Validation of the combined toxin-binding inhibition test for determination of neutralizing antibodies against tetanus and diphtheria toxins in a vaccine field study in Viet Nam

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Determination of seroconversion and measurement of protective antibody levels in children against vaccine components are essential for gauging and monitoring the efficacy of paediatric vaccination programmes.

For this purpose, we assessed the combined toxin-binding inhibition (ToBI) test for determining neutralizing antibodies to tetanus and diphtheria in a diphtheria–pertussis–tetanus (DPT) vaccine field trial in Viet Nam. A simple procedure involving collection of blood samples on filter-paper was found to be a suitable alternative to collection by venepuncture, despite a reduction in the sensitivity of the ToBI test as a result of the step necessary to elute the antibodies from the filter-paper. The results obtained demonstrate that the ToBI test can feasibly be carried out under field conditions. Preliminary results obtained with the ToBI test in DPT field trials indicate that a fourth dose of DPT vaccine one year after the third dose should be considered by developing countries.

Introduction

WHO promotes improvement of quality control and quality assurance in those countries that meet the criteria for strengthening local vaccine production.5 Viet Nam (population, 71 million) is such a country and has been selected by the Children’s Vaccine Initiative (WHO/CVI) as a priority setting for rapid implementation of vaccine self-sufficiency programmes.6

Diphtheria–pertussis–tetanus (DPT) adsorbed vaccine is one of the most important used in the Expanded Programme on Immunization (EPI) in Viet Nam. Traditionally this vaccine was prepared using a static culture method, which had the disadvantage that the production capacity was not sufficient to meet the demand. Since 1986, however, a modern production line for DPT vaccines supported by UNICEF, based upon fermenter technology, has been operational in the National Institute of Vaccines and Biological Substances (IVAC) in Nha Trang and Dalat.

After it had been confirmed in comparative duplicate quality control evaluations that various DPT vaccine lots, locally produced at IVAC, met all WHO requirements for safety and potency, they were used in a field trial. UNICEF DPT vaccine, obtained through UNICEF/WHO–EPI in Viet Nam, was included in the trial for reference purposes.

The combined toxin-binding inhibition (ToBI) test was selected to determine antibodies against diphtheria and tetanus toxins, because it has been properly validated for correlation with the in-vivo toxin neutralization test, also in the low titre range (1). Also, the ToBI test appears to be suitable for field trials since it requires only small amounts of blood; it has an established reproducibility and sensitivity, and it requires no experimental animals, unlike the in-vivo toxin neutralization test. Finally, because the ToBI test is an enzyme-linked immunosorbent assay (ELISA) it is suitable for dealing with large numbers of samples.

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A simple method involving collection of blood on filter-papers was employed for the following reasons: samples can more easily be transported under tropical conditions; and it avoids the difficulties associated with collecting blood from infants by venepuncture.

The main objective of the study was to validate the ToBI test and the use of a simple filter-paper blood collection method in a developing country. A further aim was to illustrate the test’s practical application in the preliminary assessment of the immunogenicity of locally produced DPT vaccine.

Materials and methods

Blood collection using filter-paper

Blood was collected following a method developed by the National Institute of Hygiene and Epidemiology (NIHE), Hanoi, Viet Nam, and which is routinely used in measles surveillance studies. Briefly, blood samples were collected from a finger using a needle and absorbed fully onto two squares (1 cm × 1.5 cm) of filter-paper (Whatman type 3). This enabled 50 µl of blood to be collected (approximately 25 µl of serum). The papers were dried at room temperature, placed in a small light-coloured plastic bag, and sealed before being transported to a cool, dry place until testing. Before being sealed in the bags, the papers were carefully inspected to ensure that they were completely dry. Since all blood collections took place in the hot, dry season, the papers had usually dried within an hour, although in some cases an incubator was used. All filter-paper samples were coded.

Prior to testing, each filter-paper was soaked in 250 µl of phosphate buffer solution for 1 hour, with occasional shaking, to obtain a 1:10 dilution of the serum sample. In the combined ToBI test, 1:10 diluted serum was added to the first column of the wells.

Combined ToBI test for diphtheria and tetanus neutralizing antibodies

The ToBI test was used as described previously by Hendriksen et al. (2). Briefly, 100 µl of twofold dilutions of both reference and test sera were made up in phosphate-buffered saline. Subsequently, 120 µl of a mixture containing 0.02 LF units/ml of tetanus toxin and 0.01 LF units/ml of diphtheria toxin were added. After overnight incubation, any non-neutralized toxin was detected by ELISA by transferring 100 µl of the serum–toxin mixtures to immunoassay plates coated with tetanus or diphtheria antibodies. After the plates had been incubated, bound toxin was detected by successive incubation with biotinylated horse-antitetanus or horse–antidiptheria and steptavidin biotinylated horseradish peroxidase complex followed by addition of 3,3’,5,5’-tetramethylbenzidine substrate. The enzymatic reaction was stopped after 10 minutes by addition of 100 µl of 2-mol/l H₂SO₄.

The absorbance in all the wells was measured using an automatic plate reader. Estimated antibody titres were multiplied by the pre-dilution factor (1:10).

The relative antibody activity of each serum sample was calculated by comparing the OD₅₀ values (the absorbance (A) below the value representing 50% of the total absorbance, defined as the sum of the arithmetic mean absorbance of positive control wells and the arithmetic mean absorbance of negative control wells) of the sera under test with the OD₅₀ of the National Institute of Public Health and Environmental Protection (RIVM) horse reference antitoxin. The results reported here (with the exception of those shown in Fig. 1) were calculated using this OD₅₀ method. The results shown in Fig. 1 were obtained using Biokat software (Biotek, Winooski VT, USA) to estimate the antibody levels against a calibration curve employing a four-parameter logistic fit. In this regression technique the antibody activity of the test serum is calculated and expressed in IU/ml against the RIVM horse reference antitoxin.

Negative results, i.e., those below the detection level of the test, were set at 0.001 IU/ml. The minimum antibody level that could be detected by the ToBI test in this study was, however, 0.06 IU/ml.

Serological field studies

ToBI validation. Three main groups of samples were used for validation of the ToBI test as outlined below.

- To validate the tetanus ToBI test used in IVAC against the ToBI test at RIVM, blood samples collected by venepuncture from 20 human volunteers (IVAC staff) were divided into two aliquots and tested blindly for tetanus antibodies at two different locations: the first was sent to RIVM, Bilthoven, Netherlands; the other was tested at IVAC, Nha Trang, Viet Nam.

- To validate the ToBI test and the filter-paper method two experiments were carried out: 1) 22 blood samples were collected by finger-prick and venepuncture and tested at IVAC; this was carried out prior to the start of the field studies and the
**Clinical vaccine trial**

**Study design.** A DPT vaccine field trial is being carried out by the Vietnamese Ministry of Health to evaluate various lots of DPT vaccines, both those produced nationally and those supplied by UNICEF. The trial involves three phases, as described below, but only the results of the first phase are reported here.

- **Phase I:** 15–30 infants per group (i.e., per vaccine lot) are studied mainly for side-effects and serology for the diphtheria and tetanus components.

- **Phase II:** after evaluation of phase I results, 15–100 infants per group are studied mainly for side-effects and serology (diphtheria and tetanus).

- **Phase III:** after evaluation of phase II results, 1000 infants per group are evaluated to obtain preliminary information on the efficacy of the diphtheria and tetanus components of the vaccines studied. The use of EPI programme cards (one per child) proved helpful in the assessment of the immunization status of the infants.

A total of 90 healthy unvaccinated infants aged 3–9 months with no symptoms of intestinal disease entered phase I of the study, which was performed in two distinct areas/provinces: Tien Giang and Lam Dong in the south of Viet Nam. The infants in each province were divided randomly into three groups, with about 15 infants in each group, corresponding to the three vaccines used in the study. Since the study was longitudinal, individual children could be followed throughout its course, allowing seroconversion to be monitored.

**Vaccines, immunization schedule, and administration.** Each child received three intramuscular injections (0.5 ml each of vaccine) at 1-month intervals. Three lots of DPT vaccine (one procured through UNICEF (the “UNICEF” vaccine) and two locally produced), all of which met WHO requirements for safety and potency, were included in the study. Only data on two vaccines are reported here. The locally produced vaccine (lot DPT-23K), data for which are reported here, contained diphtheria and tetanus components that had been mixed with a pertussis component procured through UNICEF. Both the UNICEF and lot DPT-23K vaccines had the following composition: 16 IU of pertussis, 15 LF of diphtheria toxoid, 10 LF of tetanus toxoid, and 1.5 mg of aluminium phosphate.

Samples of peripheral blood were collected prior to each of the three injections, as well as 1 month and 1 year after the third injection. Collection was carried out by finger-prick using filter-papers.

**Statistical tests**

Logarithmic-transformed antibody titres obtained by two different blood collection methods were compared using regression analysis.

**Results**

**Standardization of the ToBi test**

Table 1 shows the tetanus antibody titres estimated...
using the ToBI test in the RIVM and IVAC laboratories. The results demonstrate a high correlation between the two laboratories; regression analysis produced a correlation coefficient \( r \) of 0.922; \( Y = 0.967X - 0.21 \). A total of 10 samples were classified as “negative” (i.e., below the detection level of 0.06IU per ml) in both laboratories; eight samples were classified as significantly >0.1 IU per ml in both locations; in two cases a titre of 0.06IU/ml in IVAC was found to be <0.06IU/ml in RIVM. A repeat test carried out at RIVM on both these samples yielded a titre of 0.06IU per ml for one of them. It was concluded that the inter-laboratory variation fell within that in each individual laboratory.

**Blood collection by the filter-paper method**

Since finger-prick collection of blood on filter-papers was held to be convenient for use in large-scale field trials, we evaluated the effect on the ToBI test of collecting blood this way compared with collection by venepuncture using samples from human volunteers. Two validation studies were carried out as discussed below.

Table 1: Tetanus antibody titres in human sera estimated using the ToBI test in the two study laboratories

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Antibody titre in IU/ml at:</th>
<th>IVAC</th>
<th>RIVM</th>
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<tr>
<td>01</td>
<td></td>
<td>0.125</td>
<td>0.125</td>
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<tr>
<td>02</td>
<td></td>
<td>2.0</td>
<td>4.0</td>
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<tr>
<td>03</td>
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<tr>
<td>05</td>
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<td>&lt;0.06</td>
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<tr>
<td>06</td>
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<td>0.5</td>
<td></td>
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<tr>
<td>07</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
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<tr>
<td>08</td>
<td>&lt;0.06</td>
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<td></td>
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<td></td>
<td>&lt;0.06</td>
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<td>1.0</td>
</tr>
<tr>
<td>20</td>
<td>&lt;0.06</td>
<td></td>
<td>&lt;0.06</td>
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</table>

* Shown are the titres for neutralizing anti-tetanus antibodies on paired samples from 20 human volunteers from Viet Nam. IVAC = National Institute of Vaccines and Biological Substances, Nha Trang, Viet Nam. RIVM = National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands; <0.06 = too low to detect.

The first study, carried out prior to the start of the field studies, used 22 blood samples collected from human volunteers and the results were calculated using the OD\(_{50}\) method. Regression analysis of the results showed that the correlation coefficient was high for both antibodies: tetanus \( r = 0.98 \); and diphtheria \( r = 0.95 \) (data not shown). Under the conditions chosen, the method can be used reliably in field studies to estimate antibody levels greater than 0.06IU per ml; this detection limit is a consequence of the tenfold reduction in sensitivity caused by the elution of the antibodies from the filter-paper (see Discussion). Since antibody levels >0.01 IU/ml are generally held to provide clinical immunity against diphtheria and tetanus, a positive ToBI test indicates that a child is probably protected.

The second validation study involved blood samples from 49 human volunteers using a novel method for estimating titres (Biotek). The results (Fig. 1) corroborate those found in the first study discussed above, i.e., in the ToBI test, blood collected on filter-paper gives similar titre estimations to those obtained by venepuncture. A regression analysis showed that the correlation coefficients were even higher for the Biotek method than for the OD\(_{50}\) approach for both antitoxins: tetanus \( r = 0.998 \), \( n = 49 \); diphtheria \( r = 0.956 \), \( n = 49 \).

**Children with antibody titres >0.06IU/ml after vaccination**

As part of an ongoing phase-I DPT vaccine field study, in which different vaccine lots were being evaluated, the seroconversion and subsequent development of antibody titres among a group of 30 children immunized with vaccine lot 23K were followed up. The results obtained were compared with data for another group of 30 children immunized with the UNICEF vaccine.

Two groups of 30 children were immunized with either lot DPT-23K or UNICEF DPT vaccine and followed up for 1 year after the third injection. Of the 30 infants immunized with lot DPT-23K, seven (23%) had antibody titres >0.06IU/ml against tetanus, whereas four (13%) exhibited titres >0.06IU/ml against diphtheria before the first dose was given (pre-phase I) (Table 2).

In particular, we examined the development of antibody titres among children who were seronegative at the pre-phase I stage. Table 2 shows the number of children (expressed as a % of the number of seronegative children at the pre-phase I stage) with antibody titres >0.06IU/ml up to 1 year after completion of the immunization schedule.

For diphtheria and tetanus antitoxins, the proportion of children with titres >0.06IU/ml one
month after the third injection was 100%. One year after the third injection, a significant reduction in the number of children with antibody levels >0.06 IU/ml occurred for diphtheria (9/20 (45%), while for tetanus 100% (17/17) still had antibody levels above this value.

Similar trends were observed for different domestically produced DPT vaccine (data not shown) as well as the UNICEF vaccine supplied through the EPI programme (Table 2). It should be borne in mind, however, that the proportion of children actually protected against clinical disease by the vaccines studied will be greater than the 45% reported here since the cut-off applied (>0.06 IU/ml) is well above the 0.01 IU/ml level that is believed to provide clinical immunity against diphtheria.

The data in Table 2 show that for both diphtheria and tetanus the titres decreased between 1 month and 1 year after the third dose. At least for diphtheria, this may lead to a subsequent substantial increase in the number of children with antibody titres ≤0.06 IU/ml.

**Discussion**

Field studies in developing countries are hampered by the lack of reliable, reproducible screening methods that can be easily carried out under tropical conditions.

Our findings confirm the feasibility of using the ToBI test as a screening tool in field trials for evaluation of vaccines. The test was implemented at the IVAC quality control laboratory by one of us (H.A.H.) after a period of training at RIVM, where it had been developed and standardized.

Collection of blood on filter-papers was found to be satisfactory in combination with the ToBI test. Other workers have also recently reported that filter-paper collection is a reliable alternative to venepuncture for determination by ELISA of antitoxin titres to tetanus and diphtheria in blood samples. The particular advantage of the ToBI test, however, over such direct ELISA assays is its good correlation with the results of the in-vivo mouse toxin neutralization test in the low titre range (3).

In order to obtain the cooperation of mothers, all efforts should be undertaken to minimize the "trauma" associated with blood collection from neonates. The filter-paper collection method in combination with the ToBI test appears to be the optimal choice. The method proved to be very reliable and reproducible, since aliquots of the same human adult serum samples tested at different locations yielded consistently the same results (see Table 1).

Finally, from a public health point of view, the sensitivity level of 0.06 IU/ml used in this study provides a safety margin compared with other studies, which have assumed that a titre of 0.01 IU/ml would provide clinical immunity against diphtheria. A novel method of estimating antibody activity, based upon a four-parameter fit (Biotek) (Fig. 1) indicated that with this method a cut-off ≤0.06 IU/ml can be defined (Fig. 1). Further study is required to determine whether this approach in conjunction with the filter-paper/ToBI procedure would lead to reliable
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Table 3: Comparison of the geometric mean titres of diphtheria and tetanus antibodies before and after the third dose of DPT vaccine between children in the present study and studies carried out in the USA

<table>
<thead>
<tr>
<th>Study:</th>
<th>Present (lot 23K vaccine)</th>
<th>Present (UNICEF vaccine)</th>
<th>Anderson et al. (ref. 10)</th>
<th>Anderson et al. (ref. 11)</th>
<th>Edwards et al. (ref. 12)</th>
<th>Barkin et al. (ref. 13)</th>
</tr>
</thead>
</table>
| Diphtheria<br>Pre-ll | 0.001 | 0.002 | 0.04 | 0.01 | 0.06 |<br>Pre-ll | 0.03 | 0.11 | 0.23 | 0.31 | 0.03 | 0.07 |<br>1Mo-ll | 0.55 | 0.49 | 0.75 | 1.45 | 0.25 | 0.26 |<br>1Yr-ll | 0.01 | 0.03 | 0.17 |<br>Tetanus<br>Pre-ll | 0.001 | 0.01 | 0.05 | 0.04 | 0.31 |<br>Pre-ll | 0.91 | 0.92 | 1.65 | 0.42 | 0.13 | 0.15 |<br>1Mo-ll | 3.04 | 5.15 | 13.18 | 2.40 | 1.50 | 0.51 |<br>1Yr-ll | 0.22 | 0.50 | 0.23 |<br>Sample taken:<sup>a</sup> |<sup>a</sup> See footnote a, Table 2.

titre estimations in the range 0.06–0.01 IU/ml. The need for serological monitoring of vaccinees has been emphasized by studies in Sweden, which have reported that the protection level against diphtheria fluctuates considerably in individuals (4, 5). Our preliminary findings confirm those reported in studies carried out in Africa, Europe, and the USA that antibody levels against the diphtheria component of DPT vaccine tend to decrease rather quickly (4, 6–10). This is also illustrated in Table 3, which compares our geometric mean titres at certain times after immunization with those reported by various studies from the USA (10–13).

Confirmation of our findings by a larger survey would provide a scientific basis for the recommendations made by the Vietnamese Ministry of Health that a follow-up (fourth) booster dose in DPT immunization programmes should be given. This is in line with the general recommendation for diphtheria immunization policy in developing countries, i.e., should the epidemiology of diphtheria pose a significant health problem in preschool or school-age children, use of supplementary doses of diphtheria toxoid following high coverage primary immunization of under-1-year-olds is strongly favoured.<sup>c</sup>

It has been proposed that fully immunized children with antibody titres against diphtheria that gradually decrease with time (eventually falling below 0.01 IU/ml) still possess circulating memory cells and that such "primed" children probably have a basic immunity that protects them from clinical disease when challenged in nature (8).<sup>c</sup> Nevertheless, a policy advocating use of a booster dose after primary vaccination may be wise since the current diphtheria epidemic in the Commonwealth of Independent States (CIS) has revealed that (mild) cases of diphtheria occurred in persons with a full immunization history. This may be a result of the absence of natural stimuli caused by the reduction in the Corynebacterium diphtheriae reservoir, leading to a shorter post-vaccination immunity or, alternatively, may have been related to the suboptimal immune response following use of low potency vaccines for infant primary series immunization.<sup>d</sup>

Implementation of the ToBI test in a laboratory engaged in routine quality control of vaccines has the additional advantage that it could serve as an in-process control to monitor the potency of diphtheria and tetanus toxoids. Use of the ToBI test in the T-potency test has been proposed (14).

The successful introduction of the ToBI test under field conditions allows objective evaluation and comparison of locally produced vaccines, which is critical for the release by national control authorities of domestically produced vaccines to replace imported vaccines in EPI programmes. The evidence provided in Table 2 shows that 1 month after the third dose, the locally produced DPT-lot 23K conforms in general with WHO requirements for diphtheria and tetanus vaccines, i.e., that for 90% of the target population antitoxin levels against diphtheria and tetanus should be >0.01 IU/ml after the completion of primary immunization of previously unvaccinated individuals (15).


Finally, the work described here illustrates the usefulness of incorporating into technology transfer projects validated quality control methods as well as serological methods for disease surveillance in the target population.

Acknowledgements
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Résumé
Validation de l’épreuve associée d’inhibition de la liaison à la toxine pour la détermination des anticorps neutalisaants dirigés contre les toxines tétanique et diphtérique au Viet Nam

La détermination de la séroconversion et la mesure des taux d’antigènes protecteurs dirigés contre les constituants des vaccins chez l’enfant sont des outils indispensables pour évaluer et surveiller l’efficacité des programmes de vaccination pédiatrique. A cet effet, nous avons évalué l’épreuve associée d’inhibition de la liaison à la toxine (ToBI) pour la détermination des anticorps neutralisants dirigés contre les toxines tétanique et diphtérique, lors d’un essai pratique de vaccin antidiphtérique-anticoquelucheux-antitétanique (DTC) au Viet Nam. L’épreuve a été réalisée sur des prélèvements de sang recueillis sur papier filtre, méthode directe qui facilite le transport des échantillons en milieu tropical et évite les problèmes que pose le prélèvement de sang par ponction veineuse chez le nourrisson.

Le test ToBI est correctement validé du point de vue de sa corrélation avec l’épreuve in vivo de neutralisation de la toxine, également dans la gamme des faibles titres, et n’exige que de petites quantités de sang; sa reproductibilité et sa sensibilité sont établies, et aucun animal d’expérience n’est nécessaire.

D’après nos résultats, le ToBI a été mis en œuvre et validé avec succès au laboratoire de contrôle de la qualité de l’institut national de production de vaccins au Viet Nam. Le prélèvement d’échantillons de sang sur papier filtre est une alternative valable à la ponction veineuse, malgré une perte de sensibilité liée à l’étape d’élation de l’anticorps à partir du papier filtre. Plusieurs études de validation à petite échelle ont confirmé qu’avec cette épreuve le prélèvement de sang sur papier filtre donne des estimations du titre analogues à celles obtenues par ponction veineuse chez le même individu ($r = 0.988$ pour le tétanos et 0.956 pour la diphtérie).

L’épreuve ToBI utilisée en association avec le prélèvement de sang sur papier filtre représente donc une approche valable et réalisable de la surveillance de la réponse sérologique aux constituants tétanique et diphtérique du vaccin DTC lors d’essais pratiques en milieu tropical. Les résultats préliminaires d’une étude en double aveugle utilisant l’approche ToBI/papier filtre indiquent qu’une quatrième dose de DTC injectée un an après la troisième dose pourrait être envisagée dans les pays en développement.

References
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