

## Trivac HB<sup>®</sup>: a Cuban polyvalent vaccine

✉ Néstor S. Expósito<sup>1</sup>, Daniel Cardoso<sup>2</sup>, Eduardo Martínez<sup>1</sup>, Yanelis Herrera<sup>2</sup>, Karelía Cosme<sup>3</sup>, Pablo A. Díaz<sup>4</sup>, Yayrí C. Prieto<sup>1</sup>, Zoe Núñez<sup>1</sup>, Mabel Izquierdo<sup>5</sup>, Orestes Mayo<sup>6</sup>, Joel Pérez<sup>6</sup>, Luis C Hidalgo<sup>6</sup>

<sup>1</sup>Departamento de Desarrollo de Vacunas Combinadas, <sup>3</sup> Departamento de Bioterio,

<sup>4</sup> Departamento de Estudios Clínicos, <sup>5</sup> Departamento de Control de Calidad, Centro de Ingeniería Genética y Biotecnología, AP 10600, Cubanacán, Ciudad de La Habana, Cuba

E-mail: nestor.exposito@cigb.edu.cu

<sup>2</sup> Producción de Vacuna DPT, Instituto de Sueros y Vacunas "Finlay"

Ave 27 # 19805, AP 16017, La Coronela, La Lisa, Cuba

<sup>6</sup> Centro Nacional de Biopreparados, Carretera a Beltrán,

Km 1 ½, Bejucal, AP 6048, La Habana

### ABSTRACT

Here we present the technological design, development and introduction in the immunization program, of a combined vaccine of diphtheria anatoxin, tetanic anatoxin, whole cells of *Bordetella pertussis*, and recombinant hepatitis B virus surface antigen (DPT-HB<sup>®</sup>). The commercial name of the vaccine is Trivac HB<sup>®</sup>. One novel element in its design is the use of aluminum hydroxide as the adjuvant which was essential in avoiding the Glaxo Smith Kline patent for polyvalent vaccines. This is the second tetravalent vaccine registered in the world and the first in Latin America containing these antigens. The formulation of the Cuban Trivac HB<sup>®</sup> vaccine is stable for 24 months when stored at 3 ± 5 °C. Trivac HB<sup>®</sup> is an effective vaccine and well tolerated by infants. Trivac HB<sup>®</sup> was therefore registered in Cuba in 2004.

### Introduction

Global Alliance for Vaccines and Immunization (GAVI) was created with the resolution and experience of a group of researchers, experts and vaccines producers in the world. This is a historic alliance of the private and the public sectors, with the mission of saving children and protecting people from diseases with the use of vaccines.

The World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) have set three main goals in their World Vision and Strategy of Immunization (WVEI) program: a) immunize more people against more diseases, b) disseminate several new vaccines and technologies worldwide and c) accomplish decisive interventions on health through immunization.

Throughout history, vaccination has been a highly successful public health operation. It has also been one of the high cost and efficacy. For example, vaccination has eradicated smallpox; it has reduced the world incidence of poliomyelitis in 99% since 1988 and greatly reduced morbidity and mortality from diphtheria, tetanus, whooping cough and measles. In 2003 alone, immunization avoided more than 2 million deaths. However, immunization is far from been universal: many countries are retreating from their former levels of vaccination coverage. In 2003, about 27 million infants and 40 million pregnant women were unprotected against preventable diseases. "One in four children in is still deprived of lifesaving vaccines that should be within reach", pointed the director of UNICEF, Ann M. Veneman. "This new strategy recognizes that if we are to improve child survival, immunization must be sustained year in and year out".

For years WHO, UNICEF and GAVI have argued the advantages of introducing "combined vaccines" as a new generation of vaccines conferring

protection against four or five diseases at the same time instead of applying two or three vaccines separately. The benefits of this new strategy are obvious: fewer needles are used, less handling procedures and human resources are needed to guarantee vaccination and fewer visits of parents and children to the vaccination area are required, among other factors.

Vaccine supply today does not cover world demand which will require years [1]. Recommendations of these world organizations point at very important vaccines combinations: a tetravalent vaccine against diphtheria, tetanus, pertussis and hepatitis B (DPT-HB) and a pentavalent vaccine also against *Haemophilus influenzae* type b (DPT-HB-Hib). Because of a shortage of these vaccines GAVI executives express that "this has created a big challenge for us" [2].

Combined vaccines are manufactured as a physical mixture of two or more vaccines that are administered at the same time and anatomical location [3]. The first vaccine combination was made in 1949 [4], when the anti-whooping cough vaccine was mixed with the anti-tetanic vaccine. Since then and until 1983, 13 other combined vaccines were registered. Furthermore, in the last two decades the interest for combined vaccines has risen. The main reasons are:

1. There are about 30 vaccines available, three of them are parenterals: anti poliomyelitis, the vaccine against typhus fever and the cholera vaccine.
2. There are approximately 200 vaccines in the Research and Development phase and it is expected that in 2010 there will be 25 pediatric vaccines in the world market.
3. There is a concern that children are becoming "pincushions" [5].
4. Until oral vaccines for all the immuno-preventable diseases are fully available, it would be ideal to

1. Immunize Every Child, GAVI policy document, February 2000.

2. Immunization Focus - the GAVI quarterly. More combination vaccines needed. Faced with a bigger than expected shortfall in supplies of some vaccines, countries must make choices. Phyllida Brown reports. Special Report (December) 2001.

3. Rennels M. Combination vaccines. *Pediatr Infect Dis J* 2002;21:255-7.

4. Children's Vaccine Initiative. Combination vaccines- juggling with the options. *CVI Forum* 1998:16.

5. Gorlick G. Are we turning kids into pincushions? *Contemp Ped* 1991;8:23.

be able to combine the largest number of antigens in a single injection.

The advantages of combined vaccines are clear:

1. Number of injections decrease and so does the suffering and anxiety of the receptor and tutors.

2. Number of visits to the vaccination area is reduced as well as the social cost associated to vaccination.

3. Vaccination programs are better accepted by parents and sanitary personnel [6].

4. Transportation and storage cost of vaccines decline [7].

5. Vaccine delivery cost is lowered since less sanitary operations are needed as well as fewer syringes and injection needles.

Combined vaccines could also imply certain disadvantages [8]. Antigens could interfere with each other leading to a diminished potency for one or all vaccines, and antigen stability could be compromised. The interference could be of physical (maximum temperature for optimal potency) or chemical nature throughout the different adjuvants, preservative, inactivators or stabilizers [9]. There could be an incompatibility between the DPT and the VPI vaccines due to the presence or lack of thimerosal [10], immunological interference and/or biological interference between the attenuated agents [11]. Under these situations, the administration of the combined products has led to a lower immune response than through the administration of the individual components.

In spite of the difficulties encountered in development and marketing, there are many combined vaccines available, some of which are widely used. Most of them are against: diphtheria, tetanus, and either cellular or acellular *B. pertussis*.

Cuba has had the Hepatitis B vaccine Heberbiovac HB<sup>®</sup> since 1992 and two years ago the DPT vaccine was also registered. With the aim of incorporating Cuban researchers to the development of new combined vaccines so that the country may become a supplier for UNICEF and GAVI programs in the near future, a multicenter project for the development of a tetravalent Cuban vaccine was created. The project conducted by the CIGB and Finlay Institute began in 1997.

Manufacturing a combined vaccine is not the simple combination of single components. We must find the appropriate conditions for the physical, chemical and biological compatibility not only between the active components but also between these and the adjuvants, excipients and preservatives. The development of a combined vaccine requires numerous laboratory, pre-formulation, stability, toxicological and clinical studies.

Here we summarize the development stages of the product using exclusively Cuban technology in order to evade the Glaxo Smith Kline patent, the world leader of this type of vaccines. This enables the free marketing of the product avoiding patent claims.

## Materials and methods

### Pre-formulation stage

Three technological variants of the formulation were designed at the scale of 1 liter. All three variants had 3% of the aluminum hydroxide adjuvant (Alhydrogel)

(Brentag, Denmark) and the same batches of each anatoxin i.e. diphtheria (D), tetanic (T), *B. pertussis* (P), as well as the recombinant hepatitis B virus surface antigen (HBsAg) (Table 1). Every batch was approved by the Quality Control Direction of the Center for Genetic Engineering and Biotechnology (CIGB), and the Finlay Institute for Sera and Vaccines. Thimerosal was employed as the preservative.

The formulation strategies are the following:

1. In variants 1 and 2 HBsAg was added without its previous absorption to the aluminum hydroxide gel.

2. In variant 3, the HBsAg was absorbed to the gel before including it in the formulation. Hence, when *B. pertussis* cells were added, there was no interaction with the HBsAg.

3. In the three variants, the components were incorporated according to their molecular weights. Therefore, diphtheria anatoxin (63 000 Da) was added first followed by tetanic anatoxin (150 000 Da) and then HBsAg which is a particle of 42 nm. *B. pertussis* cells of 1-2 mm were added at the end. The rationale of this procedure was to reduce a possible steric hindrance between the antigens.

4. The formulation pH during storage was controlled with 4 mMol/L of phosphate buffer. This buffer solution together with a saline solution favors the isotonicity of the preparation.

The technological variants studied were the following:

Variant 1: The antigens were introduced sequentially and *B. pertussis* was incorporated in two separate additions.

Variant 2: The antigens were introduced sequentially and *B. pertussis* was incorporated in one addition.

Variant 3: HBsAg was absorbed to the aluminum hydroxide gel before its introduction to the formulation and *B. pertussis* was incorporated in one addition.

All three variants have the same final composition of each one of the ingredients.

The three formulation variants were then evaluated using the potency tests established for the four antigens and the immunogenicity assay of the HBsAg. The assays were made just after mixing the components (t=0) and after 6 months of storage at  $5 \pm 3^\circ\text{C}$ . Based on the results of these evaluations three 5 liter batches were formulated using different active pharmaceutical ingredients (AFI) which were subjected to a rigorous stability study.

The methodology for the evaluation of the potency of diphtheria, *B. pertussis*, tetanus and HBsAg, as well as the immunogenicity test for the latter is described in the following section.

Potency test for the diphtheria and tetanic anatoxins

Table 1. Batches of Diphtheria, tetanic, *B. pertussis* and HBsAg anatoxins employed in the preparations of the three technological variants evaluated in the pre-formulation study

| Antigen             | Batch     | Concentration | Purity         |
|---------------------|-----------|---------------|----------------|
| T. diphtheria       | 002/97    | 660 Lf/mL     | 3 300 Lf/mg NP |
| T. tetanic          | P-019/96  | 1 000 Lf/mL   | 1400 Lf/mg NP  |
| <i>B. pertussis</i> | A-2/97    | 788 U.O.      | not done       |
| HBsAg               | 02MPAC625 | 1.27 mg/mL    | >95%           |

6. American Academy of Pediatrics. Combination vaccines for childhood immunizations: recommendations of the Advisory Committee on Immunization Practices (ACIP), the American Academy of Pediatrics (AAP), and the American Academy of Family Physicians. Pediatrics 1999;103:1064-77.

7. Urbitzondo L, Peña A, Boldú M, Taberner J, Batalla J. Implicaciones logísticas de la evolución de los calendarios de vacunación y de las presentaciones de las vacunas. Vacunas. Investigación y práctica 2001;2: 58-63.

8. Le Ch. Combination vaccines: choices or chaos? A practitioner's perspective. Clin Infect Dis 2001;33:S367-71.

9. Vose J. Regulatory considerations for developers. In: Ronald W. Ellis (ed.) Combination vaccines. Human Press, Totowa, NJ, 1999:213-33.

10. Insel R. Potential alterations in immunogenicity by combining or simultaneously administering vaccine components. Ann N Y Acad Sci 1995;754:35-47.

11. Falk L, Arciniega J, McVittie L. Manufacturing issues with combining different antigens: a regulatory perspective. Clin Infect Dis 2001;33:S351-5.

The test was performed following the Standard Operational Procedures (SOP) established for each case [12, 13]. The potency is expressed in international units (IU) of anatoxins of diphtheria and tetanus present in the sera of animals that had received the immunogenic stimulation using each corresponding antigen.

The procedure is based on the determination of the neutralization capacity of the diphtheria and tetanic anatoxins present in the sera of animals immunized with the vaccine compared to a reference preparation of each toxin. For both anatoxins the batch is approved if the value is  $\geq 2$  IU/mL.

#### Potency test of *B. pertussis*

We used the methodology described to evaluate the degree of protection conferred by the vaccine expressed as IU of protection against and *B. pertussis*, present in the sera of the animals that had received immunogenic stimulation by each corresponding antigen.

The test consists of measuring the capacity of the vaccine preparation of protecting immunized mice against an intracranial challenge of the strain 18323 of *B. pertussis*. The test was performed according to the corresponding SOP [14]. The batch is approved if the value is  $\geq 4$  IU/doses of 0.5 mL and the lower limit is  $\geq 2$  IU/doses of 0.5 mL.

#### Relative potency test of HBsAg

##### Immunogenicity study of the HBsAg

The immunogenicity study of the HBsAg component was to compare the results of the immune response against the antigen in the three variants, measured by the antibody titer.

Five groups of 10 Balb/C mice were immunized with the three variants, a reference material of the HBsAg vaccine (HB 07-0902) and a placebo. The determination of the antibody titer was done as follows: mice were immunized with different doses of the vaccines (1.25, 0.312, 0.075, 0.039 and 0.019 mg). The immunization schedule consisted of a single dose administered intraperitoneally. Mice were bled 28 days after immunization.

A concentration of 1.25 mg was used to determine the anti-HB antibody titers. This concentration corresponds to 1/16, which is the first dilution and consequently the one that should show more animals with seroconversion. The sera of the mice at this dilution were pooled to increase the volume of the sample and to obtain a single value for the antibody titer, regardless the value of each animal. The assay was performed according to the corresponding SOP [15-16].

#### Stability study

The goal of this part of the study was to assess the stability of the Trivac<sup>®</sup> vaccine, formulated with Cuban technology and stored at  $3 \pm 5$  °C. For this we used three consecutive batches (DPT-HB/VII, DPT-HB/VIII and DPT-HB/IX). The batches were formulated according to variant 3 and the technology employed was evaluated.

#### General methodology of the study

The three batches were formulated using production batches of active ingredients of D, P, T and HB.

Samples from each formulation batch were stored in a refrigerated room at  $3 \pm 5$  °C. The stability study was performed in a time period of six months, under a defined sampling schedule. In the last year the time period was extended to 12 months. Samples were evaluated for different physical, chemical and biological parameters. The duration of the study was of 30 months.

#### Pre-clinical study

The pre-clinical study was made to assess the reactogenicity of the combined DPT-HB vaccine, by the inoculation of high doses through the intramuscular route followed by 14 days of surveillance to detect any clinical symptoms of toxicity or death of the animals. The following pre-clinical studies were conducted:

1. Evaluation of acute toxicity in Sprague-Dawley rats.
2. Evaluation of acute toxicity in Balb/C mice.
3. Evaluation of local tolerance in OF-1 mice.

Toxicity tests were made according to the guidelines approved by the Organization for the Cooperation and the Economical Development (OCED). The protocols employed corresponded specifically to the 401<sup>st</sup> OCED guideline but using the intramuscular route [17]. The samples evaluated corresponded to the final formulation used in the clinical study. The doses assayed were selected taking into account the antigen-adjuvant ratio. The lower dose used was 15 times the dose of the clinical study. The intermediate and highest doses were 30 and 60 times the doses of the clinical study respectively. Local tolerance was evaluated in OF-1 mice. Maximum dose volume was fixed to 0.5 mL as used for intramuscular route in rats. For the highest dose the total volume was divided for its administration in both legs.

#### Clinical trials

Clinical studies were conducted in two phases: phase I, for safety evaluation and phase II, to assess vaccine immunogenicity. In phase II we evaluated both, seroconversion of individuals and the antigen-specific antibody titers elicited against each antigen included in the vaccine.

Clinical studies were open, prospective, controlled and randomized. A total of 274 healthy newborn infants were initially enrolled in the study. Twenty-eight children left the study either because of the decision of their parents or tutors, because they moved out from the province, or due to migration or illness.

The Trivac<sup>®</sup> vaccine schedule consists of a series of three vaccinations given at 2, 4 and 6 months of age and it is administered in doses of 0.5 mL in the frontal region of the right thigh. The Trivac vaccine is applied after the vaccination with the Heberbiovac HB<sup>®</sup> vaccine using the same route and dose volume.

In the final reactogenicity analysis we included 246 newborn infants of both sexes which had previously received the three doses of vaccination. The sera of the same children, but collected one month after the last dose of the clinical assay, were used for the immunogenicity analysis. All parents and tutors gave their informed consent for the participation of the children in the clinical study and signed the corresponding

12. PNO 12-237 y PNO 12-131. Procedimiento para determinar la potencia de la anatoxina tetánica.

13. PNO 12-137 y PNO 12-047. Procedimiento para determinar la potencia de la anatoxina diftérica.

14. PNO 12-043. Procedimiento para determinar la potencia de la *Bordetella pertussis*.

15. PPO 4.09.060.92. Edición 05. Procedimiento para la determinación de la potencia in vivo de la Vacuna Recombinante contra la Hepatitis B y la vacuna combinada DPT-HB.

16. PPO 4.09.038.92. Edición 05. Procedimiento para determinar la concentración de los anticuerpos monoclonales anti-hepatitis B por ELISA.

17. PPO 1.27.136.96. Administración parenteral de la sustancia de ensayo a ratas.

forms. Parents were interviewed by pediatricians in order to obtain information regarding the mother's pregnancy, delivery and the first two months of life of each child. A detailed physical examination of the children was also conducted. Only healthy eutrophic newborn infants were included in the study.

Data was recorded independently by two different researchers. The information was organized using a Microsoft Excel database. Both records were carefully compared and Scomp Software (version 2.0 of 2000) was used to eliminate errors and for data validation.

#### Determination of antibody titers against tetanic toxoid

The Anti-tetanic toxin titer was determined using a commercial assay from Nova Tec Immunodiagnostica GmbH, Germany (NovaTec-Clostridium tetani IgG-ELISA Prod. No. TETG0430). The assay consists of a direct ELISA for the quantification of the anti tetanic toxin IgG in the human sera. This assay was used to assess the patient's immunological conditions in order to decide the need of a basic immunization or a booster.

#### Determination of antibody titer against the diphtheria toxin

It was employed a commercial kit from Novatec Immunostica GmbH, Germany (NovaTec-Corynebacterium diphtheriae IgG-ELISA Prod. No. CORG0090). The assay consists on a direct ELISA for the quantification of the anti diphtheria toxin IgG in the human sera. This assay was employed for the assessment of patient's immunological conditions in order to decide the need of a basic immunization or a booster.

Determination of the IgG titer against filamentous hemagglutinin and pertussis toxin from *B. pertussis*.

Antibody titers against *B. pertussis* were determined using a commercial assay from Nova Tec Immunodiagnostica GmbH, Germany (NovaTec-Bordetella pertussis IgG-ELISA Prod. No. BOPG0030). The intended use of this ELISA is the qualitative determination of IgG antibodies in human sera.

#### Determination of the IgG titer against HB

Anti-HBs were assessed by a commercial immunoenzymatic assay based on a sandwich procedure (Hepanostika anti-HBs, Organon Teknika, The Netherlands). Polystyrene stripes coated with HBsAg are incubated with serum samples. Next, a conjugate of HBsAg-horseradish peroxidase (HRP) is added. When a positive reaction occurred, the HBsAg- HRP conjugate reacts with the HBsAg-antibody complexes previously formed on the solid phase. Tetrametilbenzidine is used as enzyme substrate which yields a blue color that turns yellow upon the addition of sulfuric acid to stop the enzymatic reaction while maintaining low background color.

Protection levels for each antigen were determined as recommended by the producers of diagnostic kits employed.

Vaccinated children are considered protected against tetanus anatoxin if they reach levels of specific antibodies in serum above 0.11 UI/mL and against diphtheria when specific antibody levels are above 0.1 UI/

mL. For *B. pertussis* it is not well established a clear correlation between serum titers produced after vaccination and protection (seroproteccion). However, Nova Tec suggests considering a positive sample if total IgGs against *B. pertussis* or *B. pertussis*'s toxin are higher than 11 Novatec Units (NU). In case of Hepatitis B, it is worldwide established that titers equal or above 10 UI/mL of anti-HB in serum of vaccinated children are enough to confer protection against the disease.

#### Scale-Up

Third variant of vaccine production previously described was selected for scaled up. A total of six batches, three of 20 L and other three of 100 L were formulated and subjected to physical, chemical and biological assays for the Quality Control Department approval.

## Results and discussion

#### Pre-formulation

Potency results for each of the four antigens are shown in table 2. The technological variants were analyzed at two different times.

For all antigens potency results obtained using technological variants 2 and 3 satisfied the specification limits. This was not the case for HB antigen in technological variant 1 where the potency was consistently below the specification limits. In this variant HBsAg was incorporated to the formulation without previous adsorption to the alumina and subsequently whole cells of *B. pertussis* were added. Consisting of entire cells, this latter component is the most aggressive in the formulation due to the presence of proteases and agglutinogens among other unwanted biomolecules. When the first fraction of *B. pertussis* cells is added it could produce contacts with the HBsAg leading to chemical interference. This contact is repeated when a second fraction of *B. pertussis* is added 16 hours later potentially producing a second interference effect over HBsAg.

This phenomenon could take place also in variant 2 because HBsAg is also added to the formulation without previous adsorption. Nevertheless potency results are within the specifications limits for this assay. The incorporation of *B. pertussis* in a single addition could reduce the contact between the HBsAg and *B. pertussis* cells leading to a diminished interference.

There is an obvious greater effectiveness in the response for variant 3 probably because of the protection

**Table 2.** Potency results for the four antigens present in Trivac HB<sup>®</sup> vaccine formulation evaluated for the 3 technological variants under study. Assays were conducted at 0 and 6 months

| Assay               | Specifications | Variant 1 |       | Variant 2 |       | Variant 3 |       |
|---------------------|----------------|-----------|-------|-----------|-------|-----------|-------|
|                     |                | T=0       | T=6 m | T=0       | T=6 m | T=0       | T=6 m |
| Diphtheria          | ≥ 2.0 U/mL     | 9.60      | 5.40  | 10.00     | 8.20  | 10.00     | 11.20 |
| Tetanus             | ≥ 2.0 U/mL     | 30.0      | 42.00 | 41.00     | 28.00 | 30.00     | 38.00 |
| <i>B. Pertussis</i> | ≥ 4.0 U/dosis  | 13.70     | 8.60  | 14.50     | 12.30 | 13.60     | 15.00 |
| HBsAg               | ≥ 0.5          | 0.14*     | 0.23* | 0.80      | 1.20  | 2.74      | 2.53  |
|                     |                | 0.18*     | 0.19* |           |       |           |       |

\* This assay was made in two independent experiments.

conferred to HBsAg when it is added to the formulation previously absorbed to alumina. With variant 3 not only is fulfilled the specification, but also even better results were achieved since the values were 2 or 3 times higher than the obtained for variant 2. Hence the final procedure chosen for vaccine formulation includes both, pre-adsorption of HB antigen to alumina and incorporation of *B. pertussis* cells in a single addition.

In the three variants the potency assays for D, P and T antigens satisfied specification limits.

### HB component immunogenicity

Results of the immunogenicity study of HB component are shown in figure 1.

The anti-HB antibody titers agreed with potency test results obtained for each group. Even more, in variants 2 and 3 the anti-HB titer was higher than reference batch. This enhancing of the immunological response could be a consequence of the presence of other antigens in the formulation while in the reference batch HB antigen is alone.

For variant 1 the anti-HB response was not satisfactory indicating that this variant is neither the choice for scale up nor for the stability study.

Finally, additional physico-chemical studies performed to the formulations of the three technological variants showed no difference and satisfied the specifications established for each case.

Considering that variant 3 rendered the best results for HB antigen and the biological assays for D, P and T antigens were satisfactory, this variant was selected to be included in the stability study.

### Stability study

Three batches were stored 30 months at  $5 \pm 3$  °C. Results of the physico-chemical assays carried out to these batches satisfied the specifications regulate for each case. PH and concentration of alumina, Thimerosal and formaldehyde, affect the organoleptic characteristics of the vaccine. These factors could lead to the formation of clots, turbidity or change in vaccine color. The technology employed for the addition of the components as well as the controlled stirring used during the formulation process are also important for the organoleptic characteristics. The procedure employed in this work for the combination of the adjuvant, preservatives and excipients allowed to obtain a final product with proper organoleptic characteristics. A non toxic response was observed for the product during the evaluation period. It was also possible to identify all antigens on the vaccine.

### Potency of the antigens

In the time course of the stability study potency tests were carried out at different sampling times. In all cases, results for the four antigens present on the vaccine Trivac HB® were found within acceptance limits. Deviations observed were always associated to the utilization of biological systems which generally imply a considerable variability in the results. Potency test for tetanic anatoxin showed the greatest variability reaching sometimes values above the corresponding specification. Different from this case, for the diphtheria anatoxin the results obtained for the different

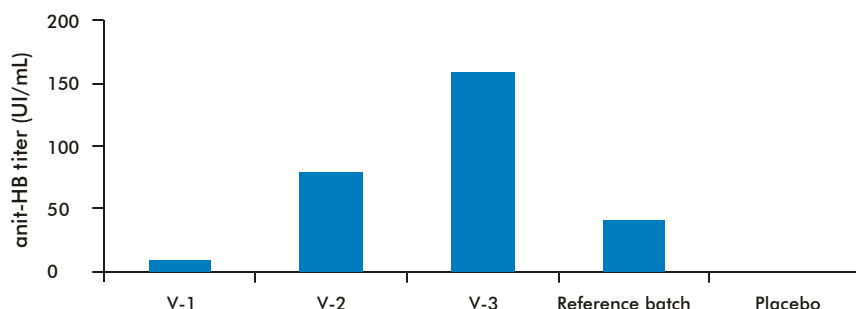


Figure 1. Anti-HB antibody titer. Variants 1, 2 and 3 are presented as V-1, V-2 and V-3. Antibody titers are expressed as IU/mL.

sampling times and batches were very similar. The variability for the HBsAg and *B. pertussis* was less noticeable.

It is important to remark that the relative humidity in the cold rooms employed for the storage was higher than 90%. This becomes a challenge for the preservation of the vaccine and the results obtained are very satisfactory since this value is higher than the average of relative humidity reported by Cartwright [18] for the tropical regions (70%).

Also during the 30 months of the stability study, the sterility test demonstrated primary package and the closing mechanism were adequate and safe for the preservation of the product.

There was good correlation between the identification results of the four antigens and the potency tests for each of them. All the antigens were consistently identified and the potency test satisfied the specifications for each case. The results for 30 months demonstrated the safety on the time of use of the product for the clinical study.

### Pre-clinical studies

The body weight of the animals immunized increased with time and there were no differences related to gender within the groups neither between the different groups evaluated.

On the clinical inspection, the animals had no signs of any toxicity effect or alterations in their behavior. There was no mortality among the animals in any of the study groups.

On the macroscopic examination of the organs there were observed calcifications in the pericardial region indistinctively in control animals as well as treated animals. Pericardial calcification is frequently found in Balb/C mice. This symptom is of genetic origin and the magnitude depends on different factors such as age, gender and diet of the animal. Hence it was considered not relevant for the study. The balanopostitis with deposited semen reported for very few animals was also considered not significant for the study. This anomaly appears spontaneously because of multiple causes not related with the object of this study [19].

### Clinical studies

#### Phase I

On this phase was evaluated vaccine reactogenicity in vaccinated children. Main findings of this part of the study are summarized below:

18. Cartwright AC. Stability Test on Active Substances and Finished Products: New European Guideline. Drug Development and Industrial Pharmacy 1989;5(10): 1743-57.

19. Faccini JM, Abbott DP, Paulus GJ. Mouse histopathology. A glossary for use in toxicity and carcinogenicity studies. Elsevier, Amsterdam-New York-Oxford, 1990.

1. Only 112 children (41.9%) showed adverse reactions and these were essentially mild.

2. In 85.5% of cases reactions were reported in the first 24 hours.

3. 74.5% of adverse reactions reported were systemic events like fever (45%) and febricula (23%).

The above mentioned results demonstrate that Trivac HB<sup>®</sup> vaccine is atoxic and well tolerated by children.

### Phase II

Two fundamental aspects evaluated in phase II of the clinical studies were: 1. percentage of seroprotection which refers to the fraction of the children that developed the necessary antibody level as to be protected against each antigen and 2. geometric mean of the antibody titers after the vaccination.

Global seroprotection was 98.8% for anti-HB antibodies, 97.9% for anti-tetanic toxin, 100% for the antibodies anti-diphtheria toxin and 93.1% for anti-*pertussis* antibodies.

Global geometric mean of antigen-specific antibody titers were: 387.07 U/mL for anti-HB antibodies, 0.56 U/ml for anti-tetanic toxin antibodies, 1.13 U/mL for anti-diphtheria toxin antibodies and 21.67 NU (Novatec Units) for anti-*pertussis* antibodies.

Results of the vaccination with Trivac HB<sup>®</sup> were satisfactory. Sero-protection due to antibodies was above 97%. This result is particularly relevant in the case of *B. pertussis* considering that for this antigen an antibody response between 80-90% is considered a very good result. It is also significant that in all cases geometric means of antigen-specific antibody titers were higher than the required to confer protection. On this respect, the lowest values of antibody titers were obtained for the tetanic anatoxin and even in this case were 5 times of the necessary titer to confer protection.

This stage of the study demonstrated that the combined vaccine Trivac HB<sup>®</sup> confers an adequate protection for vaccinated children.

### Scale up

The study with six batches at a scale of 20 and 100 liters satisfied the specifications established for their control.

The results of the study of potency of *B. pertussis* for the batches formulated at the scale of 100 liters were better than the results obtained for the batches formulated at the scale of 20 liters. This fact demonstrated that the scale up process did not impair the quality of the vaccine.

### Conclusions

After the evaluation of three technological variants for production of the combined vaccine DPT-HB, variant 3 was selected as the technological candidate to be scaled up. This variant was also used in the study of the shelf life of the formulation. The stability studies render the vaccine as stable for 24 months when stored at  $5 \pm 3$  °C. The primary package is adequate since no interactions between the vial and the product were detected. Also, it was demonstrated that the package preserve the sterility and all quality parameters of the product.

Pre-clinical studies demonstrated the vaccine is atoxic. On the other hand, evidences from the clinical study shown the four antigens included in the vaccine capable of conferring a high protection to the immunized children. It was also demonstrated that DPT-HB vaccine is safe and effective.

Results obtained in this study showed the efficacy of the procedure employed for the scale up of the formulation which is one of the most important goals in the bulk production of the tetravalent vaccine Trivac HB<sup>®</sup>. On this ground, Cuban National Regulatory Authority granted the Sanitary License for Pharma-ceutical Operations and the Trivac HB<sup>®</sup> Register for the production and commercialization of the vaccine. This achievement allowed introducing the Trivac HB<sup>®</sup> vaccine in the National Immunization Program of Cuba.