buffer exchange (10 kDa filters). The high original values were due to incomplete buffer exchange: residual sucrose disturbed the orcinol assay.

<sup>c</sup> The glass transition temperature (T<sub>G</sub>) was measured by DSC.
At first, the test was not yet fully optimized and incomplete buffer exchange resulted in a relatively high level of residual sucrose (used as bulking agent during lyophilization), which disturbed the orcinol assay. HPSEC analysis of the conjugate failed to show any significant change in peak shape. Indeed, the peak sharpness measured with a differential refractive index (RI) detector, which can show the apparition of free PRP due to the degradation of the conjugate, did not fluctuate notably. An accelerated stability study under stress conditions (37 °C, 4 weeks) was also done with the final lyophilized product (see Table 2): after 4 weeks, none of the parameters tested had significantly changed. Remarkably, the glass transition temperature (TG) of the lyophilized product, as determined by differential scanning calorimetry (DSC), remained stable (50-55 °C), and well above average room temperature, indicating the excellent stability of the final product. Indeed, the stability of the liquid bulk conjugate encouraged partners to start working on a fully liquid Hib vaccine based on the original RIVM/NVI production process. Compatibility studies with BF-produced DTwP-HepB were also performed. Negative interactions between the components of the DTwP-HepB vaccine and the Hib conjugate after reconstitution could not be demonstrated (data not shown).

Preclinical and clinical studies

The WHO recommends that a preclinical study be carried out on one of the clinical lots and results should reflect the safety of the process, not of the site where the process was performed [75]. For that reason, the preclinical study was conducted in the Netherlands with the first available clinical lot of lyophilized Hib conjugate vaccine, which was made with BF’s PRP and TTd antigens. The PRP modification step, the conjugation, and the SEC purification were done at the RIVM/NVI because at that time, these parts of the process were not yet fully implemented at BF under GMP conditions. BF performed the filling and lyophilization of the final product. Furthermore, conducting preclinical studies in the Netherlands made it easier to share the data with all the partners. The results showed that three repeated intramuscular vaccinations of male and female rats induced some minor treatment-related systemic effects, and some transient local reactive changes...
in the muscles at the site of injection. Similar effects were observed among animals vaccinated with either the clinical lot of Hib conjugate vaccine or with a comparable already licensed vaccine. The preclinical test report (RIVM/NVI proprietary data) was distributed to all partners to help them license their product.

By the end of 2003, BF had successfully completed a phase 1 clinical study in 32 Indonesian adults. The vaccine was very well tolerated, and all individuals responded with a significant increase in their anti-Hib antibody titer, from an average initial titer of 3 µg/mL, up to 75 µg/mL (BF, proprietary data). All post-immunization titers were above the minimum protective level of antibodies [76, 77], including in two subjects who had no measurable titers before immunization.

The effectiveness of the BF study prompted RIVM/NVI partners (BF, SII and BE) to start investing in new manufacturing facilities for large-scale production of pentavalent DTwP–HepB + Hib vaccines. By mid-2004, SII was ready to start its own phase 1 clinical study in India [78].

In 2004-2005, BF ran Phase 1 and 2 clinical trials in infants (6-11 months of age) with the DTwP–HepB–Hib pentavalent combination, which showed good immunogenicity and safety (see table 3). The two vaccines were administered either separately (DTwP-HepB + Hib), or in one injection (DTwP-HepB-Hib) after reconstitution of the lyophilized Hib component with the DTwP–HepB vaccine. No negative interactions between the Hib conjugate and the DTwP–HepB vaccine were found. A protective response to Hib (≥0.15 µg/ml anti-PRP serum antibodies) [76, 77] was detected in 93–100% of the vaccinated infants. The antibody response to the other components of the combination vaccines was also above protective levels in almost all vaccinated infants: 96-100% for D and T, 85-100% for wP, and 93-97% for HepB. In addition, selected serum samples were tested in an opsonophagocytosis assay to demonstrate the functionality of the induced anti-PRP antibodies, and titers were shown to correlate with the ELISA titers (G.T. Rijkers, University Medical Center, Utrecht, personal communication). Furthermore, this product was shown to be comparable (non-inferior) to a commercial PRP-T vaccine from a multinational vaccine manufacturer, which had already been marketed for several years at the time of these studies (BF, proprietary data). SII’s Phase 1 and 2 clinical trials were similarly successful [78].
Table 3. Summary of immunogenicity results of Bio Farma (BF) pentavalent vaccine clinical trials in infants (6–11 months).

<table>
<thead>
<tr>
<th>Protective antibody level</th>
<th>Phase 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phase 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Phase 2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Phase 2&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DTwP-HepB-Hib&lt;sup&gt;e&lt;/sup&gt; N (%)</td>
<td>DTwP-HepB-Hib N (%)</td>
<td>DTwP-HepB + Hib&lt;sup&gt;f&lt;/sup&gt; N (%)</td>
<td>DTwP-HepB N (%)</td>
</tr>
<tr>
<td>Diphtheria (≥0.01 IU/mL)</td>
<td>24 (100)</td>
<td>48 (100)</td>
<td>48 (100)</td>
<td>128 (96.2)</td>
</tr>
<tr>
<td>Tetanus (≥0.01 IU/mL)</td>
<td>24 (100)</td>
<td>48 (100)</td>
<td>48 (100)</td>
<td>133 (100)</td>
</tr>
<tr>
<td>Pertussis (1/dil. ≥40)</td>
<td>24 (100)</td>
<td>41 (85.4)</td>
<td>45 (93.8)</td>
<td>115 (86.5)</td>
</tr>
<tr>
<td>Hepatitis B (≥10 mIU/mL)</td>
<td>23 (96)</td>
<td>46 (95.8)</td>
<td>45 (93.8)</td>
<td>129 (96.9)</td>
</tr>
<tr>
<td>Hib (≥0.15 µg/mL)</td>
<td>24 (100)</td>
<td>45 (93.7)</td>
<td>46 (95.8)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Data presented at BF at the occasion of its 116th Anniversary Symposium (2006, Bandung, Indonesia). Results are given as the number of responders (N) four weeks after the third injection, with serum antibody titers above the established protective level, and their percentage (between brackets) relative to the total number of recruits having completed the trial. Safety surveillance was performed within four weeks after each dose. The trials were coordinated by the Child Health Department, Hasan Sadikin General Hospital (Bandung, Indonesia). The liquid DTwP-HepB vaccine (BF) was presented in ten-dose vials. Each dose (0.5 mL) contained the following antigens: diphtheria (D, 20 Lf), tetanus (T, 7.5 Lf), pertussis (P, 12 IOU), and HBsAg (HepB surface antigen, 10 µg). The lyophilized PRP-T vaccine (BF lot FPH032) was presented in five-dose vials. Each dose (0.5 mL) contained 10 µg PRP. Three doses were given to each subject who completed the study, with an interval of four weeks between injections. Blood samples were taken at t0 (before the first dose) and four weeks after the third injection. PRP immunogenicity was measured by ELISA [45] using HbO-HA (supplied by NIBSC) as coating reagent. T immunogenicity was measured with the standard ToBI test (EP). D immunogenicity was measured with the standard ELISA test (EP). P immunogenicity was measured by micro-agglutination, and HepB immunogenicity by ELISA.

<sup>a</sup> Open labeled (non-randomized, non-controlled). Subjects: 25 infants enrolled, 24 completed, 8–11 months of age.

<sup>b</sup> Open labeled (randomized, single blind, controlled). Subjects: 48 infants enrolled, 48 completed, 6–11 months of age.

<sup>c</sup> Open labeled (randomized, single blind, controlled). Subjects: 50 infants enrolled, 48 completed, 6–11 months of age.

<sup>d</sup> Open labeled (randomized, single blind, controlled). Subjects: 140 infants enrolled, 133 completed, 6–11 months of age.

<sup>e</sup> One injection: Hib vaccine (lyophilized), reconstituted with tetravalent DTwP-HepB vaccine just before immunization.

<sup>f</sup> Separate injections: tetravalent DTwP-HepB vaccine in one thigh, and PRP-T vaccine (lyophilized, reconstituted with saline just before immunization) in the opposite thigh.
Licensing of Hib conjugate vaccines

In May 2007, SII announced on the Hib initiative website that they had obtained their first Indian marketing license for a Hib conjugate vaccine developed through technology transfer from the RIVM/NVI [79]. Dr. Suresh Jadhav, executive director of SII, declared: “Thanks to this joint programme with NVI, we are now able to make the Hib vaccine ourselves and add it in the very near future to our combination vaccine against diphtheria, tetanus, pertussis and hepatitis B. Through this we can supply United Nation organizations with affordable and quality vaccines in order to contribute to the global control of vaccine-preventable diseases” [79]. In 2009, SII obtained a license to market a fully liquid pentavalent DTwP-HepB-Hib vaccine in India. That same year GAVI funds of US$ 165 million allowed more than 18 million Indian children to be immunized with a five-in-one vaccine. After the initial WHO prequalification (2008) of SII for the production of a lyophilized Hib vaccine, a tetravalent DTwP + Hib and a pentavalent DTwP-HepB + Hib combination vaccine followed suite (2010), and finally, a fully liquid pentavalent DTwP-HepB-Hib vaccine (2010) (see Table 3). This will enable UNICEF to purchase both presentations of the SII pentavalent combination vaccine at a price of less than US$ 2.00 per dose (in 2011, SII and GAVI have agreed on a price of US$ 1.75 per dose for tenders in excess of 50 million doses), against the 2010 GAVI average purchase price of US$ 2.97 per dose [80]. In 2011, it was BE’s turn to obtain WHO prequalification for a pentavalent DTwP-HepB + Hib combination vaccine.

A significant objective of the RIVM/NVI had thus been reached by enabling local manufacturers in developing countries to master conjugate vaccine technology, thus ensuring a sustainable supply of affordable quality Hib conjugate vaccines in the future [81].
Technology transfer

Intellectual property rights [40]
The use of the PRP-T conjugation method, originally developed by John Robbins and collaborators [53-55], allowed to circumvent competing conjugation patents. Due to an existing patent on the use of cross-flow filtration to separate conjugate from free polysaccharide and unconjugated carrier protein [82], the original SEC purification method had to be used instead [53]. Negotiations with the patent owner were not explored because of budget restrictions, lack of experience in legal disputes on patents, and to avoid delays in the time to market. To protect the Hib conjugate process developed at the RIVM/NVI, and to ensure that the technology could be used by our partners without legal problems, the process was patented [61, 62], and as of 2010, patent rights had been granted in the USA, Europe, India, Indonesia, and Japan.

Technical issues
RIVM/NVI contacted its partners periodically to optimize the different assays, and troubleshoot implementation of the Hib process. In particular, product quality was thoroughly checked at the RIVM/NVI during upscaling of the PRP purification, until all process steps were successfully implemented. Another important challenge concerned the presence of free PRP in the final product: this is a residual conjugation starting product (unconjugated PRP-AH), mixed with PRP hydrolyzed from the conjugate during storage [83]. The final product must contain as little free PRP as possible, which depends largely on a good implementation of the final SEC purification step [53]. Well-controlled formulation and storage conditions were also essential in keeping PRP-T intact until its use as vaccine.

Regulatory issues
In the presence of licensed safe and effective Hib vaccines, there are ethical issues which make it difficult to justify clinical trials with Hib conjugate vaccines produced with new and unknown conjugation technologies. For that reason, the PRP-T Hib conjugate was selected
because similar products (Aventis’ ActHIB® or OmniHIB®, and GlaxoSmithKline’s Hiberix®) had already been used in millions of children and appeared to be safe and effective. The experience gained by the RIVM/NVI during the development of its Hib conjugate vaccine helped design three Hib QC training courses in 2007–2008, under the auspice of the WHO GLO-VQ [84]. A total of 27 participants from NRAs and vaccine manufacturers from developing countries (mostly from the DCVMN) were trained at RIVM/NVI in technical aspects of Hib QC, to facilitate the introduction and licensing of Hib conjugate vaccines in their countries.

Strategic aspects
For the RIVM/NVI’s partners, the development of a Hib conjugate vaccine was a critical investment for several reasons. With the increasing demand for pentavalent vaccines, the DTwP and DTwP-HepB market will ultimately be replaced by DTwP-HepB-Hib combination vaccines. Moreover, investing in Hib conjugate vaccine is a first step toward new generation conjugate vaccines, including future pneumococcal and meningococcal multivalent vaccines. With the introduction of acellular pertussis vaccine combinations, multinational vaccine manufacturers are less interested in whole cell pertussis combination vaccines, which are still the vaccines of choice in many developing countries. Consequently, these represent new market opportunities for manufacturers in developing countries. The transfer of a vaccine concept at the partners’ sites instead of a fully established process contributed to a major culture change geared toward experimentation and innovation. The Hib technology transfer resulted in substantial investments in product development infrastructure, and in clinical trial and licensing experience. By limiting the RIVM/NVI project to pilot scale and proof of principle, a relatively low budget project had a major impact on the global Hib conjugate vaccine supply, because of the large-scale vaccine production capabilities and global market experience of the partners.
Marketing issues
In the absence of an assured market for Hib conjugate vaccines; there was little willingness among suppliers to make major investments in production facilities or clinical trials. As concluded in the Mercer report to the GAVI board [39], the limited number of suppliers of Hib conjugate combination vaccines resulted in relatively high prices and thus limited use of these vaccines in developing countries.
GAVI has played an important role in creating a profitable market for DTwP-based combination vaccines with HepB and Hib, by generating the necessary funds as well as supply and demand forecasts. Several emerging manufacturers, including our partners, have entered or are expected to enter this market through UNICEF. This will increase the supply of Hib vaccines and competition, which will ultimately reduce the price of vaccines [81]. In the case of DTwP-HepB + Hib vaccines supplied by UNICEF to GAVI, the price per dose was US$ 3.60 in 2007-2009, and for 2010-2012 it has been lowered to US$ 2.80-2.95 [85].
In our opinion, the other critical factor in addition to creating a market (with GAVI’s impulsion), is the access to Hib vaccine technology, as exemplified by the RIVM/NVI’s Hib project. Both mechanisms are essential in ensuring a sustainable supply of affordable Hib vaccine in developing countries.
We anticipate that a further decline of the Hib combination vaccine prices will take some time, as emerging manufacturers have made major investments for large scale production of vaccines under GMP conditions.

Financial issues
We initially explored seeking financial support for the project among international donors but this was considered as unfair competition to manufacturers who had developed their Hib conjugate vaccines without such support. However, investments by the RIVM/NVI partners could be justified because of a commercially viable vaccine concept, and the profitable market forecasts made by GAVI. In this respect, there is a fundamental difference with projects like the development of the Sabin-IPV vaccine [86], or of the MenAfriVacTM meningococcal group A conjugate vaccine within the Meningitis Vaccine Project (MVP) [87], as there are no identical products already on the market.
Critical players
GAVI has created a market for Hib conjugate vaccines and produced reliable supply-and-demand forecasts [39]. The WHO has recommended incorporation of Hib vaccines in all routine infant immunization programs in view of their demonstrated safety and efficacy: “Lack of surveillance data should not delay the introduction of these vaccines, especially in countries where regional evidence indicates there is a high burden of disease” [34]. Local vaccine manufacturers in developing countries play a critical role in meeting the MDG 4 on the reduction of child mortality. Thanks to their large scale production capabilities and global market experience [81], they are able to produce Hib conjugate vaccines for UNICEF, and for their national immunization programs. The Hib initiative has also played a critical role, by informing governments on disease burden and impact of Hib immunization.

Conclusion
The lessons learned during the development of a Hib conjugate vaccine, and the transfer of the technology to vaccine manufacturers in developing countries, have been described in this paper. At the start of the project, only around one quarter of the world’s children had access to Hib conjugate vaccines, partly because of high prices, and because local vaccine manufacturers did not master Hib conjugate technology. To address this problem, the RIVM/NVI has developed a vaccine production process based on proven publicly available knowledge, and has transferred this technology to several state-owned and private industrial partners. Clear objectives were set from the very beginning of the project and consistent financial support helped to achieve these. The simplicity and the robustness of the production process have facilitated its adoption by the partners. Between eight to ten years after inception of the project, this Hib conjugate vaccine has since been licensed as both a lyophilized, as well as a liquid formulation, either alone or as part of combination vaccines. In all instances, it has proven to be consistent, stable and immunogenic in humans. The transfer of the Hib conjugation process has resulted in access to the technology for emerging manufacturers in developing countries,
and in an increased and sustainable supply of affordable Hib conjugate vaccines, through the establishment of a level playing field and the stimulation of competition among manufacturers. Transferring the Hib technology to vaccine manufacturers in developing countries helped them in getting access to modern technologies that were not available to them before. Moreover, the acquisition of this new knowledge by the manufacturing partners paved the way to new abilities to experiment and innovate, and motivated them to invest in their research and development infrastructure, which in some cases did not exist before starting the collaboration. The threshold to start working on other conjugate vaccines of interest for developing countries, such as meningococcal and pneumococcal vaccines, was hereby reduced. Moreover, thanks to the organization of a QC course, the knowledge on Hib conjugate vaccines has also been boosted at local NRAs. Finally, it has been clearly proven that vaccine manufacturers in developing countries are capable of producing high quality modern vaccines that do meet WHO and EP requirements [56, 57]. It is our belief that the RIVM/NVI Hib conjugate vaccine project can provide a valid model for the successful transfer of modern vaccine technology to developing countries in the future.
Process development of a new *Haemophilus influenzae* type b conjugate vaccine and the use of mathematical modeling to identify process optimization possibilities

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Abstract

Vaccination is one of the most successful public health interventions being a cost-effective tool in preventing deaths among young children. The earliest vaccines were developed following empirical methods, creating vaccines by trial and error. New process development tools, for example mathematical modeling, as well as new regulatory initiatives requiring better understanding of both the product and the process are being applied to well-characterized biopharmaceuticals (for example recombinant proteins). Vaccine industry is still running behind in comparison to these industries.

A production process for a new *Haemophilus influenzae* type b (Hib) conjugate vaccine, including related quality control (QC) tests, was developed and transferred to a number of emerging vaccine manufacturers. This contributed to a sustainable global supply of affordable Hib conjugate vaccines, as illustrated by the market launch of the first Hib vaccine based on this technology in 2007 and concomitant prize reduction of global Hib vaccines.

This paper describes the development approach followed for this Hib conjugate vaccine as well as the recent mathematical modeling tool applied in order to investigate possibilities for further process improvements. The strategy followed during the process development of this Hib conjugate vaccine was a targeted and integrated approach based on prior knowledge and experience with similar products using multi-disciplinary expertise. Mathematical modeling was used to develop a predictive model for the initial Hib process (the ‘baseline’ model) as well as an ‘optimized’ model, by proposing a number of process changes.
Introduction

Regulatory guidelines for (bio)pharmaceutical products such as recombinant proteins (monoclonals, enzymes and hormones) and vaccines have been evolving to increase control on product and process. Therefore, it is necessary to apply improved process development tools based upon a maximum of scientific understanding [88] to guide a certain idea from the laboratory to the patient at affordable prices along the ‘critical path’. Examples of these science-based process development approaches, which are widely applied to proteins, (bio)therapeutics and monoclonals, include design of experiments (DOE) methods [89, 90], model based computer-aided design tools [91-95] and high-throughput screening (HTS) tools [96, 97]. Further, new regulatory initiatives gaining an increasing prominence in the pharmaceutical industry in designing and controlling processes are quality by design (QbD) [98, 99] and process analytical technology (PAT) [100] applications [101-104]. QbD and PAT initiatives encourage the use of science-based development tools for process design, optimization and control. In some cases, a combination of several of the above mentioned tools and/or applications may be considered more convenient.

The vaccine industry is still far behind in comparison with, for example, the chemical industry regarding QbD, PAT [105] and/or one of the new development tools [106,107]. Limited examples are available in vaccine related literature. The main reasons are probably that vaccines are complex, less well characterized products with an unclear structure-function relationship.

Here we describe the pragmatic approach for process development used during the development of a new Hib conjugate vaccine. In addition, more recently mathematical modeling tools were applied, using data generated during process development, to explore possibilities for process optimization as could be implemented by technology transfer partners in the future.
Development strategy followed for the new Hib conjugate vaccine

The first Hib conjugate vaccine was developed in the late 1980’s [53]. Hib vaccines were then widely used in industrialized countries. Although several Hib vaccines were proven to be effective and safe, introduction of Hib vaccines in low- and middle-income countries faced many obstacles including vaccine cost, vaccine availability and lack of information on disease burden [13]. By 1997, Gambia was the only developing country that had introduced the Hib vaccine in its national immunization program, the vaccine was donated by a manufacturer. In 1998, the National Institute for Public Health and Environment (RIVM) started a Hib project aiming at developing a scalable, non-infringing and affordable Hib conjugate vaccine process and related QC tests and transferring this technology to emerging vaccine manufacturers in low- and middle-income countries [108], in order to facilitate a sustainable supply of affordable Hib vaccine through local production.

At the beginning of the Hib project it was decided to follow a pragmatic approach for the development of this new Hib conjugate vaccine: gathering all prior knowledge (including literature, guidelines and experience with comparable vaccines) from the different relevant areas of expertise and defining a target product profile, an analytical strategy and a target process. The target process was then assessed by generating experimental data on each single process step. Where necessary a new process step was developed and/or optimized. The approach followed for the development of this new Hib conjugate vaccine is indicated as a targeted and integrated development approach. In the meantime the Hib process is already developed and transferred to emerging vaccine manufacturers in Indonesia, India and China. Several Hib vaccines, and combination thereof, using this technology are being commercialized since 2007 and have contributed to a price reduction of these vaccines [108].
Method
RIVM/NVI had prior (research and development) experience with several polysaccharide vaccines such as: pneumococcal, meningococcal and *Haemophilus influenzae* vaccines [27, 109-112]. This prior knowledge was evaluated and used whenever needed during the development of this new Hib conjugate vaccine.

Before the start of the project the target product profile (TPP) was defined based on a feasibility study, defining thereby the analytical strategy.

During the development phase the process was divided in the following sections:
- Upstream Hib polysaccharide
- Downstream Hib polysaccharide
- Conjugation process
- Formulation and Filling

For each process step of the target process the potential performance indicators (PI’s), key process parameters (KPP’s), and key raw materials (KRM’s) were defined by a multidisciplinary project team based on prior knowledge. For the development and further optimization of the process steps, experiments were needed to generate supporting data. These experiments were performed following the one-factor-at-a-time (OFAT) principle. Ultimately, the unit operations chosen to be used were tested as part of the whole process, evaluating thereby the interaction between the different unit operations (Figure 11).

![Figure 11: Process flow for the new *Haemophilus influenzae* type b conjugate vaccine ('baseline' process).](image-url)
Results

**Target product profile**

The vaccine developed was a freeze-dried PRP-T (Hib polysaccharide conjugated to tetanus toxoid), see table 4 for the target product profile (TPP). The reason for choosing to develop a PRP-T is that all potential partners had access to tetanus toxoid. Also, similar Hib conjugate vaccines were already used extensively and lot release criteria for this vaccine were already established by both EP [56] and WHO [57, 113].

**Table 4:** Target product profile (TPP) for a new *Haemophilus influenzae* type b conjugate vaccine.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indication</strong></td>
<td>Prophylactic vaccine to be administered in the national immunization program.</td>
</tr>
<tr>
<td><strong>Target population</strong></td>
<td>Infants (&gt; 2 months) and Children (&lt;5 years)</td>
</tr>
<tr>
<td><strong>Route of administration</strong></td>
<td>Intramuscular</td>
</tr>
<tr>
<td><strong>Product presentation</strong></td>
<td>Multi-dose vial (5 doses), 0.5 ml/ single human dose. Possibility of producing single-dose and a different multi-dose (10 doses)</td>
</tr>
<tr>
<td><strong>Dosage schedule</strong></td>
<td>3 doses administered 2 months apart</td>
</tr>
<tr>
<td><strong>Shelf life and storage conditions</strong></td>
<td>≥2 years at 2-8 °C</td>
</tr>
<tr>
<td><strong>Dosage form</strong></td>
<td>Freeze-dried product to be reconstituted with 0.4% NaCl solution or DTP/ DTP-HepB. One vial contains 5 doses of Hib vaccine, the vaccine has to be reconstituted in 2.8 ml (0.3 ml overfill). Bulk should be stable to allow the production of liquid formulations.</td>
</tr>
<tr>
<td><strong>Concentration and composition</strong></td>
<td>One human dose contains 10 ± 2 µg purified capsular polysaccharide conjugated to approximately 20 µg of tetanus toxoid in the presence of Tris-Sucrose.</td>
</tr>
<tr>
<td><strong>Immunogenicity, safety, reactogenicity and contra-indication</strong></td>
<td>At least comparable to a licensed PRP-T Hib conjugate vaccine.</td>
</tr>
<tr>
<td><strong>Lot-release criteria</strong></td>
<td>Both WHO and EP.</td>
</tr>
<tr>
<td><strong>Target price</strong></td>
<td>1 $/ dose</td>
</tr>
</tbody>
</table>
**Analytical strategy**

The analytical strategy applied on the new *Haemophilus influenzae* type b conjugate vaccine was decided at an early stage of the Hib project. The Hib vaccine had to meet at least EP [56] and WHO [57, 113] requirements for PRP-T production. Furthermore, internal specifications were set to achieve an optimal process robustness and consistency (table 5). Data from different experiments have been used to set the internal specifications.
Table 5: Analytical strategy used during the development of a new *Haemophilus influenzae* type b conjugate vaccine based on EP [56], WHO [57] requirements and internal specifications.

<table>
<thead>
<tr>
<th>Control</th>
<th>Requirement</th>
<th>Test</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide</td>
<td>≤ 1% (dry weight)</td>
<td>Lowry</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Protein</td>
<td>≤ 1% (dry weight)</td>
<td>UV&lt;sub&gt;260&lt;/sub&gt;</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>6.8-9.0 (dry weight)</td>
<td>Chen</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>≥ 32% (dry weight)</td>
<td>Orcinol</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Ribose</td>
<td>≤10 IU/µg % (dry weight)</td>
<td>LAL</td>
<td>WHO, EP ≤25</td>
</tr>
<tr>
<td>Molecular size distribution</td>
<td>&gt;300 KDa</td>
<td>HP-GPC</td>
<td>Internal</td>
</tr>
<tr>
<td>Identity</td>
<td>Hib-polysaccharide</td>
<td>ELISA &amp; NMR</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Residual reagents</td>
<td>Removal</td>
<td>Process validation</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Sterility</td>
<td>Sterile</td>
<td>Sterility</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Modified polysaccharide</td>
<td>20-50 RU/ NH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>ADH-groups</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Number of functional groups</td>
<td>200-50 KDa &lt; 10% PRP-ADH</td>
<td>HP-GPC</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Molecular size distribution</td>
<td>≥ 1000 kDa</td>
<td>Sterility (test)</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Free ADH</td>
<td>Sterility</td>
<td>Sterility test</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Carrier protein carrier</td>
<td>≥ 1500 LF/ mg protein nitrogen</td>
<td>Elisa</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Purity</td>
<td>Sterile</td>
<td>pH-meter</td>
<td>Internal</td>
</tr>
<tr>
<td>Bulk conjugate</td>
<td></td>
<td>Identity</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Total polysaccharide</td>
<td>-</td>
<td>Immunogenic</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Free polysaccharide</td>
<td>&lt; 20% total polysaccharide</td>
<td>Guo</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Total protein</td>
<td>-</td>
<td>Incl. bound protein</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Free protein</td>
<td>-</td>
<td>HP-GPC</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Conjugation ratio (PRP: protein)</td>
<td>0.30-0.60</td>
<td>-</td>
<td>WHO/Internal</td>
</tr>
<tr>
<td>Molecular size distribution</td>
<td>≥ 1000 kDa</td>
<td>HP-GPC</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Sterility</td>
<td>Sterile</td>
<td>Sterility test</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Specific toxicity tetanus</td>
<td>Sterility</td>
<td>Using guinea-pigs</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>pH</td>
<td>6.2-7.2</td>
<td>Sterility test</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Identity</td>
<td>Antigenic</td>
<td>Using mice</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>Immunogenic</td>
<td>Process validation</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Residual reagents &amp; reactive groups</td>
<td>Removal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final bulk</td>
<td>Osmolality</td>
<td>200-400 (mOSm/kg)</td>
<td>Internal</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.2-7.2</td>
<td>Internal</td>
</tr>
<tr>
<td></td>
<td>Sterility</td>
<td>Sterile</td>
<td>WHO/EP</td>
</tr>
<tr>
<td>Final lot</td>
<td>Total polysaccharide</td>
<td>-</td>
<td>Orcinol</td>
</tr>
<tr>
<td></td>
<td>Free polysaccharide</td>
<td>&lt; 20 % total polysaccharide</td>
<td>Guo</td>
</tr>
<tr>
<td></td>
<td>Sterility</td>
<td>Sterile test</td>
<td>WHO/EP</td>
</tr>
<tr>
<td></td>
<td>General safety</td>
<td>Satisfactory</td>
<td>Abnormal toxicity</td>
</tr>
<tr>
<td></td>
<td>Immunogenicity</td>
<td>Immunogenic</td>
<td>Using mice</td>
</tr>
<tr>
<td></td>
<td>Molecular size distribution</td>
<td>≥ 1000 kDa</td>
<td>HP-GPC</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.2-7.2</td>
<td>WHO/EP</td>
</tr>
<tr>
<td></td>
<td>Residual moisture</td>
<td>≤ 25%</td>
<td>Coulometric assay</td>
</tr>
<tr>
<td></td>
<td>Glass transition temperature</td>
<td>≥ 45°C</td>
<td>DSC</td>
</tr>
<tr>
<td></td>
<td>Identity</td>
<td>Antigenic</td>
<td>Elisa</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>≤ 10 IU/µg polysaccharide</td>
<td>LAL</td>
</tr>
<tr>
<td></td>
<td>Visual inspection</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

WHO (World Health Organization), EP (European Pharmacopeia), UV<sub>260</sub> (Ultraviolet at 260 nm), IU/µg (International Units per microgram), LAL (Limulus amoebocyte lysate), kDa (kilo Dalton), HP-GPC (High Performance Gel Permeation Chromatography); RU (Repeat Units); LF (Limit of Flocculation); mOsm/kg (milliosmole per kilogram); DSC (Differential Scanning Calorimetry).
Each lot of polysaccharide was tested for purity (protein [70], nucleic acid and endotoxin), identity, sterility, molecular size distribution [114], phosphorus [115] and ribose [68]. Modified polysaccharide was assessed for the number of adipic acid dihydrazide (ADH) groups introduced per unit of polysaccharide [71] and molecular size distribution [114].

Tetanus toxoid used for the conjugation had to meet the WHO requirements for bulk purified toxoid [57]. In addition, the purity was at least 1500 Lf/mg (nondialysable) protein nitrogen and the consistency was checked by means of high pressure gel permeation chromatography (HP-GPC). The carboxyl groups of the tetanus toxoid react with the hydrazide group of the PRP-ADH derivative, using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC) to form the conjugate. Low molecular weight by-products were removed by gel filtration chromatography. Each lot of the (concentrated) bulk conjugate was characterized by determining the total polysaccharide [68], total protein [70], free polysaccharide [74] and free protein content [70, 114]. Further, the conjugation reaction was monitored by HP-GPC and the endotoxin content was determined using the limulus amebocyte lysate (LAL) test. The polysaccharide:protein ratio was used as a conjugation marker and the pH was controlled during the conjugation reaction. The absence of tetanus specific toxicity and the reversion to tetanus toxicity had to be demonstrated during process validation. These tests were not needed for routine lot release. That was also the case for residual reagents and residual reactive groups. Initially nuclear magnetic resonance (NMR) was used to test for residual reagents and residual reactive groups but these tests could be omitted after process validation. The identity of each bulk conjugate was tested using the antigenicity test [116]. To test for the immunogenicity of the bulk conjugate a mice immunogenicity test was developed [116].

The final bulk was filled into glass vials and lyophilized using sucrose as excipient, the most important at this stage was to test for the sterility. The final lot was tested for total polysaccharide [68], free polysaccharide, sterility, molecular size distribution [114], pH, residual moisture, identity [116], and endotoxin. During process validation, the final lot was tested for immunogenicity, general safety and specific toxicity to tetanus [113]
Defining quality attributes
The performance indicators (PI’s) and key process parameters (KPP’s) identified during the development of this *Haemophilus influenzae* type b conjugate vaccine are described in table 6.

Table 6: Performance indicators (PI’s) and key process parameters (KPP’s) identified during the development of a new *Haemophilus influenzae* type b conjugate vaccine.

<table>
<thead>
<tr>
<th>Upstream Hib polysaccharide</th>
<th>Polysaccharide production</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI’s¹</td>
<td>Lysis, growth profile, polysaccharide concentration and number of repeat units</td>
</tr>
<tr>
<td>KPP’s²</td>
<td>Volume inoculum and cultivation conditions (temperature, stirrer speed, DO, pH)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Downstream Hib polysaccharide</th>
<th>Concentration/ Diafiltration</th>
<th>Precipitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI’s¹</td>
<td>Number of repeat units</td>
<td>NMR profile, number of repeat units and ribose content</td>
</tr>
<tr>
<td>KPP’s²</td>
<td>Concentration/ diafiltration factor and temperature</td>
<td>Precipitation conditions (ion strength, pH, temperature, time, centrifugation speed/time)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conjugation process</th>
<th>Activation</th>
<th>Modification</th>
<th>Conjugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI’s¹</td>
<td>-</td>
<td>Modification ratio and number of repeat units</td>
<td>Free polysaccharide, free protein and conjugate size</td>
</tr>
<tr>
<td>KPP’s²</td>
<td>Activation conditions (temperature, time, polysaccharide: CNBr ratio and number of repeat units)</td>
<td>Modification conditions (temperature, time, polysaccharide: ADH ratio)</td>
<td>Conjugation conditions (temperature, time, polysaccharide: protein ratio)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Formulation and filling</th>
<th>Formulation</th>
<th>Lyophilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI’s¹</td>
<td>Free polysaccharide and conjugate size</td>
<td>Moisture content, appearance, free polysaccharide, conjugate size and glass transition temperature</td>
</tr>
<tr>
<td>KPP’s²</td>
<td>Storage temperature</td>
<td>Freeze-drying cycle (shelf temperature, rap rate, time, pressure) and concentration PRP-T</td>
</tr>
</tbody>
</table>

¹ PI’s (performance indicators): An indicator that can be used to evaluate the performance of a process.
² KPP’s (key process parameters): A process parameter that should be maintained to ensure optimum process performance.
**Upstream Hib polysaccharide**

**Seed lot**

Within the framework of the Hib project a seed lot was produced under good manufacturing practice (GMP) and transferred to the technology transfer partners to allow the partners to set up their own seed lot system. During the development of the production process for this seed lot, the storage temperature and the process of cryopreservation were set based on prior knowledge [117]. Storage of liquid Hib seed lots at ≤-70 °C was proven to be adequate since other seed lots stored at the same temperature were still viable after more than ten years (data not shown). The Hib seed lot was preserved by adding 10% glycerol followed by (snap) flash freezing using dry ice.

The seed lot was produced in shake flasks (custom-made 1 L glass bottles with semi-permeable caps were used for this purpose) in a stove at 35 °C. The optimal harvest conditions were obtained when the culture was harvested in the logarithmic growth phase, with an optical density (590nm) between 0.6 and 1.0. Using a seed lot harvested at a much higher OD590 (2.6) resulted in a very slow growth. That was also the case when the key raw Materials were not added freshly (figure 12a).

**Media composition.**

For the cultivation of *Haemophilus influenzae* type b the impact of medium composition on growth and polysaccharide production as well as on the safety of the final product had to be evaluated. Using modified Frantz medium [118, 119] as a starting point, a cultivation medium was developed and the composition was shared with all the partners (table 7). The key raw materials were identified: cysteine, yeast extract, NAD (nicotinamide adenine dinucleotide), peptone and hemin, each of these medium components was studied separately.

As for many culture media oxidation of cysteine was prevented by substitution with cystine. High molecular weight medium components can be co-purified with the Hib polysaccharide and were omitted from the cultivation medium. Yeast extract was therefore filtered using a 30 kDa cross flow filter, only the yeast extract filtrate was used in the medium. And although it was noticed that peptone did have a positive effect on the growth, it was decided not to use it during the production. Further,
in the cases where peptone was needed, for example for the production of master seed lot, soy peptone was used instead of peptone from bovine origin. Hemin, from a bovine origin, had to be avoided because of the risks related to bovine spongiform encephalopathy (BSE). Hemin from equine (horse) or porcine (swine) origin was preferred. The source of hemin didn’t have an effect on the growth. Hemin, the only animal component used in the cultivation medium, was prepared by dissolving it in NaOH and autoclaving it (15-20 min, 121°C), minimizing thereby the chance of introducing extraneous agents through hemin. This pretreatment of hemin did not have an effect on the growth. NAD was considered to be a key raw material, based on the limited stability of this component, and had to be added freshly to the cultivation medium and stored under the proper conditions. Other raw materials used for the medium preparation were less critical from a safety and/or quality perspective.

Table 7: Composition of the cultivation medium used for the growth of *Haemophilus influenzae* type b bacteria (modified Frantz compared to Intravacc’s medium).

<table>
<thead>
<tr>
<th>Compounds (g/L)</th>
<th>Modified Frantz</th>
<th>Intravacc’s Hib medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>1.60</td>
<td>1.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Cystine</td>
<td>-</td>
<td>0.015</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>6.24</td>
<td>2.50</td>
</tr>
<tr>
<td>KCl</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.00</td>
<td>6.00</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>5.00</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5.00</td>
<td>-</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.00</td>
<td>10</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1.23</td>
<td>0.6</td>
</tr>
<tr>
<td>NAD</td>
<td>-</td>
<td>0.002</td>
</tr>
<tr>
<td>Hemin</td>
<td>-</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Polysaccharide production
The first experiments performed to develop a (upstream) process for the production of Hib capsular polysaccharide (polyribosylribitol phosphate, PRP) were performed by applying a cultivation process developed for the production of meningococcal polysaccharides [120].

Based on historical data, the following key process parameters were selected and controlled during the cultivation: temperature at 35.0±0.5°C, pH at 7.0±0.1 and DO at 30±5%. Experiments were
performed to confirm that cultivation conditions chosen did result in a product with the desired quality. Insufficient data were available on the optimal inoculation volume, therefore shake flask experiments were performed (figure 12b). An inoculum volume of 2.5±0.1% (v/v) was selected. An inoculum above 2.5% (v/v) did not lead to a higher OD while the use of more seed lot could have disadvantages such as carryover of waste materials and rapid exhaustion of the seed lot stock.

Further, the harvest quality was found to be important for subsequent downstream process steps. The harvest time and thus the harvest quality was a key process parameter, since it had an impact on the quality of the polysaccharide. During the exponential phase, the optical density increased concomitantly with the polysaccharide concentration in the supernatant [61, 62]. After reaching the maximum OD$_{590}$, just before the stationary phase, the polysaccharide concentration continued to increase. The fact that the OD$_{590}$ decreased during the stationary phase was probably an indication that the Hib bacterium released its capsule (thus polysaccharide) in the supernatant.
The optimum harvest time was therefore not based on the growth or on the concentration of polysaccharide in the supernatant but on the pH, based on experiments in which the growth and PRP secretion were monitored at several bioreactor scales (3-350 L working volume). It was concluded that harvesting at a pH between 7.3-7.5 resulted in a consistent harvest of the desired quality, with respect to PRP concentration and molecular weight (±500-800 kDa). Harvesting at a pH > 7.6 will result in a higher yield of polysaccharide due to cell lysis, but also in more contaminants such as cell debris, endotoxin and proteins, complicating the purification of the crude polysaccharide. For the determination of PRP concentration during the cultivation process the PRP-ELISA method was used [116].

_Downstream Hib polysaccharide Inactivation_

Formaldehyde was preferred as inactivating agent instead of heat inactivation to avoid any impact of heat on the stability and quality of the polysaccharide. For the development of the inactivation process the inactivation conditions (formaldehyde concentration, time and temperature) were marked as key process parameters which have to be controlled.

Several formaldehyde concentrations were tested both at 4°C and at room temperature (figure 13). At room temperature, the inactivation was completed within four hours while at 4°C more than 20 hours was needed. The following inactivation procedure was finally chosen, taking into account the stability of the polysaccharide and trying to limit the formaldehyde concentration used: the supernatant was inactivated by adding formaldehyde till a final concentration of 0.1% (v/v). The supernatant was then inactivated under gentle stirring during 2 hours at room temperature and stored at 4°C overnight (in the presence of formaldehyde).
Concentration

Directly after the inactivation, a concentration step including diafiltration was performed. A concentration and diafiltration step using a tangential flow filtration unit early in the process has a number of advantages: the purification can start using relatively comparable starting materials concerning both the polysaccharide concentration and matrix and all purification steps can be performed using relatively small volumes resulting in for example, less waste, lower cost and easier process scaling up.

For the development of the concentration step, the concentration factor, diafiltration factor and diafiltration buffer were considered of importance. Further, the optimal filter cut-off had to be determined. Therefore several filters with various cut-offs were tested. The polysaccharide concentration in both permeate and concentrate was determined. The 100 kDa cut-off filter had the best performance, since almost no polysaccharide losses occurred. This filter was considered to be suitable for concentrating the supernatant at room temperature and for removing the low molecular weight medium components and formaldehyde residues (data not shown).

The crude polysaccharide was concentrated 20-40 times. Concentrating further resulted in a viscous solution. For the diafiltration both water and phosphate buffered saline (PBS) were tested. PBS was chosen based on better stability data.

Figure 13: Inactivation of *Haemophilus influenzae* type b using formaldehyde both at room temperature and at 4°C. At room temperature (●) the inactivation is completed within 4 hours while at 4°C (¾) more than 20 hours was needed.
Precipitation steps

Based on prior knowledge, *Haemophilus influenzae* type b polysaccharide could be purified using consecutive precipitation steps [121]. Precipitation is relatively easy, inexpensive and in general no dedicated equipment is needed. This was in line with the objectives of this technology transfer project.

For the development of the different precipitation steps, the purity of the polysaccharide was of importance and had to meet the requirements. Developing an easy process resulting in high yields but not meeting the purity requirements was not an option.

Precipitation conditions, using hexadecyltrimethylammonium bromide (cetavlon), ethanol and sodium deoxycholate (DOC), were investigated in several experiments by determining the polysaccharide recovery and purity, both in the supernatant and in the pellet. Based on these experiments, the optimal conditions for each precipitation step were determined.

Conjugation process

Hib polysaccharide was conjugated to tetanus toxoid based on the conjugation method developed by Dr. J. Robbins and his group at the National Institutes of Health (NIH) [122].

First, the polysaccharide was randomly activated with cyanogen bromide (CNBr), directly followed by a modification with adipic acid dihydrazide (ADH) resulting in modified polysaccharide: PRP-ADH. Tetanus toxoid was randomly activated with N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) before being conjugated to PRP-ADH resulting in a ‘lattice conjugate’ (PRP-T): a three-dimensional framework, containing several carbohydrate molecules and several tetanus toxoid molecules.

During the development of the conjugation process, it was noticed that besides the molecular size of the polysaccharide, the ratio of polysaccharide to CNBr, ADH and protein during the activation, modification and conjugation reaction, respectively, were of importance.

It was decided to initially pretreat the polysaccharide by reducing the molecular weight to 250±50 kDa using a controlled alkaline degradation step before starting PRP modification. In this way, a consistent starting material was obtained and separation of free PRP from high molecular
weight conjugates was easier. For the conjugation step, a large excess of linker (ADH, 10-fold the CNBr amount) was needed to avoid cross-linking of PRP.

Several experiments were performed to define the optimal reaction conditions for activation, modification and conjugation reactions. The time and temperature had to be controlled (data not shown).

Formulation and filling

Development of the formulation and lyophilization process to produce a final bulk and final lot was based on prior knowledge [123]. RIVM/NVI had previous experience with the formulation and lyophilization of meningococcal and pneumococcal vaccines.

Lyophilization was used to enhance the thermostability of the vaccine and to give more flexibility concerning the final presentation. Freeze-dried Hib vaccine can be used as a stand-alone vaccine or in combination with other antigens (for example DTP-HepB). In general, during the formulation process, vaccines are buffered and then mixed with an additive before being lyophilized. The role of this additive is to provide a bulking effect (formation of a solid ‘cake’).

The freeze-drying conditions (especially the excipient used, concentration and size of the conjugate) were chosen to be key process parameters and had to be controlled during the freeze-drying process. Various excipients were tested. Sucrose was chosen based on stability data.

For logistic reasons it can be desirable not to lyophilize directly after the production of the concentrated conjugate bulk product (final bulk). Final bulk stored at different temperatures (4°C, -20°C and -70°C) was tested for stability and appeared to be most stable at 4°C (less aggregates and less free polysaccharide). Furthermore, by studying the stability of several freeze-dried products more insight was gained in the impact of conjugate molecular size and concentration of free polysaccharide before freeze-drying on the performance indicators of the freeze-drying process (for example cake appearance and moisture content).

The final bulk was filled in glass vials and lyophilized in a 5 dose presentation resulting in the final lot. Each dose of freeze-dried Hib conjugate vaccine contained 10±2 µg polysaccharide, 16-33 µg tetanus toxoid, ~0.61mgTris and ~21.4 mg sucrose.
In addition, the optimum glass transition temperature of the final lot (freeze-dried Hib conjugate vaccine) was determined at 50-55°C, indicating appropriate stability of the freeze-dried product.

Mathematical modeling and possibilities for process optimization

Method
Rational design based on mathematical modeling has been used within academia for process development, unit operation optimization, process simulation and plant wide control. However this approach is not widely used by vaccine manufacturers [124, 125]. A conjugate vaccine is a relatively well characterized product, and therefore more suitable for QbD like development and application of modeling tools.

As mentioned in this paper a relatively pragmatic targeted and integrated approach was used during the actual process development of the new Hib conjugate vaccine. At a later stage a start was made by using a rational approach as schematically illustrated in figure 14. Therefore, a mathematical model for the Hib process was developed. First a model was developed for the (pilot scale) ‘baseline’ process, as initially developed by RIVM/NVI, and transferred to several vaccine manufacturers.

The initial process steps from the ‘baseline’ process were assessed by applying rational design tools as described in the Delft manual for conceptual process and product design [126] using a stepwise approach (the Stage-Gate (table 8) Product Development Process (SGPDP) [127]). Further, more recent Hib publications were consulted [128]. This assessment resulted in a couple of potential suggestions for process optimization; therefore a model was also prepared for this ‘optimized’ process. Process modeling and simulation work was performed using a commercial batch process simulator: SuperPro Designer v9.0 from Intelligen, Inc. (New Jersey, USA). The process models provided useful data for a material balance, energy balance and economic analysis.
Figure 14: Schematic display of the design approach followed for the process development of a new *Haemophilus influenzae* type b vaccine: a classical approach compared to a rational process development approach.
Table 8: Delft method for product development approach consisting of eight key items divided in three parts: design concepts starting from supply chain, process technology and process engineering.

<table>
<thead>
<tr>
<th>Design space</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part 1</td>
<td>Supply chain</td>
</tr>
<tr>
<td>0</td>
<td>Vaccine design analysis</td>
</tr>
<tr>
<td>1</td>
<td>Input / output</td>
</tr>
<tr>
<td>2</td>
<td>Sub-process decomposition, driven by product &amp; process attributes</td>
</tr>
<tr>
<td>Part 2</td>
<td>Process technology</td>
</tr>
<tr>
<td>3</td>
<td>Task allocation and targeting</td>
</tr>
<tr>
<td>4</td>
<td>Operation procedure / units</td>
</tr>
<tr>
<td>5</td>
<td>Process integration (mass, energy)</td>
</tr>
<tr>
<td>Part 3</td>
<td>Process engineering</td>
</tr>
<tr>
<td>6</td>
<td>Equipment design</td>
</tr>
<tr>
<td>7</td>
<td>Operability integration (safety, control, availability)</td>
</tr>
<tr>
<td>8</td>
<td>Flow sheet sensitivity analysis and optimization</td>
</tr>
</tbody>
</table>

During process modeling different process sections were identified: cultivation, polysaccharide purification, polysaccharide modification, conjugation, conjugate purification, formulation and filling. Each section was divided in one or more unit operations, resulting in a total of 23 different unit operations [129] which were included in the model (figure 15). First the ‘baseline’ process (as initially developed by RIVM/NVI) was simulated. The ‘baseline’ model was prepared using experimental data and process information from Intravacc. A summary of the process steps and process data included in the ‘baseline’ model is given in the next section. This ‘baseline’ model was also used to generate information on the material balances and on the batch cycle time.
Results ‘Baseline’ model
The fermentation section consisted of a 500 ml shake flask (P1), a 15 L seed bioreactor (P2) and a 440 L production bioreactor (P3).
The following mass balance stoichiometry was assumed for the production culture. This reaction stoichiometry (the amount of substance consumed and/or produced during the production of Hib polysaccharide (PRP)) was calculated using Matlab.
\[
0.5 \text{ Medium} + 0.5 \text{ Oxygen} \\
\rightarrow 0.15 \text{ Biomass} + 0.1 \text{ Carbon Dioxide} + 0.0004 \text{ PRP} + 0.04 \text{ Impurities} + 0.71 \text{ Water}
\]

The entire fermentation process is composed by the following steps:
1. Preparation: pre-cleaning, sterilization, medium dispensing and pre-culture.
2. Actual fermentation process
3. Equipment cleaning
Volume of the cultivation broth was 350 L per batch. The actual operation time (cultivation time), excluding setup and CIP (cleaning in place), was 10 hours.
The harvest was centrifuged (P4), followed by an inactivation step (P5). Modeling of the centrifugation step is done based on the design parameters for Westfalia CSA-8, provided by the vendor. The operation time was kept at 30 minutes, excluding setup and CIP, and the flow rate was adjusted accordingly.
As the product is an extracellular capsular polysaccharide, no cell disruption is required. Further purification was done by removing residual cell biomass and extracellular impurities using diafiltration (P6 and P7).
Purification of the polysaccharide was performed by several precipitation steps (P-8 and P-9). The pellet and supernatant were separated using batch centrifugation. The purified polysaccharide was diafiltered using a tangential flow filtration system (P10 and P11). At this stage endotoxin content was preferably less than 10 IU/microgram and nucleic acid content less than 1% (w/w) with a polysaccharide recovery of more than 90%.
Figure 15: Flowchart based on a model for a new *Haemophilus influenzae* type b vaccine using SuperPro Designer (‘baseline’ process as developed by RIVM/NVI). Main operations modeled using SuperPro designer is shown in different sections. Starting with an inoculum preparation (P1), followed by production culture, inactivation, polysaccharide purification, polysaccharide modification, chemical conjugation of the polysaccharide to a carrier protein, purification of the conjugate and as last formulation including filling and visual inspection (P23).
Before modification, the polysaccharide was concentrated using a tangential flow filtration system (P12). The concentrated PRP was first partially degraded using alkaline degradation followed by CNBr activation and ADH modification (P13). Modified PRP was concentrated and purified using a tangential flow filtration unit (P14 and P15). Tetanus toxoid was randomly activated with EDC before conjugation to PRP-ADH (P16). The reaction time was 10 minutes at low temperature (2°C).

After conjugation, low molecular weight by-products were removed by microfiltration (P17) while maintaining free PRP and free tetanus toxoid content below 20 % and 5 % respectively. Removal of unreacted polysaccharide, protein and purification of Hib conjugate polysaccharide product was achieved using gel permeation chromatography (GPC) using a Sephadex G-25 M column (26 x 300 mm), with phosphate buffer at pH 7 - 8 at an elution rate of 2.5 mL/min (P18). The purified conjugate was then filtered using a 0.22 μm microfiltration unit (P19). Formulation of the Hib conjugate vaccine was done either in liquid form (liquid Hib conjugate vaccine) or lyophilized (freeze-dried Hib conjugate vaccine) with saline as a reconstitution fluid. Aseptic filling of the conjugate vaccine was done using a filling line (P20), at a rate of 12,000 vials/ hour. The batch size was 1.2 million doses. For the production of a freeze-dried product, a freeze-dryer was needed (P21), a freeze-drying cycle took about 72 hours, after which capping of the vials took place (P22). For the visual inspection (P23) 15,000 vials/ hour were considered to be inspected.

Material balances
Material balances calculated using SuperPro Designer provided input, conversion and output streams obtained during the process. Input and output streams (table 9), which were responsible for the operational cost either related to purchase or disposal, were quantified. A large amount of water and water for injection (WFI) was utilized, especially for buffer preparation and cleaning purposes. Consumables such as, filter membranes, vials and resins for chromatography were expressed separately.
Table 9: Identification of the input and output streams of a new *Haemophilus influenzae* type b conjugate vaccine process, following the Delft method for product development.

<table>
<thead>
<tr>
<th>Input</th>
<th>Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Biomass (bacteria)</td>
</tr>
<tr>
<td>Adipic acid dihydrazide (ADH)</td>
<td>Carbon dioxide (CO₂)</td>
</tr>
<tr>
<td>Inoculum (bacteria)</td>
<td>Water (produced)</td>
</tr>
<tr>
<td>Cetavlon</td>
<td>WFI</td>
</tr>
<tr>
<td>Ethyl alcohol (EtOH)</td>
<td>Product (PRP-T)</td>
</tr>
<tr>
<td>Water for injection (WFI)</td>
<td>Sodium hydroxide (NaOH)</td>
</tr>
<tr>
<td>Water</td>
<td>Tetanus Toxoid (unconjugated)</td>
</tr>
<tr>
<td>Tetanus Toxoid</td>
<td>Sodium chloride (NaCl)</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>PRP (unconjugated)</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Phosphoric acid</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>Air (oxygen &amp; nitrogen)</td>
</tr>
<tr>
<td>Air</td>
<td>Sodium bicarbonate (NaHCO₃)</td>
</tr>
<tr>
<td>Sodium bicarbonate (NaHCO₃)</td>
<td>EDTA disodium</td>
</tr>
<tr>
<td>EDTA Disodium</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Potassium chloride (KCl)</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>EDC</td>
</tr>
<tr>
<td>Cyanogen bromide</td>
<td>Monopotassium phosphate (KH₂PO₄)</td>
</tr>
<tr>
<td>EDC</td>
<td>Sodium carbonate (Na₂CO₃)</td>
</tr>
<tr>
<td>Phosphate buffer saline (PBS)</td>
<td>Adjuvants</td>
</tr>
<tr>
<td>Monopotassium phosphate (KH₂PO₄)</td>
<td>Phosphate buffer saline (PBS)</td>
</tr>
<tr>
<td>Sodium carbonate (Na₂CO₃)</td>
<td>Cell impurities</td>
</tr>
</tbody>
</table>
In addition to raw material information, SuperPro Designer was used to calculate individual streams (inputs and outputs). This information was used to verify material balances including (aqueous, solid and gaseous) waste generated (table 10). Process yields calculated using the ‘baseline’ model showed similar results to those obtained during process development.

Table 10: Overall balance obtained using a mathematical modeling tool to simulate the production process of a new *Haemophilus influenzae* type b conjugate vaccine. Minus sign indicates that the material is consumed and a plus sign that it’s produced.

<table>
<thead>
<tr>
<th>Component</th>
<th>Input</th>
<th>Output</th>
<th>Out-in</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>2,00</td>
<td>0,00</td>
<td>-2,00</td>
</tr>
<tr>
<td>Biomass</td>
<td>0,00</td>
<td>54,00</td>
<td>54,00</td>
</tr>
<tr>
<td>Carbon Dioxide</td>
<td>0,00</td>
<td>35,00</td>
<td>35,00</td>
</tr>
<tr>
<td>Cetavlon</td>
<td>1,00</td>
<td>1,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Cyanogen Bromide</td>
<td>1,00</td>
<td>0,00</td>
<td>-1,00</td>
</tr>
<tr>
<td>EDTA Disodium</td>
<td>1,00</td>
<td>1,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Ethyl Alcohol</td>
<td>75,00</td>
<td>74,00</td>
<td>-1,00</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1,00</td>
<td>0,00</td>
<td>-1,00</td>
</tr>
<tr>
<td>Impurities</td>
<td>0,00</td>
<td>14,00</td>
<td>14,00</td>
</tr>
<tr>
<td>KCl</td>
<td>1,00</td>
<td>0,00</td>
<td>-1,00</td>
</tr>
<tr>
<td>Media</td>
<td>366,00</td>
<td>2,00</td>
<td>-364,00</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>1,00</td>
<td>1,00</td>
<td>0,00</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1,00</td>
<td>1,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>113,00</td>
<td>113,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Oxygen</td>
<td>34,00</td>
<td>32,00</td>
<td>-2,00</td>
</tr>
<tr>
<td>Phosphoric Acid</td>
<td>87,00</td>
<td>87,00</td>
<td>0,00</td>
</tr>
<tr>
<td>PRP-TT</td>
<td>0,00</td>
<td>3,00</td>
<td>3,00</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>22,00</td>
<td>22,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>33,00</td>
<td>33,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Tetanus Toxoid</td>
<td>2,00</td>
<td>0,00</td>
<td>-2,00</td>
</tr>
<tr>
<td>WFI</td>
<td>9366,00</td>
<td>9366,00</td>
<td>0,00</td>
</tr>
<tr>
<td>Water</td>
<td>40641,00</td>
<td>40905,00</td>
<td>264,00</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>50748,00</strong></td>
<td><strong>50748,00</strong></td>
<td><strong>0,00</strong></td>
</tr>
</tbody>
</table>
**Batch cycle time**

The ‘baseline’ process model developed using SuperPro Designer was used to determine the process schedule (see figure 16). Frequently used equipment such as diafiltration and microfiltration units were shared across single or multiple batches to achieve higher equipment occupancy. This approach decreased the capital cost associated with the equipment. In this batch cycle diagram, main equipment is shown on the vertical axis while the occupancy time is shown on the horizontal axis. Figure 16 shows that the production process for Hib conjugate vaccine has a batch time of 252 hours. Polysaccharide purification (precipitations), formulation (freeze-drying) and filling are the most time-consuming process steps. Therefore equipment needed during the polysaccharide purification and final product formulation were identified as critical concerning process scheduling. During process modeling of the ‘baseline’ process, the batch time was optimized by rescheduling general equipment, for example storage vessels, filtration units and centrifuges. The overall equipment occupancy for a single batch was optimized to be above 80 %.

**Figure 16:** Occupancy of main equipment for the ‘baseline’ process of a new *Haemophilus influenzae* type b vaccine (SFR-101: shake flask; SBR1a: seed bioreactor; PBR1a: production bioreactor; DS-101: centrifuge; V-101: inactivation tank; DF-103: diafiltration unit; V-102 & V-103: precipitation tank; MF-103: microfilters; V-108: modification tank; V-108: conjugation tank; C-102: GPC; FL-1: filling unit; LYO-1: lyophilization unit; CPR-1: capping machine; IS-1: inspection equipment). Time (in hours and days) shown in the horizontal axis and equipment’s in the vertical axis.
‘Optimized’ process
Using the ‘baseline’ Hib process model in SuperPro it was possible to identify optimization possibilities (figure 17) for the existing process. All scenario’s mentioned below were evaluated based on the process yields, equipment occupancy and cost of goods. During the simulation of the ‘optimized’ process for a Hib conjugate vaccine the batch time and KPPs were kept within the operational window as provided by the ‘baseline’ process.

The following changes were included in the ‘optimized’ process model: using a fed-batch instead of a batch for the production of Hib polysaccharide, leaving the inactivation out, using chromatography instead of precipitation for the purification of Hib polysaccharide and production of a liquid instead of freeze-dried vaccine. During the development of the ‘optimized’ model both the process economics and product quality were taken into account. This ‘optimized’ model indicated that it should be possible to improve the polysaccharide yield to >400 – 420 mg crude PRP/L compared to 300-320 mg crude PRP/L obtained using the ‘baseline’ process. Further, the batch time can be decreased by 7%, 18 %, 26 % after implementing the proposed optimizations in

Figure 17: Alternatives studied using process simulation: the ‘baseline’ process for the production of a *Haemophilus influenzae* type b vaccine compared to an ‘optimized’ process. Process optimizations related to the following process steps were identified: upstream Hib polysaccharide, downstream Hib polysaccharide, formulation and filling. A process including the proposed optimizations was simulated in SuperPro.
respectively inactivation, Hib-polysaccharide purification and producing a liquid instead of freeze-dried product. This reduction of batch time can be due to a lower equipment occupancy and a reduced unit operation time.

**Upstream Hib polysaccharide**
For the production of the Hib polysaccharide it was proposed to use a fed-batch cultivation mode in the ‘optimized’ process. For the fed-batch mode cultivation media (table 7) were considered to be added to the bioreactor vessel at a flow rate of 60L/hour during the last 4 hours of cultivation. Initial volume of production medium in the bioreactor was considered to be equal to 100 L. Besides fed-batch, perfusion cultures are widely used for the production of biopharmaceuticals. In-situ recovery of the polysaccharide can take place during the cultivation process [129], this option was not considered in this ‘optimized’ model. Further, use of chemical inactivation is not a prerequisite from the regulatory point of view, but can give more flexibility concerning the design and the use of production area. For the simulation of the ‘optimized’ model it was considered that the inactivation step is not needed.

**Downstream Hib polysaccharide**
With respect to the purification process, different options were studied (analyzed conceptually) and evaluated using SuperPro in order to study the impact of these changes on the ‘baseline’ process. PRP purification by precipitation was used during the development of the ‘baseline’ process, because of its low capital expenditure and prior experience with comparable products. In the ‘optimized’ model, the precipitation steps needed for the purification of Hib polysaccharide were substituted by a chromatography step, anion exchange chromatography (AEC). Purification using a CarboPac® PA1 (22 x 250 mm) chromatography column was considered to be performed overnight, using acetate buffer at pH 7-8 at an elution rate of 2.6 mL/min. After the PRP purification, a concentration step using a microfilter (0.22μm) was considered.
Formulation and filling
Hib conjugate vaccine can be used as stand-alone or in combination with other antigens. For the simulation of the ‘optimized’ process, different scenarios were considered for the vaccine presentation. Different Hib vaccines available on the market were taken into consideration, there are already more than 30 different presentations of Hib vaccines prequalified by WHO [131]. Ultimately a liquid 5 dose presentation was chosen for the optimized process.

Economical evaluation
An economical evaluation was performed using SuperPro. Economical evaluation of the ‘baseline’ Hib process and COGs calculation was performed using several financial indicators of which capital expenditures (CAPEX) and operational expenditures (OPEX) are key indicators. Capital expenditures are mainly related to equipment purchase cost and its installation. Bioreactor, centrifuge, chromatography and lyophilizer were main equipment for the CAPEX (figure 18).

Operating costs are costs directly related to manufacturing of the product, which includes raw materials (example cultivation media), sterile empty vials, consumables, labor, QC/QA, utilities and other facility depended costs. The vial cost showed a significant contribution.
to the COGs (figure 19). Based on this economical evaluation using SuperPro, the cost price of the Hib conjugate vaccine produced using the initial process (‘baseline’ model) was calculated to be < $ 1,0- per dose (fill and finished product).

![Figure 19: Operational cost breakdown for the ‘baseline’ process of a new Haemophilus influenzae type b conjugate vaccine. Costs of raw materials and consumables (main contributors) is shown in the pie chart along with labor, facility dependent, laboratory and utilities costs.](image)

The effect of vaccine formulation (freeze-dried versus liquid and monodose versus multidose) on the cost of goods of the vaccine was investigated using the Hib the ‘baseline’ model. Producing a liquid instead of freeze-dried vaccine could result in a 30% reduction in the cost price of the vaccine and producing a multidose instead of monodose could reduce the cost price further up to 65% (figure 20).
Discussion and conclusions

Hib conjugate vaccines have been proven to be effective in both developed and developing country settings. In view of the demonstrated safety and efficacy, WHO recommended that Hib vaccine is introduced in routine national immunization programs. GAVI’s (financial) support has encouraged vaccine manufacturers in low- and middle-income countries to develop Hib vaccine.

Intravacc’s Hib project (previously RIVM/NVI) has contributed to WHO and GAVI’s goals by developing a new Hib conjugate vaccine, including related QC tests, and transferring the technology to a number of vaccine manufacturers in India, Indonesia and China.

The process development approach (a targeted and integrated process development approach) chosen during the process development was regulatory and quality driven showing similarities with Quality by Design tools. The process developed at a relatively small scale (3 L working volume, 5 L total volume) was scaled up by RIVM/NVI to a pilot scale (350 L working volume, 440L total volume) and appeared to be further up scalable to a manufacturing scale when implemented by the technology transfer recipients. The process was developed within a relatively short time frame (3 years) and resulted in a very competitive vaccine both quality and pricewise. This indicates that the approach
followed for process development was successful and can be used for other vaccines.
In this work, this process development approach was highlighted, and a process simulation tool was used to develop a mathematical model for the Hib conjugate vaccine process (‘baseline’ process). It was feasible to model the process using the available experimental data from Intravacc. This ‘baseline’ model for the initial process was used to explore possibilities for process optimization, focusing thereby on the equipment occupancy and on the cost price of the vaccine without compromising product quality, this resulted in an ‘optimized’ model. Based on this ‘optimized’ model using a fed-batch instead of a batch cultivation process, leaving the inactivation out and purifying the polysaccharide using chromatography instead of precipitation techniques, can help reducing the operation cost related to the production of Hib conjugate vaccine and thus lead to a lower cost price. Similarly, a multidose formulation instead of a monodose and liquid instead of freeze-dried formulation can further decrease the cost price per dose.
It was possible to prepare a highly predictive process model for the overall production process of the new Hib conjugate vaccine using SuperPro. Modeling the Hib process indicated possibilities for process improvement. Mathematical modeling tools can be used during process development but also post-licensure. A mathematical process model can be used to explore possibilities for process optimization and to perform a sensitivity analysis in order to help making certain choices (for example related to facility debottlenecking, scaling up...etc.).
Use of immuno assays during the development of a *Haemophilus influenzae* type b vaccine for technology transfer to emerging vaccine manufacturers

Ahd Hamidi
Hans Kreeftenberg
Abstract

Quality control of *Haemophilus influenzae* type b (Hib) conjugate vaccines is mainly dependent on physicochemical methods. Overcoming sample matrix interference when using physicochemical tests is very challenging, these tests are therefore only used to test purified samples of polysaccharide, protein, bulk conjugate, and final product. For successful development of a Hib conjugate vaccine, several ELISA (enzyme-linked immunosorbent assay) methods were needed as an additional tool to enable testing of in process (IP) samples. In this paper, three of the ELISA’s that have been very valuable during the process development, implementation and scaling up are highlighted. The PRP-ELISA, was a very efficient tool in testing in process (IP) samples generated during the development of the cultivation and purification process of the Hib-polysaccharide. The antigenicity ELISA, was used to confirm the covalent linkage of PRP and TTd in the conjugate. The anti-PRP IgG ELISA was developed as part of the immunogenicity test, used to demonstrate the ability of the Hib conjugate vaccine to elicit a T-cell dependent immune response in mice. ELISA methods are relatively cheap and easy to implement and therefore very useful during the development of polysaccharide conjugate vaccines.
Introduction

Enzyme-linked immunosorbent assay (ELISA) methods are widely used for the quantification of several types of polysaccharides as well as antibody responses after vaccination with polysaccharide protein conjugate vaccines [40, 132-135] for the measurement of anti-Hib antibody titers in human sera, commercially ELISA kits [136] are also available. Within the framework of the development and technology transfer related to Haemophilus influenzae type b (Hib) conjugate vaccine at the Institute for Translational Vaccinology (Intravacc, originating from the former Vaccinology Unit of the National Institute of Public Health [RIVM] and the Netherlands Vaccine Institute [NVI]) [108] several ELISA’s were developed in order to enable successful process development. The PRP (polyribosyl ribitol phosphate) ELISA was developed to determine the PRP content in in process (IP) samples generated during the cultivation of Hib organisms and purification of PRP. Quantification of PRP in the IP samples by colorimetric methods such as the Orcinol assay [68] is not possible because of interference with media components (e.g., glucose) and reagents used during purification (e.g., detergents). The Orcinol assay is only applicable on purified samples [137, 138]. Pre-treatment of the samples is possible but very laborious, since multiple precipitation steps may be needed (data not shown).

The antigenicity-ELISA was developed to evaluate the covalent linkage of PRP and TTd (tetanus toxoid) on the conjugate during the development and implementation of the Hib conjugation process. Chromatographic methods help to visualize the presence of a high molecular entity (the conjugate) in the samples but do not demonstrate the presence of both the polysaccharide and carrier protein on the same molecule. The anti-PRP IgG ELISA was used to test if the Hib conjugate is able to induce a T-cell dependent IgG antibody response against PRP in mice. A generally accepted potency test which reflects clinical efficacy is not available for Hib conjugate vaccines. Consequently such a test is not required by WHO [57] or EP [56] as lot release test. However, WHO [57] considers immunogenicity testing in animals meaningful during the development of Hib conjugate vaccines to demonstrate the ability of the conjugate to elicit a T-cell dependent immune response. Besides mice [53] other
animal models for example guinea pigs [139, 140] could also be used for immunogenicity testing of the new Hib conjugate vaccine. Mice were used for practical reasons. After successful development of the Hib conjugate vaccine and transfer of the technology to several partners, the technology transfer partners implemented the PRP-ELISA and antigenicity-ELISA to test respectively for the identity of purified PRP and that of the final product [56, 57].

In this paper the usefulness, applicability and limitations of three ELISA methods: PRP-, antigenicity-, and anti-PRP IgG ELISA are described. These ELISA’s were indispensable during the development, implementation and scaling up stage of the Hib project. Qualification and validation data generated in collaboration with or by the individual partners during the technology transfer are outside the scope of this paper.

Results

The ELISA methods described were primary developed as an additional tool to allow process development and facilitate the implementation and scaling up of the process at partner’s site. Some typical examples explaining how the different ELISA’s were used during the development of the cultivation, purification and conjugation process are presented below.

PRP-ELISA

The PRP-ELISA was developed to monitor the PRP content in samples during the cultivation of Hib organisms and purification of Hib-polysaccharide from the culture supernatant. Figure 21 shows a typical example of a PRP-ELISA curve. The concentration of the reference PRP sample was determined by the Orcinol test in advance, using D-(-)-ribose as a standard. All samples were pre-diluted to a concentration of approximately 20 ng/ml, in order to obtain a sigmoidal curve. The results of the reference PRP were used to determine the standard curve, describing the relation between the absorbance and the logarithm of the PRP concentration.
The dilutions in the linear part of the standard curve were used to calculate the PRP concentration in the samples based on the absorbance measured for the specific sample dilution, using standard regression analysis in MS Excel. The results were multiplied with the dilution-factor to get the PRP concentration in the original sample.

The PRP-ELISA was used to test in process samples from the cultivation process. Samples were taken every hour during a 40 L cultivation batch using an autosampler. A selection of these samples was tested; the results are shown in Figure 22. During the exponential phase, it is clear that the secretion of the PRP in the supernatant lags behind biomass formation. After the exponential phase of the growth (as the absorbance [OD at 590nm] decreases), PRP continued to be released in the supernatant, indicating that an optimal optical density and thus biomass formation does not correspond to an optimal PRP concentration in the culture supernatant. So the PRP-ELISA results enabled to develop a protocol to achieve optimal harvests with high PRP concentration and low waste materials (for example cell debris, proteins, and nucleic acids) in the culture supernatant, based on physical parameters.

**Figure 21.** PRP-ELISA, used to determine Hib capsular polysaccharide content in in process samples generated during the cultivation and purification process: 0.1% skimmed milk in phosphate buffered saline as a negative control (●), reference PRP sample as a positive control (■) and an experimental PRP batch (▲).
(especially pH) resulting in a very robust and easily scalable cultivation process [61]. Applying the PRP-ELISA during process development avoids harvesting too late with consequent accumulation of impurities in the culture supernatant and thus requiring an extensive purification process. The PRP-ELISA was also used in the development of the PRP purification process. It was possible to quantify the amount of PRP in the presence of medium components during the concentration step. For example when two supernatant samples of a 3.5 L culture, one just after harvesting and the other 15 times concentrated were tested with the PRP-ELISA, a quantity of respectively 960 and 940 mg PRP was detected while in both the permeate sample and in the supernatant just after inoculation a quantity of \( \leq 0.5 \) mg PRP was found. Precipitation steps in the presence of a relatively low detergent percentage (for example cetavlon, cetyltrimethylammonium bromide) could be monitored by PRP-ELISA (Table 10). Supernatant and pellet samples precipitated in the presence of respectively 0.04, 0.08, or 0.16\% w/v cetavlon were examined for PRP content. A mass balance of 85–109\% was found in the example from Table 11. At relatively 

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**Figure 22.** Cell growth and PRP concentration, determined by PRP-ELISA, as a function of cultivation time measured during the cultivation of *Haemophilus influenzae* type b at a 40 L scale: absorbance (▲) at a wavelength of 590nm (OD590), reflecting cell growth, and PRP concentration (■).
high cetavlon concentrations (>0.32%) it was clear that the presence of cetavlon disturbed the PRP-ELISA. While most of the PRP was expected to be present in the pellets, it was not possible to determine the anticipated content of PRP. Precipitation of the cetavlon pellets after dissolving in water for injection at 80% v/v ethanol, did help to determine the PRP content in the 0.32% cetavlon pellet. However for samples with a high percentage of detergent (>0.64%), one single ethanol precipitation step was clearly not enough to recover all PRP. Despite the detergent interference in the PRP ELISA, it was possible to choose the right percentages of detergent and ethanol needed in the Hib purification process.

**Antigenicity-ELISA**

To confirm the covalent linkage of PRP and TTd, the antigenicity-ELISA was used to demonstrate the presence of PRP and TTd on the same molecule of the Hib conjugate. A typical example of the antigenicity-ELISA is given in Figure 23. A mix of unconjugated PRP and TTd in a ratio equal to the test Hib conjugate vaccine was included as a negative control and a licensed Hib conjugate vaccine (PRP-T) as a positive control. Both Hib conjugate vaccines (test and positive control) consisting of PRP conjugated to Tetanus Toxoid gave a sigmoidal curve in this ELISA. As expected, the mixture of unconjugated PRP and TTd did not show a response in the test.

---

**Table 11.** PRP content in in process samples generated during the purification process of the Hib-polysaccharide, determined using PRP-ELISA. Effect of cetavlon on the PRP-ELISA results, before and after an additional ethanol precipitation.

<table>
<thead>
<tr>
<th>% cetavlon (w/v)</th>
<th>0.04</th>
<th>0.08</th>
<th>0.16</th>
<th>0.32</th>
<th>0.64</th>
<th>0.73</th>
<th>1.28</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PRP after cetavlon precipitation (pellet+supernatant)</td>
<td>109</td>
<td>103</td>
<td>85</td>
<td>53</td>
<td>9</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>% PRP recovered from the cetavlon pellet (after one 80% ethanol precipitation)</td>
<td>-</td>
<td>-</td>
<td>141</td>
<td>119</td>
<td>50</td>
<td>61</td>
<td>57</td>
</tr>
</tbody>
</table>

w/v, weight per volume.
**Figure 23.** Antigenicity-ELISA, used to demonstrate the presence of antigenic sites of the Hib polysaccharide and carrier protein (TTd, tetanus toxoid) on the Hib conjugate: a sample containing a mix of unconjugated PRP and free tetanus toxoid (●) as a negative control, a licensed Hib conjugate vaccine (■) as a positive control and the test Hib conjugate vaccine (▲).

**Figure 24.** Monitoring of the conjugation process by HPSEC analysis. Formation of the conjugate (PRP-T; experimental batch) was done at various time points during the conjugation process by UV (280 nm; t0, — black; 30 min, − − − blue; 60 min, — pink; 120 min, - - - brown; end at 280 min . . . green). The gradual disappearance of unconjugated TTd (tR ~17.5 min) occurred simultaneously to the formation of conjugate (tR 13–14 min, final Mr 1,411 kDa). ADH-modified PRP (PRP-AH) could also be detected using UV while unconjugated PRP had to be detected using Refractive Index (RI).
High Performance Size Exclusion Chromatography (HPSEC) was used to determine the molecular weight of the PRP and to monitor the conjugation process (figure 24). Molecular weight was used as a measure for visualizing the conjugation reaction of PRP to TTd and thus the production of the conjugate. Detection of the conjugate (PRP-T) is done at various time points during the conjugation by UV (280 nm); gradual disappearance of TTd and appearance of conjugate is seen. The added value of the antigenicity-ELISA in comparison with the HPSEC is the ability of this test to detect the PRP and TTd antigenic sites on the same conjugate molecule and thus in confirming that a PRP-T conjugate was produced.

Anti-PRP IgG ELISA
To examine the Hib conjugate for the ability to induce an immunological memory and consequently an IgG antibody response against PRP a mouse immunogenicity test was developed. A typical example is given in Figure 25. The results clearly show that immunization of mice with unconjugated PRP does not result in the production of IgG anti-PRP antibodies. However, immunization of mice with a Hib conjugate vaccine does result in a positive response. Five out of ten mice were identified as responders in this case. Although mice are not the best responders to Hib conjugate vaccine. The induction of an immunological memory and IgG anti-PRP antibodies could clearly be demonstrated. For that

![Graph](image)

**Figure 25.** Anti-PRP IgG ELISA, used to test if the Hib conjugate is able to induce IgG antibodies against PRP in mice. Mice were immunized with PRP-T (▲) (test conjugate vaccine) and free PRP (■) (unconjugated).
reason the mouse immunogenicity test and thus the anti-PRP IgG ELISA was considered to be useful in the development of the new Hib conjugate vaccine.

Discussion/Conclusion

Within the framework of the Hib technology transfer at Intravacc (originating from RIVM/NVI), a production process for a new Hib conjugate vaccine, including Quality Control (QC) tests, was developed. Knowhow related to the production process and QC was transferred to partners in Indonesia, India, and China. Beside the development of all (physicochemical) tests required by WHO and EP for routine lot release of the new Hib conjugate vaccine [59,143] a number of ELISA methods were developed and used to enable process development at Intravacc and process implementation, including further scaling up, at partner’s site. Being highly sensitive, specific and relatively cheap; these ELISA methods were very useful in a successful process development.

The PRP-ELISA method was used to enable the development of the cultivation and purification process by detecting Hib-polysaccharide present in several in process samples generated during these processes. Medium components do not seem to interfere with this ELISA in contrast with the commonly used Orcinol test which can only be used to test pure polysaccharide samples. No extensive attempts were made to correlate the Orcinol test with the PRP-ELISA, as the primary objective of using this ELISA was to monitor the PRP content during individual cultivation and/or purification batches. Consequently only relative values were relevant to optimize the cultivation and/or purification process. The PRP-ELISA seems to be disturbed by the presence of relatively high detergent concentrations. This didn’t limit the use of the PRP-ELISA during the development of the purification process. Additionally it’s advisable to study the impact of molecular weight on the test results, during the cultivation and purification of PRP the molecular weight of the polysaccharide may change.

The antigenicity-ELISA was used to test in process conjugation samples by demonstrating the presence of PRP and TTd antigenic sites on the same conjugate molecule.
For that reason this ELISA was considered to have an added value in the demonstration of a covalent linkage between PRP and TTd. The antigenicity-ELISA may also be useful as an additional tool throughout stability and consistency testing. However these options were not extensively explored within the project as adequate tests are already available to monitor stability and consistency. The anti-PRP IgG ELISA was very useful as a functional assay to demonstrate the ability of the conjugate to elicit an immunological memory and consequently induce IgG antibodies against PRP. For that reason mice were injected twice on days 0 and 14. A clear difference was seen with unconjugated PRP, which did not induce IgG antibodies against PRP. Sera generated in the mouse immunogenicity test were also used to test for the immunogenicity of TTd, carrier of the Hib conjugate, by using a Toxin Binding Inhibition (ToBI) assay [141]. The results showed that the TTd still had the ability to elicit anti-tetanus antibodies (data not shown). The conjugation method used to prepare the coating antigen may be of importance because of the possible presence of non-functional epitopes in case a similar method is used to the test vaccine, this need to be further investigated. In conclusion the reported antigenic and immunogenic characteristics show that the Hib conjugate vaccine production process developed by Intravacc leads to a PRP-T conjugate that expresses 300 PRP antigens that are able to induce an immunological memory and elicit IgG antibodies against PRP. In general, ELISA methods are very useful during the development, implementation, and scaling up of polysaccharide protein conjugate vaccines. When properly qualified and validated the ELISA’s can be used to test for the identity of the polysaccharide and conjugate.

Materials

Buffers: carbonate buffer (0.04 M, pH 9.6), phosphate buffered saline (PBS, 0.01 M, pH 7.2), and sodium acetate buffer (1.1M, pH 5.5). All buffers were prepared in-house.
Reagents: burro-anti-Hib (BAH) serum was kindly provided by Dr. J. Robbins [142] (NIH), this serum was purified with a protein G column and then peroxidase-labeled resulting in Burro anti-Hib~PO (BAH~PO). The labeling or conjugation of the serum took place in-house by incubating
the IgG fraction of the serum with activated peroxidase for 3 h (pH 9, room temperature). The conjugate was subsequently centrifuged and diafiltered against phosphate buffered saline. Equine anti-tetanus (HAT) was prepared in-house by immunizing horses with tetanus toxoid followed by tetanus toxin. This serum was peroxidase-labeled resulting in horse anti-tetanus~PO (HAT~PO). Biotinylated goat anti-mouse IgG: GE Healthcare, RPN1177; Streptavidin horse radish peroxidase: GE Healthcare, RPN1231, and HbOHA [143] (Hib oligosaccharide linked to human albumin, available from NIBSC).

ELISA plates: Greiner (655092).
Diluent: 0.1% skimmed milk in PBS (phosphate buffered saline).
Skimmed milk (BD Difco 232100).
Chemicals: tween-80 (Merck, 822187), ethanol (Merck, 1.00971), Hydrogen peroxide 30% (Merck, 107209), concentrated sulfuric acid (Merck, 100731), tetramethyl-benzidine (Sigma, T2885), bovine serum albumin (Serva, 11922), tween-20 (Merck, 822184). Test Hib vaccine: A vaccine produced in-house by conjugating the Hib polysaccharide to tetanus toxoid, using the conjugation method originally described by John Robbins and collaborators (National Institutes of Health) [53].
Reference PRP: WHO standard (NIBSC no. 02/208) or an internal PRP standard (meeting all WHO and EP requirements). Licensed Hib vaccine: A Hib conjugate vaccine licensed through RIVM (freeze-dried PRP-T).
Both Hib vaccines (test and licensed vaccine) were PRP-T, standalone and freeze-dried Hib conjugate vaccines.

Methods

High performance size exclusion chromatography (HPSEC)
HPSEC analysis of purified PRP samples and/or samples generated during the conjugation reaction was performed on two OHpak SB-805 and SB-804 columns (300 x 8 mm, with the addition of an SB-G guard column, 50 x 6 mm; Shodex) mounted in series, with 10 mM phosphate-buffered saline (PBS, pH 7.2) as eluent, at 1 mL/min and 35°C. Elution was monitored with a RI and a multiple-wavelength UV detector from 215 to 280 nm. The Mr value of the polysaccharides was determined relative to the retention times (tR) of defined pullulan standards (Mw 5.8–853 kDa, Mw/Mn ~1.1). The elution limits of the
column set were determined by using high Mr dextran (Mr 5–40 x 10^6 kDa) and deuterium oxide (D_2O). Detection of PRP was done by measurement of the RI signal. Impurities (proteins and nucleic acids) were detected by UV and RI.

**PRP-ELISA**

The PRP ELISA was used to monitor the PRP in culture supernatants and to monitor the purification process. The ELISA plates were coated with burro anti-Hib in carbonate buffer as catching antibody. To prevent non-specific binding of PRP samples, the plates were blocked with PBS containing 0.1% skimmed milk. The samples to be tested including reference PRP were diluted 2-fold in series of eight dilutions using PBS containing 0.1% skimmed milk and 0.05% tween-80, making sure that the starting concentration was approximately 20 ng/mL. The plates were incubated for 2 h at room temperature. On each ELISA plate, a negative control (PBS with 0.1% skimmed milk) was included. The bound PRP was detected with peroxidase labeled burro anti-Hib (burro anti-Hib~PO). Tetramethyl-benzidine was used as substrate; Tetramethyl-benzidine was being used as a replacement for carcinogenic compounds such as benzidine [144, 145]. This reaction was stopped after ten minutes using 2 M sulfuric acid. The intensity of the color was directly related to the amount of PRP present in the sample. The absorbance was measured at 450 nm using an ELISA reader (Bio-tek Reader Elx808). Intermediate washing steps were performed after each treatment except for the last incubation with the substrate. Washing could be done either manually using a multi-channel pipette or by means of a special washing apparatus, the plates were washed using a solution of 0.05% Tween-80 in PBS and turned upside down between the washing steps. The PRP content in the samples was calculated by plotting the absorbance as a function of the sample dilution and by comparing the sample with the PRP reference (known PRP content) in a parallel line analysis in Excel. For this calculation only the linear part of the curve was used.

**Antigenicity ELISA**

The antigenicity ELISA was used to demonstrate covalent linkage of PRP and TTd in Hib conjugate. Unconjugated PRP and TTd were used
Immuno assays during Hib vaccine development

as negative controls. A licensed Hib conjugate vaccine was used as positive control. The ELISA plates were coated with burro anti-Hib in carbonate buffer as catching antibody. To prevent non-specific binding of samples, PBS containing 0.1% skimmed milk was used as a blocking agent. The samples tested were diluted 2-fold in series of eight dilutions with PBS containing 0.1% skimmed milk and 0.05% tween-80. The plates were incubated for 2 h at room temperature. Bound Hib conjugate was detected using peroxidase labeled antibodies directed against TTd (horse anti-tetanus-PO). Intermediate washing steps were done as described under PRP ELISA. Tetramethyl-benzidine substrate was used to react with the peroxidase. This reaction was stopped after ten minutes using 2 M sulfuric acid. The absorbance was measured at 450 nm using an ELISA reader (Bio-tek Reader Elx808).

Anti-PRP IgG ELISA

The ability of the Hib conjugate vaccine to induce a T-cell dependent immune response was investigated in mice. A group of 10 mice (NIH mice, 10–14 g, same sex) was injected subcutaneously with 1/10 of a human dose of the test Hib conjugate vaccine corresponding to 1 mg PRP, on day 0 and 14. The mice were bled on day 28 (eye extraction under anesthesia). Serum was prepared by allowing blood to coagulate for approximately 2 h at 37°C and overnight at 4°C. After coagulation, the serum was separated by centrifugation. The serum samples were stored at -30°C after inactivation for 30 min at 56°C. The individual serum samples were analyzed for IgG anti-PRP antibodies. A licensed Hib conjugate vaccine was included as positive control in each test. Unconjugated PRP was used as a negative control. Immunization of mice using either a licensed vaccine or unconjugated PRP was done following the same procedure as the one followed for the test vaccine. The ELISA plates were coated with a solution of 1 mg/mL HbO-HA antigen in PBS. The serum samples were diluted 2-fold using PBS containing 1% BSA and 0.05% Tween-20 and incubated for 2 h at 37°C. To detect IgG anti-PRP antibodies, biotinylated goat anti-mouse IgG was added. Streptavidin-peroxidase was added and TMB used as a substrate. This reaction was stopped after ten minutes using 2 M sulfuric acid. The absorbance was measured at 450 nm using an ELISA reader (Bio-tek Reader Elx808).
Preclinical evaluation of a *Haemophilus influenzae* type b conjugate vaccine process intended for technology transfer

Ahd Hamidi
Pauline Verdijk
Hans Kreeftenberg
Abstract

Introduction of *Haemophilus influenzae* type b (Hib) vaccine in low- and middle-income countries has been limited by cost and availability of Hib conjugate vaccines for a long time. It was previously recognized by the Institute for Translational Vaccinology (Intravacc, originating from the former Vaccinology Unit of the National Institute of Public Health (RIVM) and the Netherlands Vaccine Institute [NVI]) that local production of a Hib conjugate vaccine would increase the affordability and sustainability of the vaccine and thereby help to speed up Hib introduction in these countries. A new affordable and a non-infringing production process for a Hib conjugate vaccine was developed, including relevant quality control tests, and the technology was transferred to a number of vaccine manufacturers in India, Indonesia, and China. As part of the Hib technology transfer project managed by Intravacc, a preclinical toxicity study was conducted in the Netherlands to test the safety and immunogenicity of this new Hib conjugate vaccine. The data generated by this study were used by the technology transfer partners to accelerate the clinical development of the new Hib conjugate vaccine. A repeated dose toxicity and local tolerance study in rats was performed to assess the reactogenicity and immunogenicity of a new Hib conjugate vaccine compared to a licensed vaccine. The results showed that the vaccine was well tolerated and immunogenic in rats, no major differences in both safety and immunogenicity in rats were found between the vaccine produced according to the production process developed by Intravacc and the licensed one. Rats may be useful to verify the immunogenicity of Hib conjugate vaccines and for preclinical evaluation. In general, nonclinical evaluation of the new Hib conjugate vaccine, including this proof of concept (safety and immunogenicity study in rats), made it possible for technology transfer partners, having implemented the original process with no changes in the manufacturing process and vaccine formulation, to start directly with phase 1 clinical trials.
Introduction

*Haemophilus influenzae* type b (Hib) conjugate vaccines have been highly efficacious in reducing Hib-related disease incidence in developed countries, mainly meningitis and pneumonia. Although safe and effective Hib conjugate vaccines have been available since the late 1980s, the introduction of these vaccines in low- and middle-income countries did not start until 20 y later. The availability of Hib conjugate vaccines could be improved by assuring a sustainable local production in low-and middle-income countries; this was only possible by enabling access to (Hib) conjugate technology. The Institute for Translational Vaccinology (Intravacc, originating from the former Vaccinology Unit of the National Institute of Public Health (RIVM) and the Netherlands Vaccine Institute (NVI)) developed an up-scalable, non-infringing, and affordable production process of Hib conjugate vaccine plus the related quality control (QC) tests and transferred the technology to several vaccine manufacturers in India, Indonesia, and China [40, 42, 78, 108, 146].

Hib vaccine developed by Intravacc consists of purified polyribosylribitol phosphate (Hib capsular polysaccharide, PRP) conjugated to a carrier protein (tetanus toxoid, routinely prepared by Bio Farma). Tetanus toxoid, when covalently bound to PRP (PRP-T), is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide. The Hib technology developed resulted in stable intermediate and final products and allowed both freeze-dried and liquid formulations of several Hib vaccines. Based on this technology, the Indian partners have succeeded in producing, marketing, and prequalifying both standalone and combined Hib conjugate vaccines [147-148]. Prequalification is a service provided by WHO to United Nations Children’s Fund (UNICEF) and other United Nations agencies to assure that vaccines considered for purchase by such agencies meet global standards of quality, safety, and efficacy [150]. Local production by emerging manufacturers as a result of the technology transfer has contributed to an effective and sustainable way in increasing the Hib conjugate vaccine supply [151,152]. Nonclinical evaluation, including a safety and immunogenicity study in rats, was part of the Hib technology transfer project at Intravacc. The first clinical lot was produced in collaboration between Intravacc and Bio
Farma: the production of polysaccharide, production of tetanus toxoid, freeze-drying and filling of the final lot took place at Bio Farma, and the conjugation of the Hib-polysaccharide to tetanus toxoid took place at Intravacc. The safety and immunogenicity of this clinical lot, produced under Good Manufacturing Practices, was tested in a repeated dose toxicity and local tolerance study in the Netherlands according to WHO guidelines on nonclinical evaluation of vaccines [75]. Based on the preclinical data from this study, phase 1 clinical trials could be initiated in Indonesia using the same lot [108].

Although Intravacc as the owner/transferee of the technology had no intention of marketing the Hib conjugate vaccine, it chose to evaluate the new Hib conjugate vaccine and thereby the developed process nonclinically and to provide the data to all technology transfer partners in order to achieve an efficient and cost-effective technology transfer. The nonclinical data provided by Intravacc could be used by all technology transfer partners to build their registration dossier, to obtain approval from independent ethics committees and regulatory authorities to perform clinical trials with the product produced in their own facilities and to obtain marketing authorization. In this way, it was not necessary for each individual partner to generate similar data, an approach supported by WHO [75]. This approach can be set as an example for other technology transfer projects, assuring that the local requirements are taken into account. Since relatively few publications are available on preclinical evaluation of Hib conjugate vaccines, it was chosen to publish the design and results of the preclinical safety study performed within the framework of the Hib technology transfer project.

The primary objective of this preclinical study was to assess the systemic and local toxicity/reactogenicity of this new Haemophilus influenzae type b conjugate vaccine after repeated intramuscular vaccinations in male and female rats in comparison to a commercial Hib conjugate vaccine. In addition, the antibody response was measured in rat sera to confirm that the Hib conjugate, produced by the production process developed by Intravacc was immunogenic in rats.
Results

Toxicity study
No major abnormalities were observed in the appearance, general condition, growth, food consumption, hematological and clinical chemistry values, organ weights and gross necropsy, and histopathological findings in any of the groups. Temporary signs of local irritation, including erythema and hematomas, were observed at the injection sites among animals of all groups indicating an effect induced by the injection procedure. Twenty-one days after the last injection, no signs of local reactions were apparent at the injection site, except for localized myodegeneration in a single animal treated with the licensed vaccine. In both Hib conjugate vaccine–treated groups, the mean body weights at the end of the study were lower in comparison with that of the placebo group, but the differences were only statistically significant for female rats (5%). Body weights at day 63 and mean food conversion efficiencies at day 77 are summarized in Table 12. Other effects were observed alternatively in rats treated with the test and licensed vaccine: increase in neutrophils and lymphocytes, decrease in the albumin/globulin ratio, weight increase of the popliteal lymph nodes. These effects may be indicative for a normal immune response following vaccination. The degree and incidence of these effects were comparable between both groups and were not statistically significant.

Table 12: Study results of the repeated-dose toxicity study with *Haemophilus influenzae* b vaccine, summary of injection site reactions and body weights at day 63 (sacrifice of main group) and mean food conversion efficiencies at day 77 (total study period).

<table>
<thead>
<tr>
<th>Injection site reaction</th>
<th>Mean body weights (d63) (g) ± sem</th>
<th>Mean food conversion efficiency² ± sem (d0-d77)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythema</td>
<td>Haematoma</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Hib Vaccine</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Licensed Hib Vaccine</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviations: d, day; sem, standard error of the mean.

(1) Number of animals showing the observation for both male (♂) and female (♀) rats.
(2) Gram weight gain/g food consumed.
In addition, a slight increase was observed in the weight of the draining popliteal lymph nodes only in animals treated with the test vaccine. This effect was no longer observed at day 21 after the last vaccination and was likely caused by the activated immune response following vaccination. Table 13 summarizes the most important Haematology and Clinical chemistry data.
Table 13: Study results of the repeated-dose toxicity study with *Haemophilus influenzae* b vaccine, summary of main Haematology and Clinical chemistry data

<table>
<thead>
<tr>
<th>Time point</th>
<th>Placebo</th>
<th>Test Hib vaccine</th>
<th>Licensed Hib vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>N m/f</td>
<td>d1</td>
<td>d29</td>
<td>d57</td>
</tr>
<tr>
<td>Haematology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total white blood cells (*10^9/L±sem)</td>
<td>9.7 ±0.6</td>
<td>11.5 ±0.6</td>
<td>12.2 ±0.6</td>
</tr>
<tr>
<td>Eosinophils (%±sem)</td>
<td>0.5 ±0.3</td>
<td>0.6 ±0.3</td>
<td>1.6 ±0.3</td>
</tr>
<tr>
<td>Neutrophils (%±sem)</td>
<td>8.3 ±1.7</td>
<td>6.0 ±1.7</td>
<td>7.2 ±1.7</td>
</tr>
<tr>
<td>Lymphocytes (%±sem)</td>
<td>88.3 ±1.3</td>
<td>91.6 ±1.3</td>
<td>90.9 ±1.3</td>
</tr>
<tr>
<td>Monocytes (%±sem)</td>
<td>3.0 ±0.2</td>
<td>2.1 ±0.2</td>
<td>1.6 ±0.2</td>
</tr>
<tr>
<td>Albumin/Globulin ratio (±sem)</td>
<td>1.72 ±0.03</td>
<td>1.68 ±0.02</td>
<td>1.58 ±0.02</td>
</tr>
</tbody>
</table>

Statistics: (Two-Sided) Anova + Dunnett’s tests, * P≤5%; ** P≤1% (both bold).
Abbreviations: d, day; N m/f, Number of rats male/female; sem, standard error of the mean; P, probability value.
Immunogenicity
Blood was collected at necropsy from all animals and used to evaluate the immune response after vaccination by determining the level of serum IgG specific for Hib-polysaccharide (PRP). PRP-specific IgG was detected in both sera from the test Hib group and the licensed vaccine group, but not in sera from the placebo group (Figure 26). Some difference was seen between male and female rats, especially for the test vaccine. This difference was not further investigated since the primary objective of the assay was to prove that the Hib-vaccines were immunogenic in rats.

Figure 26: PRP- specific IgG antibodies in serum after vaccination. Rats were vaccinated with 3 doses of a licensed Hib vaccine (square) and the test Hib vaccine (triangle) on day 0, 28, and 56. As a negative control a group of rats was vaccinated using a placebo (circle). The level of PRP- specific IgG antibodies were measured in a rat-anti-PRP ELISA at OD450.
Discussion/Conclusions

Technology Transfer has been proven to be one of the fastest routes in getting access to know-how. Intravacc has a longstanding history in supporting manufacturers to establish production capacity by providing access to the required technologies. Transferring the technology of Hib conjugate vaccine has helped a number of emerging manufacturers to establish local Hib conjugate vaccine manufacturing capacity, to get access to innovative conjugation technology and to acquire more regulatory expertise on (Hib) conjugate vaccines. The strategy followed by the technology transferee (Intravacc) in the Hib technology transfer project was to develop a pilot-scale process as soon as possible, to generate all necessary supportive data and to provide the data to all technology transfer partners. This included immunogenicity and safety data, generating thereby a proof of concept for the Hib technology to be transferred independent of the site were the product is produced, an approach that can be followed for other technology transfer programs. In this case, it was not needed to generate preclinical data by each individual partner, a time and money saving approach. Knowing that some local authorities may not accept this WHO approach, intensive communication with the Indonesian regulatory authorities was needed before being able to start with the preclinical study in the Netherlands. Ultimately, the Indonesian partner was able to start with the clinical trials without repeating the preclinical study in Indonesia [108]. Further, both the Indian partners and the Chinese partner were able to rely on this preclinical data when submitting their clinical plans and registration dossiers to the local authorities. Rats were chosen because of their proven suitability as an animal model for toxicological studies and for Hib conjugate vaccine immunogenicity studies [153,154]. Beside rats, several other animal models have been described in the literature to be used successfully to study the immunogenicity of Hib vaccines, including mice, guinea pigs, and rabbits [155,156]. Despite that, there are a limited number of publications describing the preclinical evaluation of Hib conjugate vaccines [140, 155, 157-159]. Because of the absence of major abnormalities in the appearance, general condition, growth, food consumption, hematological and clinical chemistry values, organ weights and gross necropsy, and histopathological findings, it can be
concluded that the investigational Hib conjugate vaccine was equally well tolerated by the animals as the licensed vaccine. In addition, both the new Hib conjugate vaccine and the licensed vaccine induce PRP-specific IgG antibodies. The animal model used during this study is thus considered to be suitable for preclinical evaluation of Hib conjugate vaccines. The Hib conjugate vaccine produced using Intravacc’s technology is already licensed and prequalified as part of several combination vaccines, containing other childhood vaccines such as DTP [18, 148, 149]. Therefore, it can be stated now only with near certainty that the approach followed in this Hib technology transfer project can be followed for other technology transfer projects, provided that the local regulatory authorities are involved from the very beginning of the project, including in the design of the preclinical study.

Materials and Methods

The repeated-dose toxicity and local tolerance study was performed by an external and independent research laboratory: TNO Nutrition and Food Research in Zeist, the Netherlands, and conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Principles for Good Laboratory Practice [160]. Table 14 summarizes the study design including the most important observations, analyses, and measurements performed during the study.

Vaccines

Both the investigational Hib conjugate vaccine (lot number FPH012) and the licensed Hib conjugate vaccine (lot number U1059, RIVM) were stand-alone freeze-dried vaccines consisting of Hib capsular polysaccharide conjugated to Tetanus Toxoid (PRP-T). Both Hib conjugate vaccines had the same composition: an equivalent of 10 mg polysaccharide and approximately 20 mg tetanus toxoid per human dose in the presence of sucrose. The test Hib vaccine used was a clinical lot produced by Intravacc and Bio Farma; based on the process developed by Intravacc and transferred to the technology transfer partners [61,62] meeting all WHO and EP requirements [56, 57, 113]. The Hib polysaccharide and tetanus toxoid were produced and purified at Bio Farma, the polysaccharide was conjugated to tetanus toxoid at Intravacc.
Tetanus toxoid was routinely produced by Bio Farma and was meeting all WHO Hib requirements [56] for a carrier protein. The polysaccharide was conjugated to tetanus toxoid at Intravacc using the conjugation method originally described by John Robbins and collaborators (National Institutes of Health, Bethesda, USA) [53]. The purified concentrated conjugate bulk was sent to Indonesia for formulation, freeze-drying, and filling [108]. Part of this (pre)clinical lot was sent to the Netherlands to be used for the preclinical study and part was used at a later stage for a clinical study in Indonesia under the auspices of Bio Farma [108].

**Table 14**: Study design of the repeated-dose toxicity study with *Haemophilus influenzae* b vaccine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Signs looked for</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical observations</td>
<td>Erythema</td>
<td>0-63</td>
</tr>
<tr>
<td></td>
<td>Haematoma</td>
<td>At least once daily</td>
</tr>
<tr>
<td></td>
<td>Oedema</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body weights</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Food intake</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skin changes</td>
<td></td>
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<tr>
<td>Haematology</td>
<td>haemoglobin</td>
<td>1, 29, 57, 63, (77)</td>
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<tr>
<td></td>
<td>packed cell volume</td>
<td></td>
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<td></td>
<td>red blood cell count</td>
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<td></td>
<td>reticulocytes</td>
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<td></td>
<td>thrombocyte count</td>
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<td></td>
<td>total white blood cell count</td>
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<td></td>
<td>differential white blood cell count</td>
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<td></td>
<td>Prothrombin time</td>
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<td></td>
<td>mean corpuscular volume</td>
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<td></td>
<td>haemoglobin</td>
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<td></td>
<td>mean corpuscular</td>
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<td></td>
<td>concentration</td>
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<td>Clinical Chemistry</td>
<td>alkaline phosphate activity</td>
<td>1, 29, 57, 63, (77)</td>
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<td></td>
<td>alanine aminotransferase activity</td>
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<td>aspartate aminotransferase activity</td>
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<td></td>
<td>gamma glutamyl transferase activity</td>
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<td></td>
<td>albumin</td>
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<td>ratio Albumin to Globulin</td>
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<td>creatinine bilirubin</td>
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<td>Cholesterol</td>
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<td>sodium</td>
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Each vial of the freeze-dried Hib vaccines, containing the equivalent of one human dose, was reconstituted in 0.25 mL phosphate-buffered saline (PBS, Gibco BRL, Life Technologies Ltd). Reconstituted vaccines were used within 4 h after reconstitution. PBS was used for the control animals (placebo).

Test animals
Fifty-four animals (27 males and 27 females) young adult Wistar outbred rats (Charles River) were obtained from a colony maintained under Specific Pathogen Free (SPF) conditions. The age of the animals at the time of commencement of the study was 8 to 10 wk. The animals were randomly assigned to one of the following groups: test Hib conjugate vaccine, placebo, and licensed vaccine. To correct for differences in the mean weight of animals between groups after computer randomization, 2 animals from the test group had to be exchanged with 2 animals from the group to be used for the licensed vaccine. None of the animals showed any signs of illness and/or anomalies. A serological investigation of the microbiological status was also conducted. All animals examined were assigned to the study. The animals were acclimatized to the laboratory conditions for 10 d before the start of the study.

Injections
Rats received a total of 3 injections with the test Hib conjugate vaccine, the placebo, or the licensed Hib conjugate vaccine. The vaccines and the placebo were administered on day 0, 28, and 56 by intramuscular route alternatingly in the gastrocnemius or thigh muscle in the hind legs. At each immunization, 0.125 mL of the Hib conjugate vaccine, the placebo, or the reference vaccine was injected in both the left and right limb, which in total was equal to one human dose. The following parameters were assessed during the study: clinical signs, body weight, food intake, hematology and clinical chemistry of the blood, examination at necropsy for gross macroscopic changes, organ weights, and histopathology of various tissues and organs (including the injection site). Of each group a subgroup of animals was sacrificed on day 63, consisting of 12 animals (6 males and 6 females) per dose group. The remaining animals (3 males and 3 females/group) were sacrificed on day 77 (recovery group).
Collection of blood
Blood samples were collected at necropsy from all animals and transferred to the Laboratory of Control of biological products (LCB, at that time part of NVI), for evaluating the immunogenicity of the vaccines by determining antibody production against Hib-polysaccharide. Serum was prepared by allowing blood to coagulate for approximately 2 h at 37 °C and overnight at 4 °C. After coagulation the serum was separated by centrifugation. The serum samples were stored at -30 °C after inactivation for 30 min at 56 °C.

Serology
An enzyme-linked immunosorbent assay (ELISA) was performed to measure antibodies specific for Hib-polysaccharide. For this ELISA, Immulon II plates were coated with Human Serum Albumin conjugated to Hib-polysaccharide (HbO-HA). 2-fold serial dilution ranges were made of the rat sera, starting at 1/10 till 1/1280. Following overnight incubation at room temperature, 100 mL of a peroxidase-labeled sheep-anti-rat IgG solution, in BSA (1% w/v) and Tween 20 (0.05% v/v), was added to each well and the plates were incubated for one hour at 37 °C. After adding 100 mL Tetramethyl-benzidine (TMB) substrate (in sodium acetate) to each well, the plates were incubated for 10 min at room temperature; sulphuric acid was used to stop the reaction. The Optical Density (OD) was measured at 450 nm using an ELISA reader. On each ELISA plate, a positive control was tested. As positive control a pool of rat sera was used, these rats were immunized using 2 injections at day 0 and day 7 of the licensed Hib vaccine.

Reagents and chemicals
The following reagents and chemicals were used for the ELISA: HbO-HA: Lederle Praxis Biologicals, lyophilized, 1 mg/vial, obtained from NIBSC.
BSA: Sigma A-4503.
Tween 20: Merck 822184.
TMB: Sigma T2885.
Sheep anti rat IgG solution: Amersham.
Sulphuric acid, concentrated: H2SO4, MW 98.07, 95–97%, (Merck no. 100731).
Lessons learned during the development and transfer of technology related to a new Hib conjugate vaccine to emerging vaccine manufacturers

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C. Boog
S. Jadhav
H. Kreeftenberg
Abstract

The incidence of *Haemophilus influenzae* type b (Hib) disease in developed countries has decreased since the introduction of Hib conjugate vaccines in their National Immunization Programs (NIP). In countries where Hib vaccination is not applied routinely, due to limited availability and high cost of the vaccines, invasive Hib disease is still a cause of mortality. Through the development of a production process for a Hib conjugate vaccine and related quality control tests and the transfer of this technology to emerging vaccine manufacturers in developing countries, a substantial contribution was made to the availability and affordability of Hib conjugate vaccines in these countries. Technology transfer is considered to be one of the fastest ways to get access to the technology needed for the production of vaccines. The first Hib conjugate vaccine based on the transferred technology was licensed in 2007, since then more Hib vaccines based on this technology were licensed. This paper describes the successful development and transfer of Hib conjugate vaccine technology to vaccine manufacturers in India, China and Indonesia. By describing the lessons learned in this process, it is hoped that other technology transfer projects can benefit from the knowledge and experience gained.
Introduction

The World Health Organization (WHO) estimated *Haemophilus influenzae* type b (Hib) to cause at least 3 million cases of serious disease and 400,000–700,000 deaths each year in young children in 1998 [32], almost all in developing countries. Hib was the leading cause of non-epidemic bacterial meningitis and the second leading cause of bacterial pneumonia [161]. Developing countries have been hesitant to introduce the vaccine in their NIP’s because of its relatively high price and their limited awareness about the disease burden. In addition, in the absence of sufficient clinical evidence about the effectiveness of Hib vaccine in developing countries there was no clear strategy of WHO about the use of the vaccine in these countries [32]. Local production of Hib vaccine would solve the supply problem and lower the price. However the Hib technology was not accessible to Developing Countries Vaccine Manufacturers due to patents and proprietary know how about the Hib production technology. Given the longstanding history of Intravacc (The Institute for Translational Vaccinology, originating from the former Research and Development Unit of the National Institute of Public Health (RIVM) and the Netherlands Vaccine Institute (NVI)) in technology transfer [40, 86, 109, 141, 162–167], it was decided to develop a process for the production of a Hib conjugate vaccine and transfer the technology to manufacturers in developing countries. The Hib technology transfer project, started at Intravacc in 1998. The primary objective was to develop and transfer Hib conjugate vaccine technology to a number of emerging manufacturers in Indonesia, India and China in order to ensure a sustainable supply of affordable Hib conjugate vaccine. This project was unique compared to other technology transfer activities organized by Intravacc until then. The main difference being that the technology that had to be developed was not directly needed for the Dutch Immunization Program. The project was mainly financed by the technology transfer partners after a seed capital was made available by Intravacc to start. This paper will focus on the lessons learned within the framework of the Hib conjugate vaccine development and technology transfer project at Intravacc.
**General aspects**

Project feasibility and project management are two aspects that can be considered general, relatively independent of the project type, but of great influence on the progress.

**Project feasibility**

Before starting the Hib project at Intravacc a feasibility study was performed in order to evaluate the national and international importance of the Hib project and to see if it was possible to start such a project. All technologies transferred until then were already being used routinely to produce and/or test vaccines for the Dutch NIP. What made the Hib project different was that it was solely intended for technology transfer. As part of the feasibility study (Figure 27), different aspects were studied: freedom-to-operate, Prior Knowledge, expected cost price of the vaccine, project continuity including impact on on-going projects, funding, potential partners and available market.

![Figure 27: Schematic representation of the feasibility study performed before starting the *Haemophilus influenzae* type b project at Intravacc: by studying the project feasibility at an early stage, taking several aspects into consideration (including freedom-to-operate, prior knowledge, vaccine cost price, position of the project both for Intravacc and for the partners, funding, available market and potential partners), it was possible to define a realistic Product Target Profile and thereby acceptable project deliverables.](image-url)
Based on this feasibility study it was concluded that it was possible to develop a non-infringing, scalable, transferable, robust and cost effective Hib process within 2–3 years and to transfer the process and related quality control tests and documentation to several partners. Based on the cost-price calculations performed at that stage of the project it was concluded that a very competitive cost price was possible (≤1.00 $/dose). The Hib project was considered feasible and the main preconditions were defined:

- Assuming that the potential partners did not have any Prior Knowledge related to polysaccharide/conjugate vaccines, the Hib vaccine had to be developed solely by Intravacc.
- Technology transfer had to start at an early stage of the project to allow the partners to make certain choices that may be of importance for their own situation: depending on the partner; attention was required for retention of staff, setting up a research and development infrastructure and/or building a production facility.
- Intravacc had Prior Knowledge on polysaccharide and conjugate vaccines [28, 168–170], this knowledge had to be evaluated and used as much as possible for the benefit of this project.
- The process to be developed had to be non-infringing with existing patents.
- The concentrated conjugate bulk had to be stable in order to allow the possible production of a liquid stand-alone or combination vaccine at a later stage; therefore stability testing of intermediate products had to be part of the project.
- The process had to be as much as possible free from raw materials from animal origin.
- The process had to be scalable since it was decided that not all unit operations could be scaled up at Intravacc. This was mainly due to a limited availability of large-scale capacity because of other high-priority projects.
- The vaccine had to comply with both European Pharmacopeia and WHO requirements [56, 57, 113]; local requirements did not exist at that time.
- The process had to use as much as possible “conventional” equipment that is already available at partner’s facility.
It was decided to develop a PRP-T vaccine (Hib polysaccharide conjugated to tetanus toxoid), as similar vaccines were already licensed and extensively used in various NIP’s worldwide. In addition, lot release criteria were already available for this vaccine and the conjugation process was published by Robbins [54, 171] and not patented. Further, tetanus toxoid was being routinely produced by most of the potential partners and clinical trials, to demonstrate the effectiveness of this vaccine, were relatively simple to perform as a serological correlate of protection was available.

**Project management**

In order to manage the project in an efficient way a dedicated project team was assigned at Intravacc (seven FTE’s for at least 2–3 years). Further, a project management software (“Microsoft-project”) was used to plan and manage the tasks and activities related to the project. An integrated project planning, including both the activities at Intravacc and at partner’s site, was updated regularly in coordination with each individual partner. The deliverables, milestones and go-no-go decisions were clearly defined in the planning and discussed with the partners. Further, both Intravacc’s and partner’s management team had regular meetings in order to discuss the progress of the project. Thus, in general the management approach followed was partner- and regulatory-driven: every decision taken was evaluated from an applicability point of view, at the partner’s site, as well as from a regulatory point of view and possible impact on the time-to-license.

**Project specific aspects**

Beside the general aspects mentioned above, some specific aspects were found to be very important for a successful Hib technology transfer project. These aspects are mainly related to the nature of this project.

**Intellectual property**

The Hib technology that was developed and transferred had to be non-infringing with existing patents in order to guarantee freedom-to-operate after the completion of technology transfer. A preliminary patent search took place at a very early stage of the project followed by a regular consultation of the relevant patent literature during different
development stages of the process. A couple of patents were considered to be of relevance in the development of the Hib process. Several conjugation methods used in the production of licensed Hib conjugate vaccines were already patented [172–176], so these methods were not used. A conjugation method patented by Intravacc was not used as this method was never applied for the production of a licensed vaccine [112]. A conjugate purification method that gave relatively high yields appeared to be patented as well [82], a different purification method had to be chosen, accepting thereby a lower process yield. Processes for the formulation of combination vaccines including Hib were also patented [177]. Therefore, the partners were advised to stay as close as possible to their own DTP and/or DTP-HepB formulation, which was also preferred for regulatory reasons. Ultimately, an infringing process was developed and implemented [108]. It was decided to file a “protective” patent application on the Hib process developed by Intravacc since the innovative aspects of this specific process were not covered in the (patent) literature. This was also done in order to protect this new knowledge from being patented by a third party and to guarantee the access of the Hib partners to the technology developed by Intravacc during and after the finalization of the transfer of the technology. In the meantime, the patent is granted in all countries where one or more of the Hib partners are present, in Europe and US. The partners were given a license on this patent [61, 62, 178].

Regulatory aspects
When Intravacc started with the Hib project, a number of safe and effective Hib conjugate vaccines were already licensed [179]. One of these vaccines (PRP-T) was produced according to the Robbins conjugation method [54, 171]. As this technology was not patented and already well-established resulting in safe and effective Hib conjugate vaccines, this conjugation method was selected in order to produce a ‘me-too’ product, following the published information. Further, lot release criteria had been already established for this Hib conjugate vaccine [56, 57, 113]. In case a new conjugation method would have been chosen, which was technically quite feasible, as Intravacc owned a patent, new product specifications should have been investigated in extensive clinical trials to support the lot release criteria. That was
Lessons Learned during development and technology transfer of a Hib vaccine

Considered as unethical, costly and time consuming. Further, during the process of development and transfer of the Hib technology it was noticed that local regulatory authorities did appreciate additional information on conjugate vaccines. This was provided by Intravacc through Hib training courses and workshops on quality control and lot release aspects in collaboration with WHO. In addition, local regulatory authorities were involved in crucial decision-making’s from the very beginning of the project.

Strategic aspects

Investing in a Hib conjugate vaccine was very critical for most of the emerging vaccine manufacturers, since DTP-HepB-Hib combination vaccines were replacing DTP and DTP-HepB. Further, in most developed countries acellular pertussis vaccine was being introduced while in developing countries, whole cell pertussis was still the vaccine of choice. To anticipate any shortage of combinations based on whole cell pertussis vaccine, local production of these vaccines was needed. This was the incentive for many emerging vaccine manufacturers to expand their product portfolio by introducing new combination vaccines [41]. Further, it was very important to decide on the selection of potential partner(s).

The first selected partner was Bio Farma, Indonesia. Bio Farma’s management was very committed to the Hib project and competent staff was available. In addition, Bio Farma was WHO pre-qualified and had thereby access to the global market. Intravacc decided together with Bio Farma about the Target Product Profile: a freeze-dried stand-alone Hib conjugate vaccine to be combined with a quadravalent vaccine (DTP-HepB) just before injection. In addition it should be possible in the future to develop a liquid stand-alone or liquid pentavalent (DTP-HepB-Hib) combination vaccine starting with concentrated bulk conjugate. Since the partner was willing to produce the Hib vaccine both for local as well as for global (through UNICEF) use, it was decided that the product should meet the European Pharmacopeia and WHO requirements. Local requirements did not exist at that time. None of the potential partners had previous experience with conjugation technology, getting access to Hib conjugation technology have given Hib partners the opportunity to get familiar with conjugate vaccines in general and invest in the necessary infrastructure [180,181]. Further, one of the conditions to
enter into a Hib technology transfer agreement with Intravacc was
to invest in the research and development infrastructure locally. As
Intravacc is not a routine producer of Hib vaccine, troubleshooting had
to be taken over by the partners after a couple of years from finalizing
the technology transfer. In addition, Intravacc identified many triggers
for further process optimizations, but not all could be implemented
because of the relatively short duration of the project. Partners were
therefore advised on aspects related to optimization and scaling up of
the process. Intravacc did develop the process at pilot scale (cultivation
up to a 500 L bioreactor scale and conjugation up to 20,000 doses)
including related quality control tests and transferred this technology
to all partners. The partners were responsible for further scaling up
in their own facilities. The large scale technology developed by the
individual partners was considered to be proprietary know how and not
shared between partners. Intravacc was involved in the implementation
and scaling up of the process at partner’s site. The choice to follow this
approach was made taking the available resources into account; a turn-
key project up to commercial scale (including all clinical data) would
take more time and money and was only relevant in case the product to
be developed was to be licensed through Intravacc. Further, Intravacc
generated relevant materials, documentation and data, including
preclinical data, and transferred all the materials and information to the
individual partners. Comparability of the batches produced at different
scales and different production sites was assessed based on the lot
release criteria. Whenever needed, data were duplicated by Intravacc
and additional tests were used to assess consistency and comparability
of the batches. To facilitate the comparability studies performed by
Intravacc, all the technology transfer partners used (raw) materials of
the same quality (documents related to (raw) material specifications
were provided by Intravacc in the framework of the Hib project). Further,
Intravacc provided the individual partners with seed lot, reference
samples and reagents and Bio Farma (the first partner) produced the
first clinical lot (used for (pre)clinical studies) in close cooperation
with Intravacc [108], this lot was used as a “golden standard” for all
technology transfer partners.
Financial aspects
Getting external financial support was not possible. After exploring several possibilities, it was clear that receiving financial support would have been seen as an unfair competition vis-à-vis Hib conjugate vaccine manufacturers who already had a licensed product. Consequently the whole project was financed by the partners and a seed capital from Intravacc.

Marketing aspects
The Global Alliance for Vaccines and Immunization (GAVI) has played a very important role in creating a Hib market, resulting in the routine use of the vaccine in many developing countries. 83% of the GAVI eligible countries were using Hib conjugate vaccine in 2009 [13, 182]. This approach has attracted new manufacturers and created more competition, to supply Hib vaccine at a lower price to developing countries. Although GAVI has played an important role in making the necessary funds available, to allow UNICEF to buy the vaccine (the so called “pull strategy”), GAVI did not support new manufacturers to develop Hib vaccines (“push strategy”). This was done by Intravacc by means of the Hib project. At least two partners of the Hib project contribute significantly to the global supply of Hib vaccine to UNICEF. So both strategies are needed to achieve a sustainable supply of affordable and quality vaccines to developing countries. Further, the availability of data from multiple burden assessment studies followed by the publication of a revised WHO position paper [34] in 2006, recommending global use of Hib vaccine, played a critical role in a growing demand for Hib vaccine in developing countries.

Technology Transfer aspects
The technology transfer approach chosen for this project was one that covers all the phases of a technology transfer process: preparation, start-up, implementation, evaluation and trouble-shooting (Figure 28). During the preparation phase enough information was exchanged in order to have a license agreement in place, the partner had to make sure that funding is secured and that the continuity of the project could be guaranteed. This was followed by appointing a dedicated project team, preparing an integrated project planning and a work
Figure 28: Schematic representation of the “overall technology transfer approach” followed for the *Haemophilus influenzae* type b project at Intravacc including main activities: this approach covered all the phases needed for a technology transfer process; preparation, start-up, implementation, evaluation and troubleshooting.
Lessons Learned during development and technology transfer of a Hib vaccine

Intravacc’s Hib technology was ultimately transferred to four different partners [108]. Bio Farma (BF, Indonesia), Biological E Ltd. (BE, India), Serum Institute of India (SII, India) and Shanghai Institute of Biological Products (SIBP)/Glovax (China). Many other vaccine manufacturers showed interest in this project but it was decided from the very beginning to transfer the technology to a selected group in order to have enough focus and to achieve the preset goals. Unnecessary competition at an early stage would not encourage emerging manufacturers to invest in a new product. Further, within the framework of the Hib project, people from Birmex (Laboratorios de Biologicos y Reactivos de Mexico) and UMC (Universitair Medisch Centrum Utrecht) were trained on more generic conjugation know-how. In addition, a Hib course was developed in collaboration with WHO to train both production and regulatory staff from several countries on quality control aspects related to conjugate vaccines. During the technology transfer of Hib technology from Intravacc to the individual partners, a couple of key features were determined that can be considered to be decisive factors for the success of the Hib technology transfer project: commitment
of all personnel involved, including the management, competent and dedicated team both at Intravacc and at each individual partner and clear communication between Intravacc and the partners including timely sharing of data and information.

Discussion

The Hib project was the first technology transfer project at Intravacc which was solely meant for technology transfer purpose and not for the Dutch National Immunization Program. For that reason many lessons were learned in the course of this project. A committed, multidisciplinary, competent and dedicated team was needed both at the technology owner and at the technology recipient side. Having such a team at Intravacc made it possible to develop a non-infringing, scalable, transferable, robust and cost effective Hib production process including quality control tests, within 2–3 years. In addition, preclinical data were generated within 4 years realizing a proof-of-concept for the process to be transferred. A committed management willing to invest in the new product was crucial in order to have a fast and efficient technology transfer. An example was Serum Institute of India (SII). The transfer of technology started in 2001 and already in 2007 SII was able to license and prequalify Hib conjugate vaccine. To achieve this, SII had to invest in its production facilities and research and development infrastructure. An open communication with the Hib project team in SII did help to insure an efficient transfer of the technology. The delay that other partners experienced was mainly because of the difficulties to keep competent/trained personnel and managerial issues. In line with the primary objective of the Hib project; two partners have played an important role in the global price reduction and sustainable supply of large quantities of Hib conjugate vaccine: Bio-logical E Ltd. (BE) and Serum Institute of India Ltd. (SII). SII supplies pentavalent vaccine (DTP-HepB-Hib) through GAVI for $1.75 starting from 2011 and BE decided to cut the GAVI price to $1.19 per dose starting from 2013. SII and BE are committed to supply about 600 Million doses of this vaccine in the coming years. A decade ago, GAVI had only one European supplier and the price of pentavalent vaccine was $3.56 per dose [15,183]. Through this Hib project, Intravacc’s partners were
provided with general conjugation knowhow, and related quality control tests, resulting in a broadening of their research and development portfolio with several conjugate vaccines including meningococcal and pneumococcal vaccine, beside several Hib combination vaccines. Emerging vaccine manufacturers consider technology transfer to be the fastest route to develop a vaccine and to get access to the know-how needed [41]. In general technology transfer has been proven to be an efficient tool in increasing a sustainable vaccine supply and consequently in improving population health [151]. The approach followed for this Hib technology transfer project was successful and can be followed for other technology transfer projects. Before starting the project an extensive and detailed feasibility study was needed in order to evaluate most important aspects: freedom-to-operate, Prior Knowledge, expected cost price of the vaccine, project continuity including impact on on-going projects, funding, potential partners and available market. Based on the outcome of the feasibility study, key factors influencing the success and effectiveness of the project were defined. By defining most of the elements having an impact on the project and identifying the project priority position for both the transferee and transferor, it was possible to decide on the project preconditions and thus the mechanism to be followed during the process development and technology transfer. The partner selection has taken place at an early stage and was mainly based on the management commitment to the project, the capability to keep competent personnel and the willingness to invest in the new product. The Target Product Profile and thereby the product requirements to be met were decided in collaboration with the technology transfer partners and could be partly based on the anticipated market. The availability of an integrated project planning facilitated the communication between the technology owner and recipient. Further, the data, materials and documentation that was generated and transferred by the technology owner was discussed with the local regulatory authorities at an early stage of the project to avoid unnecessary delays in the licensure of the vaccine. Training of staff from local regulatory authorities was considered because the product was relatively new.
Economical evaluation of a production process for a new *Haemophilus influenzae* type b conjugate vaccine

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S. Ghimire  
R. Tiesjema†
Introduction

Since 1987, several *Haemophilus influenzae* type b (Hib) conjugate vaccines have been licensed for use in infants. Safety and immunogenicity of Hib conjugate vaccines is demonstrated worldwide [85] including in low- and middle-income countries. For example in Gambia and Uganda [86, 87] near elimination of the Hib disease was achieved with high vaccination coverage rates. Although most of the high-income countries introduced this safe and immunogenic vaccine in their national immunization programs quickly after licensure, low- and middle-income countries (supported by GAVI) started introducing the vaccine one decade later. The slow uptake of Hib vaccine in these countries was mainly due to the relatively high cost.

The National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM, in Bilthoven, The Netherlands) started a Hib project in 1998 to address the global shortage of Hib vaccine and the slow introduction of this vaccine in low- and middle-income countries. By developing a cost-effective and commercially viable production process for a Hib conjugate vaccine and transferring this process to emerging vaccine manufacturers in low- and middle-income countries a sustainable price reduction of Hib vaccine could be assured. The cost of goods (COGs) target set before the start of the Hib project was $US 1,- per dose whereas the UNICEF price was >$US 3,- per dose at that time [85]. Nowadays, the Hib-technology is fully developed and transferred to vaccine manufacturers in India, Indonesia and China. Hib conjugate vaccine is being commercialized by Intravacc's\(^1\) (Institute for Translational Vaccinology) Hib technology transfer partners.

In this chapter first the initial calculation of the COGs\(^2\) of this new Hib conjugate vaccine will be discussed. This calculation was performed as part of the project feasibility study using Excel (before starting the Hib project) and was based on a number of assumptions. Second, more recently the Hib production process was simulated using mathematical

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\(^1\) RIVM tasks related to technology transfer were transferred to the Netherlands Vaccine Institute (NVI) between 2003 and 2010. In 2013, these tasks were transferred to Intravacc (Institute for Translational Vaccinology).

\(^2\) The COGs being the direct cost attributable to the production of Hib conjugate vaccine, indirect expenses such as distribution and marketing cost are not included.
modeling tools and COGs was recalculated. Besides confirmation of COGs, the Hib process model allowed to perform a sensitivity analysis and to investigate possibilities for further price reduction.

Initial calculation of the cost of goods of a new *Haemophilus influenzae* type b conjugate vaccine

The following assumptions were made during the initial calculation (before the start of the Hib project) of the expected COGs of the new Hib conjugate vaccine:

- The technology transfer partner was assumed to produce tetanus toxoid routinely at approximately a scale of 800 liter with a yield of 25 Lf/ ml. The partner produced tetanus in a dedicated facility (following WHO and EP guidelines).
- The technology transfer partner would not need to build a new facility for the production of Hib vaccine. The Hib polysaccharide can be produced campaign wise using for example a pertussis facility. All selected partners would have experience with producing DTP (Diphtheria, Tetanus and Pertussis).
- The yield of purified polysaccharide was set equal to 20 mg per liter.
- The conjugate vaccine to be produced was assumed to contain 10 µg polysaccharide and 20 µg tetanus toxoid (equal to 10 Lf) per single human dose.
- The Hib vaccine to be produced would be lyophilized, 20-dose presentation using 11 ml vials.
- The lyophilization run would take one week and a lyophilizer with a capacity of approximately 25,000 of 11 ml vials was considered to be needed.
- One production batch was set equal to 500,000 doses (25,000 vials).
- A weekly rate of about US$ 3000,-/ person was considered concerning labor cost. This includes overhead, equipment and facility depreciation.
- Estimation of material cost was based on experience with other (conjugate) vaccines.
The production process of this new *Haemophilus influenzae* type b vaccine was considered to be comparable to that of a meningococcal vaccine [27, 109].

Taking these assumptions into account the expected COGs was initially, before the start of the Hib project in 1998, calculated using Excel. To calculate the COGs, the production process for the Hib conjugate vaccine was divided in the following phases: polysaccharide production, tetanus toxoid production, conjugation, lyophilization and final lot production. Material and labor costs were calculated for each phase, calculating thereby the total cost for each individual phase. Further, cost related to quality control and quality assurance were also calculated in the same way (total cost is the sum of material and labor cost). By dividing the total cost for a single production batch by the number of doses assumed per batch (500,000), the COGs of one single human dose was calculated. Contribution of quality control and quality assurance, polysaccharide production, conjugation, final lot and tetanus toxoid production to the COGs was 36%, 20%, 20%, 5% and 3% respectively. Further, lyophilization related cost was equal to 16% of the COGs. The calculated COGs of one single human dose of a lyophilized vaccine was US$ 0.67, - whereas that of a liquid vaccine was US$ 0.56, -.

**Cost of goods analysis provided using the process model of a new *Haemophilus influenzae* type b conjugate vaccine**

The production process of a Hib conjugate vaccine was recently simulated using SuperPro Designer v9.0 [129] from Intelligen, Inc. (New Jersey, USA). First, a mathematical model was prepared for the ‘baseline’ process, being the process developed and transferred by RIVM/NVI to the technology transfer partners [108] in order to produce a lyophilized Hib conjugate vaccine. Later a second model was prepared including some process optimizations (‘optimized’ model). Both models were used to perform an economical evaluation of the Hib process. Economical evaluation of the Hib process and COGs calculation was performed using several financial indicators of which capital expenses
CAPEX) and operational expenses (OPEX) are key indicators. The cost of equipment was estimated by the software using build-in cost correlations based on data from vendors and literature. Capital expenses were calculated based on equipment cost and using various multipliers which are both equipment (e.g., installation cost) and process specific (e.g., buildings cost). Assuming that the Hib vaccine was produced in an existing facility, the CAPEX would mainly consist of direct cost related to the equipment including installation, piping, electrical... etc.

In table 15 the distribution of the cost of equipment used during the production of Hib conjugate vaccine using the ‘baseline’ process are described. Based on this ‘baseline’ model the fill and finish equipment is the biggest cost contributor (39% of the total equipment cost) followed by the bioreactors (21%) and chromatography equipment (19%).

Operating cost or operational expenses (OPEX) are costs directly related to the manufacturing of the vaccine, which include raw materials, consumables, labor-dependent, laboratory/QC/QA, utilities, waste treatment and other facility dependent costs. Costs associated with raw materials (e.g. tetanus toxoid and cultivation media) and consumables (e.g. empty vials) contributed significantly to the total operating cost, with 42% and 22% respectively (table 15).
Table 15: Equipment cost and operational cost breakdown of the ‘baseline’ process obtained using SuperPro Designer v9.0 from Intelligen to simulate Haemophilus influenzae type b conjugate vaccine process.

<table>
<thead>
<tr>
<th>Cost item</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equipment cost</td>
<td></td>
</tr>
<tr>
<td>Chromatography</td>
<td>19</td>
</tr>
<tr>
<td>Microfilter(s)</td>
<td>4</td>
</tr>
<tr>
<td>Diafiltration unit(s)</td>
<td>4</td>
</tr>
<tr>
<td>Centrifuge(s)</td>
<td>11</td>
</tr>
<tr>
<td>Bioreactor(s)</td>
<td>21</td>
</tr>
<tr>
<td>Containers</td>
<td>2</td>
</tr>
<tr>
<td>Filling</td>
<td>3</td>
</tr>
<tr>
<td>Lyophilizer</td>
<td>26</td>
</tr>
<tr>
<td>Capping</td>
<td>8</td>
</tr>
<tr>
<td>Visual inspection</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operational cost</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consumables</td>
<td>22</td>
</tr>
<tr>
<td>Utilities</td>
<td>2</td>
</tr>
<tr>
<td>Laboratory/QC/QA</td>
<td>3</td>
</tr>
<tr>
<td>Other facility dependent</td>
<td>7,4</td>
</tr>
<tr>
<td>Labor cost</td>
<td>23</td>
</tr>
<tr>
<td>Raw materials</td>
<td>42</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
</tr>
</tbody>
</table>

Sensitivity analysis

Process simulation tools like SuperPro can help to evaluate an existing process and to investigate alternatives, based on cost analysis and project economics. After a model is developed for a certain process, a sensitivity analysis can be carried out by investigating the impact of different scenarios on for example the production cost and/or cost of goods. The results of a sensitivity analysis help to improve the understanding of the process and can assist in a more efficient planning and in making thoughtful choices.

The model developed for the Hib process in SuperPro Designer was used to carry out a sensitivity analysis for certain variables. By normalizing the COGs (setting the COGs of one single human dose produced using the ‘baseline’ scenario at 100%), the COGs of one single
human dose calculated using the ‘baseline’ scenario can be compared to that calculated using other scenarios. The impact of final formulation (monodose vs. multidose and lyophilized vs. liquid) and labor cost on COGs are depicted in figure 29 and 30 respectively.

**Figure 29:** Impact of the formulation of *Haemophilus influenzae* type b conjugate vaccine on the COGs of one single human dose and on the annual operational cost, monodose compared to a multidose and a lyophilized (Lyo) compared to a liquid (Liq) product. The annual operating cost and the unit production cost related to the production of one dose lyophilized Hib conjugate vaccine using the ‘baseline’ model was set equal to 100%.

**Figure 30:** Impact of a different labor-related cost on the COGs of one single human dose of *Haemophilus influenzae* type b conjugate vaccine. The label-related cost and unit production cost, when producing in low-income countries was equal to 100%.
A multidose lyophilized presentation appears to result in a lower price per single human dose than a monodose. One single human dose obtained using a 10-dose lyophilized vaccine is half the price of a monodose lyophilized vaccine. The cost of one dose liquid Hib conjugate vaccine is approximately 40% cheaper than one dose lyophilized vaccine. The ‘baseline’ scenario is when producing Hib conjugate vaccine in a low-income country (Hib technology transfer partners are emerging manufacturers in low-income countries), therefore the cost was set equal to 100% for this case. Approximately a 10% reduction is seen in the COGs of one single human dose when producing in a low-income compared to a high-income country.

Besides the sensitivity analysis presented in this chapter, the process model developed can be used to investigate other key drivers, for example process yield (upstream, downstream and conjugation), raw materials cost, and capital expenses (building a new facility compared to using an existing facility). Further, it may be also interesting to investigate the impact of scaling up on the cost price, scaling up the polysaccharide production process (bioreactor volume) and/or the conjugation process step can be of influence on the cost of goods of the vaccine.

Process optimization
Next to a predictive model for the ‘baseline’ process of Hib conjugate vaccine, a model was developed for an ‘optimized’ Hib conjugate vaccine process. The proposed process changes were mainly at the cultivation part namely using a fed-batch cultivation mode instead of a batch mode, leaving inactivation out (from the process point of view the inactivation is not really needed but introducing an inactivation process step is useful from the facility design point of view) and using ion exchange chromatography instead of precipitation at the polysaccharide purification part for recovering the polysaccharide from the fermentation broth. Two different models were created and evaluated separately for the targeted final product without making any compromise in the final product quality.
The impact of the proposed process optimizations on the COGs was studied. For example using chromatography instead of precipitation for the purification of the Hib polysaccharide did have an impact on the overall cost but not specifically (less than 2% reduction) on COGs of one single human dose (figure 31).

Figure 31: Impact of a different purification process for *Haemophilus influenzae* type b polysaccharide on the COGs of a single human dose. The total cost and cost of goods calculated using the ‘baseline’ model was set equal to 100%.

The impact of the proposed process optimizations on the COGs was studied. For example using chromatography instead of precipitation for the purification of the Hib polysaccharide did have an impact on the overall cost but not specifically (less than 2% reduction) on COGs of one single human dose (figure 31).

Figure 32: Cost of goods of several formulations (1, 2, 5 and 10 doses), lyophilized (Lyo) and Liquid (Liq), of Hib conjugate vaccine calculated using the ‘baseline’ model compared to the ‘optimized’ model. COGs related to the production of 1 dose lyophilized Hib conjugate vaccine using the ‘baseline’ model is set equal to 100%.

Figure 32: Cost of goods of several formulations (1, 2, 5 and 10 doses), lyophilized (Lyo) and Liquid (Liq), of Hib conjugate vaccine calculated using the ‘baseline’ model compared to the ‘optimized’ model. COGs related to the production of 1 dose lyophilized Hib conjugate vaccine using the ‘baseline’ model is set equal to 100%.
The proposed process optimizations would result in a reduction of COGs, e.g. a lyophilized monodose produced using the optimized process can cost approximately 25-30% less than when using the ‘baseline’ process (see figure 32). This cost reduction, using the optimized process, can be achieved because of an expected higher polysaccharide yield in the fermentation broth (25% more when using a fed-batch) and because of a lower batch time. The batch time can decrease by 7%, 18 %, 26 % when implementing the proposed optimizations related to inactivation, Hib-polysaccharide purification and producing a liquid instead of lyophilized product respectively.

It’s worth to mention that besides the optimizations mentioned in this chapter; other process optimizations would also be of interest to study using the models developed: for example a continuous cultivation mode instead of a fed-batch, and the use of newer conjugation chemistries. A different conjugation chemistry may result in better conjugation yields leading to further reduction in cost and to an improved stability profile.

### Conclusions and discussion

Before starting the Hib project at RIVM, in 1998, the UNICEF price of *Haemophilus influenzae* type b conjugate vaccine was >$US 3,- per dose [85]. The target COGs of the new Hib conjugate vaccine was set at $US 1,- per dose.

Based on a preliminary COGs calculation using Excel, before the start of the project, the COGs of one single human dose lyophilized Hib conjugate vaccine (20-dose) was calculated to be equal to US$ 0.67,- and that of a liquid Hib conjugate vaccine was equal to US$ 0.56,-. The Hib project was considered to be feasible.

Recently, Hib production was simulated using mathematical modeling tools and the COGs was recalculated. First a model was simulated for the ‘baseline’ process, the process developed and transferred by RIVM/NVI, followed by a model for an ‘optimized’ process including a couple of process optimizations as mentioned before. Using the model for the ‘baseline’ process the COGs of one single human dose was calculated to be equal to US$ 0.66,-. Using the model for the ‘optimized’ process the COGs of one single human dose was calculated to be between US$ 0.51,- (mono-dose lyophilized vaccine) and US$ 0.26,- (10-dose
liquid vaccine). The proposed change in the polysaccharide purification (chromatography instead of precipitation) reduced the price by 2% only while producing a liquid vaccine instead of a lyophilized product resulted is a relatively high price reduction (≥25%). Besides a decrease in the batch time, producing a liquid vaccine instead of a lyophilized one and producing a multidose instead of a monodose does help in reducing the secondary cost (filling and packaging). However, using a multidose is not advisable when wastage is expected during vaccination. Further, a lyophilized vaccine has many advantages over a liquid vaccine e.g. the possibility to formulate with different antigens just prior to vaccination and better stability.

The preliminary calculation using Excel did give a good estimation of the COGs to be expected after process development and product commercialization. Even when a number of initial assumptions turned out at a later stage to be less realistic, for example a typical tetanus toxoid yield is equal to 50-100 Lf/ml, a 20-dose presentation will be less attractive from a wastage point of view and based on the lyophilization cycle it should be possible to perform two runs each week instead of one. COGs calculated using the Hib model was in the same range as COGs calculated before the start of the project. In 2013, the lowest UNICEF price of a pentavalent vaccine (DTP-HepB-Hib), 10-dose liquid formulation was $US 1.19,- per dose [183]. Taking the price of the other components (DTP-HepB) into account (the UNICEF price of a ten dose DTP-HepB vial was in 2012 equal to $US 0.69,- [183]), it can be concluded that the calculated COGs for the Hib component/ product are very realistic and that both Excel and mathematical modeling can be used for calculation of COGs. The advantage of mathematical modeling tools is that a sensitivity analysis can be made to be able to make certain choices (e.g. related to upscaling, using an existing facility vs. building a new one, adjusting the process by using a different conjugation chemistry...etc.). In addition, possibilities for process optimization can be explored. Hence, at the initial stage of process development Excel can perfectly be used while a mathematical process model can be used during process development or whenever process optimization or facility debottlenecking is desirable.
CHAPTER 8

General discussion
Out of approximately nine million children under the age of five year who die each year, about 1.5 million die of vaccine preventable infectious diseases for which safe and effective vaccines are available [184]. Besides death these infectious diseases can also cause lifelong disability. One of the main reasons why these underused vaccines are not globally used is the relatively high cost. Local vaccine manufacturers in low- and middle-income countries have been producing traditional vaccines [185] like DTP, measles, polio and BCG, with a relatively low profit margin. The total price of vaccines protecting against “traditional diseases” is about $ 1,-. Thus while most of the vaccines, in terms of doses, are being produced by local manufacturers, the more expensive ones have been produced for years by vaccine manufacturers in high-income countries. It can be stated that the market of underused and new vaccines was for a long time dominated by “Big-Pharma” [186]: GSK, Sanofi, MSD and Novartis. One of the reasons why local manufacturers didn’t invest in their research and development infrastructure and thus in new and underused vaccines is the low profit margin of the traditional vaccines they used to produce [186]. Another reason is that new vaccine production technology is often proprietary and protected by patents [187]. In addition, due to lack of information on the disease burden it is difficult for local authorities to set priorities or to make a cost benefit analysis [37]; therefore underused vaccines were for a long time not part of the national immunization programs in those countries. From the moment it was recognized that the spread of infectious diseases in the developing world is a major threat for global security, several “pull” and “push” mechanisms were applied to help interrupting this closed circle and thus introducing underused and new vaccines in national immunization programs of low- and middle income countries. Push mechanisms aim at lowering the risks and cost for example by providing grants for product development while pull mechanisms aim at providing a purchase guarantee. Applying these mechanisms is not sufficient; an adequate local capacity to produce vaccines that are required is indispensable. Emerging vaccine manufacturers are ready to fill the “gap” created by vaccine manufacturers who have a little incentive to develop vaccines against diseases that are limited to the developing world and/or improving the accessibility to technology related to under-used vaccines.
One of these underused vaccines is *Haemophilus influenzae* type b conjugate vaccine (Hib). In 2000 Hib disease caused approximately 400,000 deaths among children aged 1-59 months [12], predominantly in low- and middle-income countries. Hib can cause, among others, pneumonia, meningitis, epiglottitis and cellulitis. To address this problem, the National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM, in Bilthoven, The Netherlands) took the initiative to develop a production process for a new Hib conjugate vaccine, including related quality control tests, and transfer the technology to emerging vaccine manufacturers in low- and middle-countries. The Hib technology developed by RIVM is scalable, non-infringing, simple and affordable. The Hib project shows that both the creation of a viable vaccine market (pull mechanism applied by GAVI) and the access to vaccine technology by local manufacturers are critical to ensure a sustainable supply of affordable vaccines to low- and middle-income countries.

Two of Intravacc’s (Institute for Translational Vaccinology) partners have succeeded in playing an important role in the Hib vaccine market worldwide by producing affordable and quality Hib vaccines in large quantities. Consequently, the GAVI price of a pentavalent vaccine (DTP-HepB-Hib), Hib is the most expensive of all five antigen, has been reduced to almost one third compared to a couple of years back.

Although resources for traditional pull and push mechanisms are finite, technology transfer can be considered as an infinite “push” mechanism that assures not only a sustainable introduction of under-used and new vaccines in low- and middle income countries but also helps generating an adequate R&D, production and regulatory infrastructure and thus broadening the product portfolio of the local vaccine manufacturers.

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1 RIVM tasks related to technology transfer were transferred to the Netherlands Vaccine Institute (NVI) between 2003 and 2010. In 2013, these tasks were transferred to Intravacc (Institute for Translational Vaccinology).


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Haemophilus influenzae type b (Hib) conjugate vaccine is a safe and effective vaccine that can prevent meningitis and pneumonia caused by Hib disease. Hib vaccine is recommended for all children under 5 years [32, 188].

Despite the availability of safe and effective Hib vaccines since early 1987, Gambia was the only low-income country that had introduced Hib vaccine in its national immunization program. In 2000 it was reported that the disease accounted for about eight million illnesses each year resulting in approximately 400,000 deaths of children aged 1-59 months almost all in low-income countries [12]. The introduction of Hib vaccines in these countries was limited because of the relatively high price of the vaccine and limited availability. Further, many countries lack information on the disease burden and consequently on the cost-effectiveness of the vaccine.

To address this problem the National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM, in Bilthoven, The Netherlands) decided in 1998 to help emerging vaccine manufacturers in gaining access to Hib technology through technology transfer and thereby contributing to a sustainable supply of affordable Hib vaccines to low- and middle-income countries and to the United Nations Millennium Development Goals (MDGs) in reducing child mortality. There was no need to develop an in-house Hib vaccine for the Dutch population, as an imported Hib vaccine was already being used for years in the Dutch national immunization program. So the decision to develop a Hib vaccine was solely for technology transfer to local manufacturers (chapter 2). The Hib technology developed by RIVM was successfully transferred to Biological E (BE, India), Serum Institute of India (SII, India), Bio Farma (Indonesia) and Shanghai Institute of Biological Products (SIBP, China). The technology transfer to SIBP was facilitated by Glovax (Korea). All four partners were able to implement the process in their facilities. SII succeeded in obtaining a marketing license for their Hib vaccines in 2007 and BE was able to WHO prequalify a pentavalent vaccine including Hib in 2011. Several Hib vaccines produced by SII are now also WHO prequalified. Bio Farma decided in 2009 to use a different conjugation technology from
the one transferred by RIVM/NVI¹ and has licensed its Hib vaccine in 2013. Technology transfer to SIBP started in 2006, till date SIBP didn’t commercialize Hib vaccine, as the Chinese authorities decided not to allow clinical studies using this Hib vaccine since there are already sufficient other public institutes producing Hib vaccines locally.

RIVM succeeded in developing an innovative, scalable, non-infringing (patents and proprietary technology), simple and affordable process for the production of quality Hib vaccines (chapter 3). The process was developed based on public information and prior knowledge on conjugate vaccines. Further, additional experiments were performed in order to support the choices made during the process development and to decide on the critical quality parameters. Recently a predictive mathematical model was developed for the Hib process. This model helped to evaluate the existing process and to study possibilities for process optimization.

Besides the process and all related quality control tests, additional immuno assays were developed in order to support the development of the new Hib conjugate vaccine (chapter 4). The immuno assays developed by RIVM/NVI were very helpful during process development. All these test methods were transferred to the partners as part of the technology transfer package. Besides for the purpose of process implementation and scale up some ELISA methods were used by technology transfer partners for lot release purpose, after proper qualification and validation.

The technology transfer package included all documentation related to the process, quality control tests, raw materials and equipment specifications, materials (e.g. seed lot and reference samples) and (pre)clinical data (chapter 5). The preclinical study was performed by RIVM/NVI on a clinical lot produced in close collaboration between Bio Farma (the first Hib technology transfer partner) and RIVM/NVI.

Many lessons were learned during the development and technology transfer of the new Hib conjugate vaccine (chapter 6). The technology

¹ RIVM tasks related to technology transfer were transferred to the Netherlands Vaccine Institute (NVI) between 2003 and 2010. In 2013, these tasks were transferred to Intravacc (Institute for Translational Vaccinology).
transfer partners who have used this Hib technology have made a considerable contribution to ensure sustainable supply of affordable Hib vaccines worldwide. Serum Institute of India (SII) made pentavalent vaccine (DTP-HepB-Hib) available to GAVI for $1.75,- per dose starting from 2011 and Biological E (BE) decided to cut the GAVI price to $1.19,- per dose starting from 2013. SII and BE are committed to supply about 600 Million doses of this vaccine in the coming years to GAVI. Till 2007, GAVI had only one European supplier and the price of pentavalent vaccine was $3.56,- per dose. The approach followed for the Hib project can be applied to other technology transfer projects. Before starting the project an extensive and detailed feasibility study was performed to evaluate the following aspects: freedom-to-operate, prior knowledge, expected cost price of the vaccine, project continuity including impact on on-going projects, funding, potential partners and available market. Based on the outcome of this feasibility study the product target profile was defined and decisions were made on the approach to be followed during process development and technology transfer. The process developed had to allow freedom-to-operate (non-infringing with existing patents and proprietary rights) and to result in very stable intermediate and final products. Although the product to be produced using this process was a freeze-dried Hib conjugate vaccine, it was taken into consideration that technology transfer partners would eventually be aiming at a liquid formulation of both standalone and combination vaccines. As the developed process was not going to be used for routine production of the Hib vaccine in the Netherlands, all Hib technology transfer partners were advised to invest in a research infrastructure for conjugate technology in order to be able to perform further scaling up and troubleshooting after the finalization of the technology transfer. The scalability and the GMP applicability of the process were taken into consideration during process development.

As part of the feasibility study performed by RIVM before being able to start the Hib project the expected cost of goods was calculated (chapter 7). The cost of goods was recalculated at a later stage using a mathematical model. COGs calculated using both methods was in
the same range. The availability of a process model for Hib conjugate vaccine is advantageous because it makes it possible to perform a sensitivity analysis if certain decisions are to be taken and/or process optimization is considered.

Through this Hib project, Intravacc’s partners were provided with general conjugation knowhow, and related quality control tests, resulting in a broadening of their research and development portfolio with several conjugate vaccines including meningococcal, and pneumococcal vaccine. Further, The Hib technology allowed producing freeze-dried and liquid formulations of several standalone and combination vaccines. The Hib project has also demonstrated the importance of technology transfer to local manufacturers ensuring a sustainable supply of under-used vaccines to low- and middle-income countries.
Nederlandse samenvatting
Haemophilus influenzae is een bacterie die bij veel mensen in de neuskeelholte voorkomt. Haemophilus influenzae komt uitsluitend bij mensen voor; er is geen andere natuurlijke gastheer bekend. Als de bacterie het lichaam binnendringt, kan het ernstige infecties veroorzaken waarna de ziekte vrij snel kan verlopen en in het ergste geval binnen een paar dagen de dood tot gevolg heeft. De ziekte kan ook blijvende schade veroorzaken zoals gehoorverlies.

Haemophilus influenzae bacteriën kunnen onderverdeeld worden in stammen met of zonder kapselpolysacharide. Van de gekapselde bacteriën zijn zes verschillende serotypes bekend (a-f) waarvan type b de meeste virulente is. Haemophilus influenzae type b (Hib) kan door hoesten en niezen overgebracht worden en kan voornamelijk hersenvliesontsteking (meningitis), longontsteking (pneumonie), bloedvergiftiging (sepsis), middenoorontsteking (otitis) en ontsteking aan de gewrichten tot gevolg hebben. Alle ziektes die veroorzaakt worden door Hib worden “Hib-ziektes” genoemd. De beschermende antistoffen zijn gericht tegen het kapselpolysacharide, een polyribosylribitol fosfaat (figuur 33).

**H. influenzae b**

![Structure of Hib capsular polysaccharide](image)

$\text{C}_{10}\text{H}_{18}\text{O}_{11}\text{P} = 346.228$

$\text{C}_{10}\text{H}_{19}\text{NaO}_{11}\text{P} = 368.210$

**Figure 33:** Structuur Hib kapselpolysacharide [189, 190].

Hib vaccin geeft volledige bescherming tegen de ziekte. Het Hib vaccin is een polysacharide-eiwit conjugaat vaccin dat bijvoorbeeld bestaat uit gezuiverd kapselpolysacharide van Hib gekoppeld aan tetanus toxoid. Het koppelen (conjugeren) van de kapselpolysacharide aan een dragereiwit is nodig om langdurige immuniteit door middel van immunologisch geheugen na vaccinatie te bewerkstelligen. In tegenstelling tot het gezuiverde kapsel polysacharide induceert het geconjugeerde vaccin een T-cel afhankelijke immuunrespons. Het polysacharide is een thymus-onafhankelijke (TI)-2 antigeen. Door het koppelen van het polysacharide aan een eiwit verandert het polysacharide van een (TI)-2 antigeen in een thymus-afhankelijk (TD) antigeen dat in staat is om een immuun reactie en een immunologisch geheugen in kinderen te bewerkstelligen. Dat is de reden waarom Hib polysacharide vaccin niet werkzaam is in zeer jonge kinderen (onder de twee jaar). Deze antistoffen zorgen voor opsonisatie en complement activatie waardoor het micro-organisme wordt opgeruimd door fagocyterende cellen.

Er zijn verschillende Hib conjugaat vaccins op de markt. Deze verschillen vooral in het dragereiwit en de gebruikte conjugatie methode. Sinds kort is er ook een Hib vaccin op de markt dat geproduceerd is met een chemisch gesynthetiseerd polysacharide. Hib vaccins zijn vanaf begin 1987 geïntroduceerd in verschillende geïndustrialiseerde landen. Sinds de invoering daarvan in de Verenigde Staten en in Europa is de incidentie van invasieve Hib-infecties sterk afgenomen. De introductie van het vaccin in ontwikkelingslanden is echter achterwege gebleven. Sinds 1993 maakt Hib vaccin deel uit het Nederlandse Rijksvaccinatieprogramma. De eerste prik wordt gegeven als het kind ongeveer 6 weken oud is. Daarna zijn er inenting op de leeftijd van ongeveer 3, 4 en 11 maanden. De voornaamste reden voor het niet gebruiken van het Hib vaccin in ontwikkelingslanden was de relatieve hoge prijs van het vaccin.
Verder, er was weinig informatie beschikbaar over de ziektelast en over de prijseffectiviteit van het Hib vaccin. Ook hadden lokale producenten geen toegang tot de noodzakelijke productie technologie.

Het Rijksinstituut voor Volksgezondheid en Milieu (RIVM, Bilthoven, Nederland) besloten in 1998 om een Hib project te starten met als doel de productie technologie te ontwikkelen voor het Hib vaccin en die beschikbaar te stellen aan producenten in ontwikkelingslanden. Op deze manier zou bijgedragen kunnen worden aan de duurzame beschikbaarheid van betaalbare Hib vaccins voor ontwikkelingslanden (hoofdstuk 2). De Hib technologie ontwikkeld door RIVM werd succesvol overgedragen aan Biological E (BE, India), Serum Institute of India (SII, India), Bio Farma (Indonesia) en Shanghai Institute of Biological Products (SIBP, China). Overdracht naar SIBP verliep via Glovax (Korea). Alle vier partners hebben het proces kunnen implementeren. SII en BE hebben hun marktlicentie en WHO prequalificatie gekregen respectievelijk in 2007 en 2011. Bio Farma besloot in 2009 een andere conjugatie methode te gebruiken en heeft in 2013 een marktlicentie gekregen. SIBP, dat in 2006 gestart was met het implementeren van de Hib technologie, heeft tot nu toe geen toestemming gekregen van de lokale overheid voor klinisch onderzoek omdat er andere publieke producenten in China zijn die Hib vaccins produceren.

Het RIVM is erin geslaagd om een innovatief, opschaalbaar, eenvoudig en betaalbaar proces te ontwikkelen voor de productie van een nieuw Hib vaccin (hoofdstuk 3). Het proces werd ontwikkeld op basis van openbare informatie en bij het RIVM (later NVI (Netherlands Vaccine Institute) en nu Intravacc1 (Institute for Translational Vaccinology) ontwikkelde kennis op het gebied van conjugaat vaccins. Recentelijk is een mathematisch model van het Hib proces ontwikkeld dat heeft geholpen bij het identificeren van mogelijkheden voor proces optimalisatie.

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Naast het productie proces zijn er ook de nodige testen ontwikkeld, inclusief additionele testen die van belang waren voor proces ontwikkeling (hoofdstuk 4).

Het pakket dat is overgedragen aan de partners omvatte niet alleen de protocollen voor het productie proces en kwaliteitscontroles maar ook de specificaties voor de grondstoffen en de te gebruiken apparatuur en de nodige materialen (waaronder referentie monsters en seed lot). Verder is een (pre)klinische lot, geproduceerd in samenwerking met Bio Farma (de eerste Hib partner), (pre)klinisch getest (hoofdstuk 5) en de data beschikbaar gesteld aan de partners.

De ervaringen die opgedaan zijn tijdens de ontwikkeling en kennis overdracht gerelateerd aan het Hib vaccin zijn beschreven in hoofdstuk 6, daar ze van nut kunnen zijn bij andere projecten op het gebied van kennisoverdracht. Twee partners die de ontwikkelde Hib technologie hebben overgenomen van het RIVM/NVI leveren een grote bijdrage aan de duurzame beschikbaarheid van betaalbare Hib vaccins wereldwijd. SII verkoopt vanaf 2011 pentavalent vaccin (DTP-HepB-Hib) aan UNICEF via GAVI voor $US 1.75,- per dosis en BE heeft in 2013 de prijs verlaagd naar $US 1.19,- per dosis. Het Hib component is de duurste van alle vijf antigenen. SII en BE hebben zich aan GAVI gecommitteerd tot het leveren van ongeveer 600 Miljoen doses in de komende jaren. Tot aan het moment waarop nieuwe spelers inclusief Intravacc’s Hib partners (SII en BE) op de Hib vaccinmarkt kwamen, tot 2007, kon GAVI pentavalent vaccin alleen kopen van één Europese leverancier voor de prijs van $US 3.56,- per dosis.

Een gedetailleerde haalbaarheidsstudie werd uitgevoerd, rekening houdend met verschillende aspecten, voordat het Hib project gestart werd. Gebaseerd op de uitkomst van deze studie is de strategie voor proces ontwikkeling en kennis overdracht vastgelegd waaronder ook het product profiel. Behalve toegang tot Hib technologie heeft het project de partners ook geholpen om hun onderzoeksportfolio uit te breiden met andere conjugaat vaccins.
Als onderdeel van de haalbaarheidsstudie werd ook een schatting gemaakt van de te verwachten vaccine kostprijs (hoofdstuk 7). De kostprijs werd later nauwkeuriger berekend gebruikmakend van een mathematisch model.

De aanpak die gevolgd is voor het Hib project was succesvol en laat het belang zien van kennisoverdracht aan lokale producenten bij het beschikbaar komen van betaalbare vaccins op een duurzame manier.
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Curriculum vitae
Ahd Hamidi was born on September 9, 1971 in Tetouan, Morocco. After her final year of high school in 1989 (Hassan II in Tetouan, Morocco) she moved to Rotterdam, the Netherlands. During her first year in the Netherlands she followed several Dutch language courses and passed entrance tests (mathematics, physics, chemistry, English and Dutch) for Delft University of Technology (TUDelft in Delft, The Netherlands) and for Wageningen University (Wageningen, the Netherlands). She chose to follow a study at the faculty of Chemical engineering at TUDelft, in 1995 she graduated with a master in engineering (Ir.). The final post-graduation project was at the department of Microbiology and Enzymology. This project was an assignment from Unilever (Rotterdam, the Netherlands): the use of mathematical modeling tools to simulate several fermentation methods for the production of a heterologous protein in yeast and optimization of the fermentation process and the expression of the protein using a continuous culture. The work placement that preceded the post-graduation was at the Department of Downstream processing of Diosynth (Oss, the Netherlands) and aimed at investigating the purification and stability of different glycoprotein hormones. In 1996, Ahd started a two-year Post-MSc programme (Designer in Bioprocess Engineering) at the TUDelft to graduate in 1998 as a Professional Doctorate in Engineering (PDEng). The Individual design project during this programme was for the TUDelft and aimed at designing a multipurpose high pressure counter-current extraction system, including recirculation of auxiliary materials. The group design project was for Gist-Brocades (Delft) and aimed at designing an Amoxicillin production unit. In 1998, she worked as a researcher at the department of Bioprocess technology (TUDelft) to investigate the hydrodynamics of liquid two-phase systems in a continuous counter current sieve column. October 1998, she started her career at the National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM, in Bilthoven, The Netherlands) and worked both at the Office of International Collaboration, among other as a training coordinator (for example to coordinate courses through WHO on Diphtheria-Pertussis-Tetanus (DTP) production) and at the Process Development department
on the development of a new *Haemophilus influenzae* type b (Hib) conjugate vaccine. Part of this work is described in this thesis. In 2001, she became the project manager of the Hib project. Between January 2007 and August 2009 she ran a consultancy company together with her husband, Abdelkhalek Daoudi, (BioMarc). During this period she worked among others as a General Manager at the Bacterial research and development department of Biological E. limited (Hyderabad, India) and for the WHO (participation in a workshop on the implementation of procedures for Rapid Review of Imported pre-qualified vaccines for use in National Immunization Programs in Oman, Jordan). August 2009 she returned to the NVI (RIVM tasks related to technology transfer were transferred to the Netherlands Vaccine Institute (NVI) and at a later stage to Intravacc (Institute for Translational Vaccinology)) to work as a training coordinator for the Inactivated Polio Vaccine project, based on Sabin strains (Sabin-IPV) and Influenza project. Since 2011, she worked as a project manager for several IPV projects (for example Sabin-IPV technology transfer projects and Salk-IPV optimization project). Besides project management, Ahd is within Intravacc the technology transfer expert and works closely with both the process and the business development department.
List of publications


• Hamidi A., Kreeftenberg H. Use of immuno assays during the development of a Haemophilus influenzae type b vaccine for technology transfer to emerging vaccine. Human Vaccines &Immunotherapeutics 2014; 10(9): 2697-703.

• Hamidi A., Boog C., Jadhav S., Kreeftenberg H., Lessons learned during the development and transfer of technology related to a new Hib conjugate vaccine to emerging vaccine manufacturers. Vaccine 2014; 32(33):4124-30.