

RIVM report 623860008

**The Pertussis Serological Potency Test
collaborative study to evaluate the replacement
of the Mouse Protection Test**

A.A.J. van der Ark, I. van Straaten - van de
Kappelle, R.M. Ölander, K. Enssle, S.S. Jadhav,
H.J.M. van de Donk, C.F.M. Hendriksen

June 1999

This investigation has been performed by order and for the account of Alternative to Animal Experiment Platform within the framework of project 623860, "Coordination Alternatives to Animal Testing ", sub-project 190060, "Validation of a serological model (PSPT) as an alternative to the lethal challenge procedure in potency testing of pertussis whole cell vaccines".

National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands
Telephone: + (31) 30 247 91 11 , Telefax : +31 30 274 29 71

Abstract

A small-scale collaborative study was performed to establish the validity and reproducibility of the Pertussis Serological Potency Test (PSPT). The PSPT was developed as an alternative to the Mouse Protection Test (MPT). In an in-house validation study it was shown that the PSPT is easy to perform and should warrant the expectation of a reliable potency testing of pertussis whole cell vaccines.

The collaborative study was divided in 3 phases: the pre-Phase to try out the 18323-whole cell ELISA (18323-WCE), Phase I to assess the precision of the 18323-WCE for each participant, and Phase II to evaluate the implementation of the PSPT, and to compare and correlate the PSPT with the MPT.

The intra-assay and inter-assay precision varied per laboratory (from 8.9 to 27.9% and 13.4 to 24.5%, respectively) and the inter-laboratory precision has been determined at 25.1%.

However, the ranking in levels of antibodies of the serum pools corresponded well between the laboratories.

The overall correlation between the PSPT and MPT was demonstrated by means of a χ^2 -test of homogeneity: $p > 0.05$ and a PSPT/MPT ratio of 0.877 (0.738-1.065). Compared to the MPT, the PSPT is more reproducible and thereby reduces the chance of re-testing compared to the MPT. The somewhat higher frequencies of products meeting the lower limit of the potency (2.0 IU/human dose) in the PSPT compared to the MPT was due to the smaller 95% confidence intervals.

Contents

Samenvatting 4

1. Introduction 6

2. Laboratory animals, Materials and Methods 8

2.1 *Laboratory animals* 8

2.1.1 Mice 8

2.2 *Materials* 8

2.2.1 Vaccines 8

2.2.2 Reference sera 8

2.2.3 Serum samples 8

2.2.4 18323-Whole Cell ELISA 9

2.3 *Methods* 9

2.3.1 Pertussis Serological Potency Test 9

2.3.2 Intracerebral Mouse Protection Test 10

2.3.3 Collaborative study design 10

2.3.4 Statistics 10

3. Results 12

3.1 *Pre-phase: 18323-WCE try-out* 12

3.2 *Phase I: 18323-WCE validation* 12

3.3 *Phase II: comparative study of the PSPT and MPT* 13

4. Discussion 15

References

Appendix 1 Participants collaborative study on PSPT

Appendix 2 Tables

Appendix 3 Mailing list

Samenvatting

De Pertussis Serological Potency Test (PSPT) is gebaseerd op het *in vitro* meten van de humorale afweerrespons tegen *Bordetella pertussis* bacteriën en ontwikkeld als een alternatief voor de muisbeschermingstest (MBT) voor het kinkhoest “whole cell” vaccin (WCV). Middels een internationale ringstudie van beperkte omvang (5 laboratoria) is de relevantie en betrouwbaarheid van de PSPT bestudeerd. De studie is opgedeeld in drie verschillende fases met elk hun eigen doelstelling. De pre-fase is toegevoegd als trainingssessie voor de participanten, die geen ervaring hadden met de antilichaam detectie assay, de 18323-whole cell ELISA (18323-WCE). Zestien serumpools zijn op 5 verschillende dagen getest, hetgeen resulteerde in significante verschillen in de extinctiewaarden en antilichaamconcentraties tussen de laboratoria. Tijdens de fase I studie werd de herhaalbaarheid (plaat en dagverschil) en de reproduceerbaarheid (verschil tussen laboratoria) bestudeerd. De deelnemende laboratoria werd gevraagd 16 serumpools op 5 verschillende dagen in vijfvoud te testen. De gewenste precisie van minder dan 20% werd niet altijd gehaald en significante verschillen in antilichaamconcentraties werden gedurende de hele fase I-studie gevonden. Echter, de rangschikkingen van de serumpools op basis van de antilichaamconcentraties van de laboratoria komen goed met elkaar overeen, waardoor een betrouwbare potency bepaling van WCV's in de PSPT gewaarborgd lijkt. In fase II was een vergelijkend onderzoek van de PSPT en de MBT, om de implementatie van de PSPT in andere laboratoria te evalueren door de reproduceerbaarheid en betrouwbaarheid van beide modellen te vergelijken en een correlatie tussen beide modellen aan te tonen. Door vier van de vijf participanten werden 4 WCV's van verschillende herkomst tweemaal in beide modellen getest. De gemiddelde antilichaamconcentratie per vaccindosis in de PSPT, maar ook de overleving van de muizen in de MBT verschilde significant binnen en tussen de laboratoria. Desalniettemin, zijn er voor de vaccins in beide werkzaamheidstesten geen significante verschillen in de potencies gevonden ($p>0,05$). Met behulp van de χ^2 -test is aangetoond dat de PSPT en de MBT goed correleren zowel binnen als tussen de laboratoria. De potencies komen goed met elkaar overeen (ratio = 0.877), echter de PSPT is beter reproduceerbaar en verlaagt de kans op hertesten van het vaccin omdat de betrouwbaarheidsintervallen kleiner zijn dan bij de MBT. Met deze studie hebben we aangetoond dat de PSPT een valide test is voor het bepalen van de werkzaamheid van kinkhoest WCV's, afkomstig van verschillende producenten.

Summary

The Pertussis Serological Potency Test (PSPT) - based on *in vitro* assessment of the humoral immune response against *Bordetella pertussis* - was developed as an alternative for the Mouse Protection Test (MPT). A small-scale collaborative study was carried out in five laboratories to evaluate the relevance and reliability of the PSPT. The study has been divided into three separate phases, each with its own objective.

A pre-phase study of the antibody detection assay, the 18323-whole cell ELISA (WCE) was included for training purposes. Sixteen serum samples were tested on 5 different days, resulting in significant differences in absorbance and antibody concentrations between the laboratories.

In the Phase I study, the intra-assay, inter-assay and inter-laboratory precision of the 18323-WCE was assessed. The 5 participants assayed sixteen other serum pools 5 times on 5 different days. Although a precision of less than 20% was not always established and significant differences in antibody concentrations were found at random throughout the Phase I study, the ranking of the antibody concentrations corresponded well between the laboratories and should warrant a reliable potency estimation of whole cell vaccines (WCV's) in the PSPT.

Phase II was a comparative study of the PSPT and the MPT to evaluate the implementation of the PSPT, to demonstrate correlation and to compare the reproducibility and reliability of both tests. Four of the 5 participant have tested 4 different WCV's twice in the PSPT and the MPT. The mean antibody concentrations per vaccine dose in the PSPT and the survival of mice in the MPT differed significantly within and between the laboratories. Nevertheless, the potencies of the vaccines under test estimated in both test models did not differ significantly ($p > 0.05$). The PSPT and MPT correlated well in a χ^2 -test of homogeneity within and between the laboratories. The potencies were almost similar (overall ratio = 0.877), but the PSPT is more reproducible and reduces the chance of re-testing due to the smaller 95% confidence intervals. In conclusion, the PSPT is a valid model to estimate the potencies of pertussis WCV's from different manufacturers.

1. Introduction

Replacement, reduction and refinement of the use of laboratory animals testing - the three R's of Russell and Burch¹ - is a general recognized worthwhile goal for reasons of animal welfare. However, there are others reasons to develop alternatives for the vaccine quality control tests currently used. Improvements of reliability, reproducibility, and safety in the laboratory are also important considerations to replace traditional animal tests. In recent years serological models have been developed,^{2,3,4,5} which can be used as a replacement for lethal challenge procedures in potency testing of Diphtheria- and Tetanus Toxoid vaccines. The MPT⁶ is a lethal challenge model for the potency testing of pertussis WCV's, which has a significant intra-, and inter-laboratory variation,⁷ and requires large numbers of mice. We reported before on the PSPT^{8,9} as an alternative to the challenge procedure. The PSPT is based on the *in vitro* assessment of the humoral response against the wide range of surface-antigens of *B. pertussis* in mice after immunization with WCV. Mice are immunized intraperitoneally (i.p.) with graded doses of vaccines under test and bled after four weeks. Antibodies against *B. Pertussis* in sera are measured in the 18323-WCE with strain 18323 whole cells as coating. The potency of a vaccine under test is based on vaccine dose-dependent antibody responses and estimated by means of parallel line analysis. Good correlation with the MPT was demonstrated in an in-house validation study.⁸ Compared to the MPT, the PSPT is more precise, better reproducible, reduces the number of mice with at least 25%, and the test is less distressful to the animals. Eventually, the number of mice used could be reduced even more by simplifying the multiple dose design to a single dose model and by combining *in vitro* assays for potency testing of tetanus, diphtheria and pertussis components in one animal model.

This report describes the results of a small-scale collaborative study to demonstrate the relevance (correlation with mouse protection) and the reliability (intra- and inter-laboratory variation) of the PSPT. Four laboratories were invited to participate in this study: two laboratories in Europe and two laboratories from outside Europe, selected with the help of the World Health Organization (Appendix 1). The study was divided into separate phases, each with its own specific objective. The pre-phase or 18323-WCE try-out was included for the participants to become acquainted with the 18323-WCE. Validity parameters¹⁰ were recorded during the whole study to evaluate the performance of 18323-WCE. In the Phase I study the intra-assay, inter-assay, and inter-laboratory precision or variation of the 18323-WCE was assessed using a set of 16 serum pools. Our preliminary goal was to achieve a

precision of 20% or better, within a 99% confidence interval (coefficient of variation (CV) = 20%, p = 0.01). In the Phase II study 4 vaccines were tested in the PSPT and MPT, to demonstrate a correlation between both tests and to compare the reproducibility and reliability of the PSPT and MPT. In addition, the influence of local practicalities, such as the mouse strain, housing, and animal diet, on the WCV-induced humoral antibody responses were evaluated. Detailed instructions, procedures and essential materials for this study, such as ELISA coat, ELISA plates, conjugates etc. were provided by the RIVM. The vaccines were a generous gift of 3 of the participants. All data processing and statistical analysis were performed at the RIVM to avoid bias of results.

2. Laboratory animals, Materials and Methods

2.1 Laboratory animals

2.1.1 Mice

For the PSPT and the MPT, equal numbers of both sexes were used according to the specifications as given in Table 1.

2.2 Materials

2.2.1 Vaccines

The reference Kh 85/1 is a lyophilized *B. pertussis* whole cell preparation with a potency of 30 International Units (IU) per ampoule and contains 40 Opacity Units per millilitre (OU/ml) after reconstitution in 5 ml Phosphate Buffered Saline (PBS). Vaccine A is a Diphtheria-Pertussis-Tetanus-polio (DTP-polio) vaccine and vaccine B is an expired batch DTP-polio from the same manufacturer. Both vaccines contain 16 OU/ml pertussis whole cells.

Vaccine C and D are DTP-vaccines produced by two other manufacturers, containing 32 OU/ml. Potencies were previously estimated at the manufacturer's laboratories.

2.2.2 Reference sera

The standard reference serum (PSPTst) of the 18323-WCE contains 415 ELISA Units (EU)/ml. PSPTpc and PSPTnc are used as positive and negative control sera, containing 360 and 0 EU/ml, respectively. The reference sera were obtained from NIH mice immunized i.p. with a protective dose of reference Kh 85/1, DPT-polio or saline for PSPTst, PSPTpc and PSPTnc, respectively. Mice were bled on day 28, sera pooled and calibrated against the former reference serum hyper immune serum (HIS) 4.⁸

2.2.3 Serum samples

For the pre-phase, a set of 16 serum pools from excess PSPT-sera with known antibody concentrations were used. For the Phase I study 16 other serum pools were used, each obtained from one of the vaccine dilutions used in the PSPT. These serum pools were

divided over 80 samples (50 µl/sample) in such a way that each serum pool was measured randomly in the 5 ELISA-plates needed. The serum pools covered the range of antibody concentrations seen in the PSPT.

2.2.4 18323-Whole Cell ELISA

The 18323-WCE was carried out as described previously,⁸ according to detailed instructions. Some alterations were made for this collaborative study. Briefly, polystyrene polysorp immuno plates (Nunc, Denmark) containing 18323-whole cell suspension (0.25 OU/ml) were evaporated overnight. Non-specific binding-sites were blocked. PSPTst was serially diluted twice in a 3-fold dilution range of 8 dilutions, starting at a 1/1000 dilution. PSPTpc, PSPTnc and 16 serum samples under test were serially diluted in a single 5-fold dilution range of 4 dilutions, also starting at a 1/1000 dilution. IgG titers were assessed using the biotin-streptavidin labeling system (Amersham, UK). Finally, binding was visualized by addition of a tetramethylbenzidine (TMB; Sigma, USA) substrate solution; H₂SO₄ stopped the coloring reaction. Absorbance was measured at 450 nm and antibody concentrations were calculated by means of a 4-parameter fitting analysis and expressed as ELISA Units (EU)/ml. The following parameters were recorded during the whole study: the minimum and maximum absorbance of the PSPTst, PSPTpc and PSPTnc, and the antibody concentrations of PSPTpc and PSPTnc. Two other parameters were the limit of detection (LOD) and the limit of quantitation (LOQ). The LOD is the minimal amount of antibodies, which can be distinguished from the background and the LOQ is the minimal antibody concentration, which can be measured with conventional precision.

2.3 Methods

2.3.1 Pertussis Serological Potency Test

The PSPT was performed as described before,⁸ according to detailed instructions. Briefly, mice (n=12) weighing 20-24 grams were immunized intraperitoneally (i.p.) with 0.5 ml of a two-fold serial dilution of reference Kh 85/1 (50, 25, 12.5, and 6.25 µl), vaccines A and B (80, 40, 20, and 10 µl) or vaccines C and D (50, 25, 12.5, and 6.25 µl). Mice were bled after a four-week interval. The serum samples were measured in a random order according to the 18323-WCE protocol and the validity parameters were recorded. The antibody

concentrations were calculated and used to estimate the potency by means of parallel line analysis with log transformation.

2.3.2 Intracerebral Mouse Protection Test

The MPT was performed according to detailed instructions based on the WHO guidelines.¹¹ Mice (n=16) of 10-14 grams in weight were immunized i.p. with 0.5 ml of five-fold serial dilution of reference Kh 85/1 (62.5, 12.5, 2.5, and 0.5 µl), vaccines A and B (100, 20, 4, and 0.8 µl), or vaccines C and D (50, 10, 2, and 0.4 µl). Animals were challenged i.c. after 14 days with virulent *B. pertussis*, strain 18323 using the local challenge culture and procedure. The number of mice that died was recorded daily until day 28. For the potency calculation, only mice dying from day 17 to 28 were taken into account. Based on the percentage of surviving mice per vaccine dilution, the potency of vaccines was estimated by means of probit analysis.

2.3.3 Collaborative study design

The pre-phase or 18323-WCE try out was included for training purposes. The antibody concentrations of 16 pre-phase serum samples were measured on 5 different days and raw data was sent to the RIVM for evaluation. Phase I is a validation study of the 18323-WCE. The participants were requested to measure the antibody concentration of 80 serum samples on 5 different days. The serum samples were from 16 serum pools, which were distributed in such a way that all 16 pools were assayed in random order on each of 5 plates. Raw data were sent to the RIVM for processing and statistical evaluation. Phase II is a comparative study of the PSPT and MPT and included the testing, in duplicate in both tests, of 2 batches of DPT-polio vaccine (A and B) from one manufacturer and 2 batches of DPT vaccine (C and D) produced by two other manufacturers. Raw data were processed and statistically evaluated at the RIVM to avoid bias in results.

2.3.4 Statistics

The precision of the 18323-WCE has been determined by the variation in antibody concentrations (CV in %), and the reliability (p-value) of a series of measurements of each tested serum sample. The intra-assay precision or repeatability expresses the variation in results under the same operation conditions over a short interval of time: differences within or between plates. The inter-assay or intermediate precision expresses the variation within the

laboratories: different days, different technicians etc. The inter-laboratory precision or reproducibility expresses the variation between laboratories in collaborative studies. The CV is calculated by dividing the standard deviation by the average. To determine the intra-assay, inter-assay or inter-laboratory variation, the mean CV of all individual serum samples was used. The reliability (p-value) is estimated by means of analyses of variation (ANOVA). The ranking in antibody concentrations of the serum samples per plate, per day and per laboratory was determined by means of the Kruskal-Wallis method.

Correlation between the corresponding potencies from the MPT and PSPT was estimated by means of a modified chi-square (χ^2)-test of homogeneity.¹² The ratios of both estimates, in which the variances of individual potencies of a test are used as a weighing coefficient, were calculated and used in the χ^2 -test of homogeneity. Both test systems correlate if the ratio does not differ significantly from 1.00 ($p > 0.05$).

The reproducibility and reliability of the PSPT and MPT were determined by means of the geometric mean, the mean variance, and the derived p-value (from the calculated χ^2) of a series of potencies. A test is more reproducible when the mean variance is smaller than the corresponding test. Potencies are significantly different when $p \geq 0.05$.

3. Results

3.1 Pre-phase: 18323-WCE try-out

Three of the 5 laboratories performed the pre-phase as requested. Laboratory 3 measured the serum samples on 4 different days and laboratory 5 measured the serum samples five-times on 5 different days. The absorbance data of the laboratories 1, 2 and 3 were comparable, maximal optical densities (OD's) of 1.500 up to 2.300. The OD's of laboratory 4 were significantly higher (up to 3.400) and the absorbance data of laboratory 5 varied considerably per day, maximal OD's of 0.660 up to 1.300. As is shown in Table 2, the antibody concentrations of PSPTpc varied also per day and per laboratory (CV between 18.6 and 44.6%). The LOD was between 0.5 to 5.9 EU/ml and the LOQ between 1.1 to 10.6 EU/ml. The antibody concentrations of the 16 pre-phase serum samples measured by the participants corresponded poorly with the given antibody concentrations (Table 3). The laboratories 1, 2, and 3 measured antibody concentrations, which were consistently lower (\pm 90, 70, and 60%, respectively) and the antibody concentrations of laboratory 4 and 5 were consistently higher (\pm 167.3, and 112.1%, respectively) compared to the mean antibody concentration of all laboratories.

3.2 Phase I: 18323-WCE validation

The Phase I study was carried out according to the detailed instructions; the 80 serum samples were measured on 5 different days. The absorbance data of the laboratories 1, 2, 3 and 5 corresponded well, while the OD's of laboratory 4 remained clearly higher. Table 2 shows the variation in antibody concentrations of PSPTpc for each of the participants (CV between 11.5 and 20.8%). The LOD and LOQ diverged slightly and were determined at 1.3 and 1.5 EU/ml, respectively.

The antibody concentrations of the Phase I serum pools differed also per laboratory. In contrast with the pre-phase, the antibody concentrations of laboratory 1 were comparable to the mean antibody concentrations of all participants, whereas the antibody concentrations of laboratory 2 and 5 were approximately 20 and 30% lower, respectively (Tables 4,5,8 and 9). The laboratories 3 and 4 found antibody concentrations, which were \pm 20 and 10% higher, respectively (Tables 6 and 7).

The intra-assay precision for laboratory 1, 2, and 4 was less than 14% and for laboratory 5

less than 20% (Table 10). The intra-assay precision of laboratory 3 varied per day, from 15.3 to 27.9%. In general, two of the 16 serum samples were out of the 20% variation range, except for laboratory 3. The inter-assay precision for laboratory 1, 2, 3, 4, and 5 was 16.6, 13.7, 24.5, 18.6 and 20.1%, respectively. The number of serum samples, which did not meet the 20% variation range and/or the intended 99% confidence interval ($p = 0.01$) varied per laboratory. The inter-laboratory precision was determined at 25.1%. Fifteen of the 16 serum samples had a CV above the intended 20% and the antibody concentrations of 6 serum samples were significantly different.

The ranking of the antibody concentration of serum samples corresponded well between the laboratories, as is shown in Tables 11 and 12. Throughout the Phase I study significant differences were found in random order, varying per day and per laboratory ($p < 0.05$).

3.3 Phase II: comparative study of the PSPT and MPT

Four of the 5 laboratories participated in the comparative study of the PSPT and MPT. Four WCV's were tested twice on different occasions in the PSPT and MPT. The PSPT's were performed according to detailed instructions, only the mouse strain in use (Table 1) and the animal husbandry differed per laboratory. The sera were tested for antibodies against *B. Pertussis* in the 18323-WCE and the OD-files were sent to the RIVM for data processing. The inter-assay precision of the 18323-WCE was based on the antibody concentrations of PSPTpc and varied per laboratory from 9.8 to 19.8% (Table 2). The inter-laboratory precision was determined at 18.1%.

The mean antibody concentration of the serum samples per vaccine dose varied considerable between the laboratories (up to a factor 10) and to a lesser degree between the tests within the laboratories (up to a factor 4), as illustrated in Tables 13a and 13b. The number of non-responding mice also varied considerable per test and per laboratories. Less than 3% of the mice in laboratory 1 and 2 did not induced a proper pertussis antibody response, while 4.3% (0.7 to 8.3% per test) of the mice in laboratory 3, and 7.5% (0.7 to 13.2% per test) of the mice in laboratory 4 responded poorly or not at all. Antibody concentrations of sera, of which OD's were below the OD's of the PSPTnc, were left out of the potency calculations.

The MPT's were also performed according to detailed instructions. Besides the mouse strain and animal husbandry, each laboratory used its own challenge culture and procedure. The survival of mice was recorded and the raw data was sent to the RIVM. The Tables 14a and 14b show clearly the differences in the survival of mice per laboratory, as can be seen in the

ED50's, slopes, and LD50's of the tests. The ED50's of the reference vaccine in laboratory 1, the LD50's of laboratory 3, and the slopes of the vaccine dose-responses in laboratory 2 and 3 differed considerably.

Despite, the differences in antibody concentrations (PSPT) or the survival of mice (MPT), the potencies of each vaccine under test did not differ neither significantly in the PSPT nor in the MPT, within or between the laboratories (Tables 15 and 16). As is shown in Table 17, the PSPT and MPT correlated well within and between the laboratories, as is indicated by the PSPT/MPT-ratios and the p-values. The reproducibility of the PSPT and MPT is determined by the pooled mean variances of both tests (Table 16). The pooled mean variance of the MPT is about twice the pooled mean variance of the PSPT, indicating a better reproducibility of the PSPT.

4. Discussion

According to WHO guidelines thoroughly validated and standardized assays are required for the detection of antibodies in immunogenicity models. To this end, a validation study of the 18323-WCE has been included in the collaborative study on the PSPT to evaluate the replacement of the MPT. Four of the 5 laboratories had no previous experience with the 18323-WCE at the beginning of the study. For this reason, the 18323-WCE try-out as pre-phase was included. Essential materials and detailed instructions were supplied by the RIVM to standardize the 18323-WCE as much as possible. Validity parameters were recorded to evaluate the performance of the 18323-WCE during the study. The large differences in OD's between the laboratories underpinned the need to establish feasible validity criteria for each laboratory separately. Despite the standardization of the 18323-WCE, the antibody concentrations were consequently higher or lower per laboratory, probably due to the local laboratory conditions. The same phenomenon was observed in Phase I of the collaborative study on alternative methods for potency testing of tetanus toxoid vaccines for human use.¹³ In a solid-phase enzyme immuno assay such as the 18323-WCE, the antibody activity is measured rather than its concentration¹⁴ and is influenced by for example the temperature, humidity and time of incubation.

In Phase I, the intra-assay, inter-assay, and inter-laboratory precision of the 18323-WCE has been studied. Tijsen stated that dose-response curves for antibodies are influenced by a variety of factors; their range is generally larger than 4-6 log10 dilutions and the response curves for various antisera are rarely parallel, due to differences in concentrations and affinity of the antibodies.¹⁴ A curve obtained from a single reference serum cannot take both parameters into account and is hardly representative for other sera. Our goal was to achieve a variation in the antibody concentrations less than 20% within a confidence interval of 99% range (CV = 20%, p = 0.01) for sera containing different amounts of antibodies. The precision of the 18323-WCE (CV and, if possible, p-value) has been determined by using the individual antibody concentrations of each serum pool.

As is shown in Table 10, the intra-assay and inter-assay precision varied per laboratory (from 8.9 to 27.9% and 13.4 to 24.5%, respectively). The inter-laboratory precision has been determined at 25.1%. Except for laboratory 3, the intra- and inter-assay precision but not the inter-laboratory precision of the 18323-WCE were within the intended range (CV ≤ 20%).

The high CV's of laboratory 3 were probably due to errors in the coating procedure, the whole cell suspension in the wells was not totally evaporated. Without the results of

laboratory 3 the inter-laboratory precision hardly changed (23.6% instead of 25.1%). Significant differences ($p < 0.01$) in the antibody concentrations for the serum pools were found at random throughout the whole Phase I study, randomly distributed per plate, per day and per laboratory. Equal distribution of the serum samples per vaccine dose over the required number of plates may improve the intra- and inter-assay precision. On the other hand, the antibody concentrations of immunized mice in the PSPT are used to estimate the relative potency of WCV's by means of a parallel line assay with log transformation. Therefore, the relative antibody concentration per vaccine dose may be of more importance than the absolute antibody concentration. We have ranked the antibody concentrations per plate, per day and per laboratory, to show whether the proportion between the serum pools corresponded between the laboratories (Table 12). The ranking of the serum pools corresponded well between laboratories, despite the significant differences found throughout the study ($p < 0.05$). Although a precision of less than 20% for the 18323-WCE was not always established, the intra-laboratory ranking of the serum pools should warrant the expectation of a reliable potency estimation of WCV's in the PSPT.

In the Phase II study, 4 batches of pertussis WCV's were tested in duplicate by both test methods, but not always simultaneously, according to detailed instructions by 4 of the 5 participants. The results of the PSPT and MPT were sent to the RIVM and evaluated for reproducibility and correlation.

In particular, the obvious significant differences in the mean antibody concentrations per vaccine dose of the PSPT's, within and between the laboratories may indicate a variation in immune status of the mice. The origin of the differences in antibody responses of mice within the laboratories was not clear, while the differences in immunity found between the laboratories may be due to different mouse-strains¹⁵ used, diet¹⁶ and other aspects of animal husbandry. To circumvent these differences in immune responses, it may be considered to use a more homologous reference, which is produced from the same mouse strain as the test mice, instead of the international reference serum, which is generated from different mice housed under different conditions. To this end, each laboratory should produce its own in-house reference – according to a detailed protocol using an international reference vaccine – to which a pre-determined antibody concentration has been designated.

The number of non-responding mice also varied considerably per test and laboratory, which might be partly due to an inconsistent i.p. injection procedure.¹⁷ We have noticed in our own laboratory that the number of non-responders was reduced after a period of time, because the number of non-responders due to improper injection is perceptible in a serological test but not

in a challenge model, and corrective measures could be taken. Nevertheless, the potencies of the vaccines in the PSPT's did not differ significantly within or between the laboratory ($p \geq 0.05$).

The protection of mice against a lethal i.c. challenge in the MPT also varied considerably per laboratory. Clear differences in the survival of mice and consequently in the slopes, ED50-, and LD50-values were observed. The differences in survival may be due the challenge strain, challenge procedure, and mouse strain used or animal husbandry. The challenge culture used by laboratory 3 is clearly more virulent than the challenge cultures of the other laboratories, of which the LD50's are comparable. Differences in immunity of the mice may be indicated by the moderate survival of mice in laboratory 2 after immunization with protective doses of vaccine and the low ED50's of most of the vaccines in laboratory 1. Although there were differences in survival for each vaccine, the potencies in the MPT's were not significantly different within or between the laboratories ($p \geq 0.05$).

The PSPT and MPT correlated well within and between the laboratories ($p \geq 0.05$ in a χ^2 -test of homogeneity). The pooled results of all potency determinations in the PSPT and MPT ($n = 32$) were almost similar, as is indicated by a PSPT/MPT ratio of 0.877 (0.738-1.065).

Within the laboratories, the ratio's varied per vaccine ($n = 2$) and per laboratory ($n = 8$) due to the limited number of tests. Strict compliance to the protocols have improved the reproducibility of the MPT considerably, compared to the variation in potencies of the vaccines under test in the MPT in the collaborative study by I. Van Straaten-van de Kappelle *et al.*⁵ Nevertheless, the PSPT is still more reproducible than the MPT and reduces the chance of re-testing due to the smaller confidence intervals. We deliberately included low potency products to verify whether the products pass the WHO and European Pharmacopoeia requirements for pertussis WCV's in both tests. Vaccine A and B should have a potency of more than 4.0 IU/ml with a lower limit of 2.0 IU/ml and vaccine C and D a potency of more than 8.0 IU/ml with a lower limit of 4.0 IU/ml. Eight of the 32 MPT potency determinations and 7 of the 32 PSPT potency determinations did not meet the required potency of 4.0 IU/human dose. However, when looking at the lower limit of 2.0 IU/human dose it can be seen that in the MPT 5 of the 32 tests did not pass this limit while in the PSPT only one test failed. Taking in account the average results per laboratory in the MPT, the number of vaccines passing the requirements in the PSPT correlates well. We, therefore, have demonstrated that the PSPT is a valid alternative for the MPT to estimate the potencies of pertussis WCV's of different manufacturers.

Appendix 1 Participants collaborative study on the PSPT*

Dr. Maria Luisa Brero,
Instituto Nacional de Biologica,
Servicio Vacunas Bacterianas,
Buenos Aires, Argentina.

Dr. Suresh S. Jadhav,
Serum Institute of India,
Quality Assurance,
Hadapsar, India.

Dr. Rose-Marie Ölander,
National Public Health Institute,
National Vaccine Quality
Control Laboratory,
Helsinki, Finland.

Dr. Karlheinz Enssle,
Chiron - Behring,
Quality Control,
Marburg, Germany.

Dr. Coenraad Hendriksen and Arno van der Ark,
National Institute of Public Health and the Environment,
Laboratory for Control of Biological Products,
Bilthoven, The Netherlands.

* The laboratories are encoded as lab. 1 to 5, but not in the order as listed above.

Appendix 2 Tables

Table 1. Specifications of mouse strains used by the participants in the PSPT and MPT.

Laboratory	mouse strain	PSPT sex (m/f)	weight (g)	MPT sex (m/f)	weight (g)
1	N:NIH	m+f	20-24	m+f	10-14
2	NIH Swiss Albino	m+f	20-24	m+f	10-14
3	Hsd:NIH/S	m+f	20-24	m+f	10-14
4	NMRI	f	20-24	f	10-14

m male

f female

Table 2. Precision of the 18323-WCE during the collaborative study based on the antibody concentrations of the positive control serum PSPTpc.

	Lab.	antibody concentration mean*	range*	CV (%)	n
Pre-phase	1	390	266 – 487	22.1	5
	2	302	236 – 376	18.6	5
	3	263	177 – 366	29.6	4
	4	431	207 – 673	44.6	5
	5	467	336 – 619	21.8	25
Phase I	1	344	279 – 423	13.6	25
	2	296	242 – 365	14.1	24
	3	272	217 – 331	11.5	24
	4	335	230 – 455	14.4	25
	5	441	292 – 818	20.8	25
Phase II	1	386	301 – 449	9.8	40
	2	321	252 – 439	13.0	39
	3	275	190 – 402	19.8	39
	4	321	238 – 408	11.6	40

* EU/ml

Table 3. Mean antibody concentrations (EU/ml) of pre-phase samples

serum sample	given	lab.1	lab.2	lab.3	lab.4	lab.5	avg.	c.v. (%)
A	633	354	326	735	422	621	492	30.3
B	305	192	174	617	239	320	308	41.5
C	189	69	87	249	112	118	127	38.4
D	65	38	42	118	65	45	62	39.1
E	2271	2563	1920	6526	2927	4938	3775	41.5
F	2334	1545	1392	3773	1860	3125	2341	38.0
G	987	805	605	1529	861	1323	1024	31.3
H	787	424	423	1087	526	834	659	36.6
I	3678	2058	1626	4256	1961	3470	2674	35.6
J	2433	1305	837	2278	1379	2019	1564	29.9
K	1411	582	472	1245	903	1756	992	41.1
L	676	328	291	859	543	538	512	31.6
M	945	632	672	1208	903	1151	886	26.5
N	723	376	382	886	501	638	557	29.5
O	369	165	171	458	227	288	262	34.0
P	236	82	93	254	100	160	138	40.2
PSPTnc	0	1	2	1	0	1	1	
PSPTpc	360	320	261	431	364	453	362	17.8

Table 4. Intra-assay precision of the 18323-WCE by laboratory 1. Antibody concentrations (EU/ml) of Phase I serum samples were measured in 5 plates on 5 different days.

serum sample	plate					c.v. (%)	serum sample	plate					c.v. (%)				
	1	2	3	4	5			1	2	3	4	5					
1	day 1	168	219	183	279	321	234	22.6	10	day 1	270	242	311	313	259	279	9.5
	day 2	230	312	264	240	322	249	14.4		day 2	396	442	728	325	379	454	24.1
	day 3	256	264	289	252	226	257	5.7		day 3	473	398	383	395	440	418	7.4
	day 4	236	240	263	256	269	253	4.7		day 4	346	457	448	383	461	419	10.4
	day 5	279	322	209	240	292	268	13.1		day 5	406	456	427	446	483	444	4.8
2	day 1	133	93	114	123	103	113	10.9	11	day 1	238	250	263	281	318	270	8.7
	day 2	161	130	104	131	170	139	15.2		day 2	371	289	420	366	345	358	9.1
	day 3	213	118	117	122	131	140	20.7		day 3	392	334	460	409	329	385	11.0
	day 4	111	133	143	120	114	124	8.9		day 4	318	364	302	304	380	334	9.3
	day 5	128	132	147	122	98	125	9.6		day 5	375	358	288	279	505	361	17.4
3	day 1	64	85	60	64	56	66	11.4	12	day 1	71	83	103	91	82	86	10.2
	day 2	90	103	120	94	88	102	8.3		day 2	136	145	124	129	100	127	9.4
	day 3	84	67	97	74	75	79	10.9		day 3	138	122	106	89	117	115	11.8
	day 4	60	85	78	71	66	72	9.9		day 4	102	109	124	117	118	114	6.1
	day 5	74	82	90	66	60	75	12.4		day 5	112	121	85	120	110	109	9.0
4	day 1	33	44	36	30	53	39	19.0	13	day 1	346	366	394	399	349	371	5.6
	day 2	42	59	52	50	57	52	9.5		day 2	417	569	746	590	451	555	17.5
	day 3	43	51	41	33	54	44	14.3		day 3	601	476	515	586	480	532	9.3
	day 4	53	43	42	88	39	53	26.2		day 4	587	441	600	598	502	546	10.9
	day 5	42	36	37	39	33	37	6.7		day 5	503	589	601	468	525	537	8.6
5	day 1	404	356	342	279	359	348	8.6	14	day 1	238	317	278	287	296	283	7.1
	day 2	378	669	441	272	504	452	23.7		day 2	465	438	484	396	461	449	5.7
	day 3	407	535	457	252	627	456	22.1		day 3	549	442	571	376	362	460	17.4
	day 4	400	441	536	396	657	486	18.1		day 4	412	367	396	460	361	399	7.3
	day 5	387	423	485	378	438	422	7.5		day 5	404	372	378	523	414	418	10.0
6	day 1	258	263	275	316	249	272	6.9	15	day 1	133	157	198	154	139	156	11.0
	day 2	466	446	537	483	398	466	7.6		day 2	161	235	234	189	196	203	12.3
	day 3	555	364	383	326	370	400	15.6		day 3	213	165	188	204	192	192	6.7
	day 4	310	454	368	448	478	412	14.1		day 4	218	183	235	208	361	241	20.0
	day 5	337	423	451	401	395	401	7.1		day 5	130	175	214	209	364	218	26.7
7	day 1	95	107	134	97	181	123	22.8	16	day 1	6	1	1	4	2	3	
	day 2	150	197	183	90	187	162	20.5		day 2	0	2	0	0	0	0	
	day 3	179	129	160	124	143	147	12.4		day 3	0	0	0	0	0	0	
	day 4	135	136	115	126	149	132	7.1		day 4	0	0	0	0	0	0	
	day 5	211	169	155	131	124	158	16.1		day 5	0	0	0	0	0	0	
8	day 1	65	59	67	55	65	62	6.4	PSPT nc	day 1	0	1	2	1	1	1	
	day 2	87	101	100	81	124	99	11.8		day 2	0	0	0	0	0	0	
	day 3	96	78	91	70	89	85	10.1		day 3	0	0	0	0	0	0	
	day 4	87	103	87	87	68	86	8.3		day 4	0	0	0	0	0	0	
	day 5	73	81	79	69	63	73	7.6		day 5	0	0	0	0	0	0	
9	day 1	377	421	430	409	554	438	10.6	PSPT pc	day 1	514	493	589	607	640	568	9.2
	day 2	612	737	943	742	833	774	11.9		day 2	327	418	386	361	286	356	11.0
	day 3	661	630	642	693	656	656	2.5		day 3	307	279	282	289	298	291	3.1
	day 4	477	659	642	693	656	615	9.0		day 4	304	375	338	311	368	339	7.7
	day 5	597	862	741	792	612	721	12.9		day 5	380	423	386	369	387	389	3.5

Table 5. Intra-assay precision of the 18323-WCE by laboratory 2. Antibody concentrations (EU/ml) of Phase I serum samples were measured in 5 plates on 5 different days.

serum sample	plate					c.v. (%)	serum sample	plate					c.v. (%)				
	1	2	3	4	5			1	2	3	4	5					
1	day 1	198	209	197	201	191	199	2.3	10	day 1	230	373	213	317	277	282	17.8
	day 2	212	272	241	187	229	229	10.2		day 2	255	418	344	250	323	323	16.2
	day 3	193	267	231	293	209	239	13.6		day 3	278	333	346	416	224	319	17.1
	day 4	189	207	203	293	209	204	7.4		day 4	245	277	339	265	316	288	10.9
	day 5	222	176	193	222	183	199	9.1		day 5	276	268	265	261	278	269	2.3
2	day 1	89	95	112	105	96	99	7.4	11	day 1	216	190	211	219	280	223	10.1
	day 2	93	122	155	103	118	118	13.6		day 2	220	284	298	275	269	269	7.3
	day 3	103	116	118	150	104	118	10.6		day 3	184	232	324	263	330	267	18.1
	day 4	112	109	110	130	126	117	7.1		day 4	283	264	263	246	312	274	7.0
	day 5	95	96	105	110	87	98	7.4		day 5	231	253	238	247	250	243	2.9
3	day 1	56	59	52	53	60	56	4.9	12	day 1	65	69	72	89	83	82	11.2
	day 2	60	80	77	—	76	78	1.9		day 2	86	90	428	84	82	82	6.5
	day 3	55	62	53	66	70	61	9.4		day 3	74	86	86	75	91	82	7.5
	day 4	58	65	69	56	79	65	10.5		day 4	76	78	90	71	94	82	1.9
	day 5	56	55	61	59	55	57	3.9		day 5	73	70	94	94	77	81	12.3
4	day 1	35	32	35	39	34	35	4.7	13	day 1	325	335	281	379	358	336	7.8
	day 2	31	41	42	43	39	39	8.5		day 2	458	448	439	361	427	427	6.2
	day 3	31	35	35	48	46	39	16.3		day 3	365	470	357	662	420	455	19.6
	day 4	35	35	44	39	44	39	9.1		day 4	402	402	434	609	511	471	15.0
	day 5	36	35	39	35	41	37	6.2		day 5	355	369	418	352	378	375	5.1
5	day 1	356	363	295	228	362	321	14.7	14	day 1	246	291	213	345	358	291	16.9
	day 2	426	341	517	400	421	421	9.6		day 2	325	412	334	361	356	356	6.3
	day 3	404	314	394	320	388	364	10.3		day 3	268	377	320	401	348	343	11.4
	day 4	382	329	496	318	418	389	14.0		day 4	295	302	318	331	359	321	6.0
	day 5	288	352	375	284	364	333	11.2		day 5	284	283	284	284	300	287	1.8
6	day 1	217	249	336	247	285	267	13.0	15	day 1	149	135	136	144	358	184	37.8
	day 2	276	320	361	308	316	6.2		day 2	108	158	180	355	231	231	43.0	
	day 3	399	273	276	332	405	337	15.5		day 3	154	147	158	193	348	200	29.6
	day 4	264	325	266	270	348	295	11.4		day 4	145	128	162	148	359	188	36.3
	day 5	241	288	269	298	235	266	8.4		day 5	130	127	162	127	300	169	30.9
7	day 1	106	87	147	104	123	113	15.2	16	day 1	2	2	2	2	2	2	2
	day 2	137	128	125	138	132	3.4		day 2	2	0	2	2	2	2	2	
	day 3	127	108	123	138	145	128	8.4		day 3	2	4	3	4	3	3	3
	day 4	124	101	127	103	126	116	9.7		day 4	3	3	4	2	4	3	3
	day 5	105	83	112	98	116	103	9.5		day 5	2	4	5	4	3	3	3
8	day 1	60	55	50	53	71	58	10.6	PSPT	day 1	2	1	2	2	2	2	2
	day 2	58	60	71	64	63	52	5.2		day 2	2	3	0	2	2	2	2
	day 3	64	57	71	74	68	67	7.5		day 3	2	4	5	4	4	4	4
	day 4	59	65	71	64	63	65	5.1		day 4	3	3	3	1	3	3	3
	day 5	59	58	56	63	54	58	4.6		day 5	30	3	4	4	3	3	3
9	day 1	450	526	440	542	446	480	8.9	PSPT	day 1	310	242	257	244	263	250	8.9
	day 2	426	564	562	498	516	8.4			day 2	327	418	386	361	286	356	11.0
	day 3	485	481	452	606	485	502	8.3		day 3	291	357	342	277	321	15.9	
	day 4	436	492	529	450	554	492	8.0		day 4	245	287	287	242	281	6.0	
	day 5	355	335	438	537	392	412	14.8		day 5	282	312	361	363	365	355	12.6

Table 6. Intra-assay precision of the 18323-WCE by laboratory 3. Antibody concentrations (EU/ml) of Phase I serum samples were measured in 5 plates on 5 different days.

serum sample	plate					c.v. (%)	serum sample	plate					c.v. (%)				
	1	2	3	4	5			1	2	3	4	5					
1	day 1	259	372	340	240	280	300	15.0	10	day 1	446	465	449	413	431	440	3.3
	day 2	197	225	264	229	487	280	29.4		day 2	355	397	375	302	123	311	25.3
	day 3	238	318	173	517	386	326	30.6		day 3	1165	288	324	567	313	532	50.4
	day 4	389	282	331	233	254	298	16.8		day 4	524	410	713	557	512	543	13.5
	day 5	244	338	231	297	275	277	11.8		day 5	502	410	379	419	481	438	9.7
2	day 1	185	134	202	112	145	155	19.5	11	day 1	364	343	535	410	401	410	12.1
	day 2	125	141	472	125	367	189	37.5		day 2	445	368	360	351	379	381	6.8
	day 3	218	157	153	233	88	170	26.5		day 3	934	319	273	328	341	439	45.1
	day 4	238	159	227	165	168	191	17.3		day 4	491	393	238	292	467	376	23.6
	day 5	252	107	144	149	145	159	23.4		day 5	491	297	453	261	384	377	20.8
3	day 1	75	87	85	68	75	75	11.4	12	day 1	104	133	154	73	94	112	23.1
	day 2	62	68	64	71	117	78	20.5		day 2	107	120	119	89	98	107	9.7
	day 3	194	83	58	85	96	103	35.3		day 3	390	171	133	239	145	216	36.6
	day 4	84	70	61	76	88	76	10.8		day 4	200	140	190	144	223	179	16.6
	day 5	74	83	59	136	90	88	22.3		day 5	122	121	95	112	168	126	14.4
4	day 1	39	47	49	36	46	44	10.5	13	day 1	470	601	608	443	550	534	11.6
	day 2	36	36	40	43	41	39	6.5		day 2	389	530	404	501	283	418	18.8
	day 3	42	32	82	121	29	61	52.5		day 3	395	171	133	239	145	780	35.5
	day 4	62	58	79	63	45	61	13.1		day 4	200	140	190	144	223	1016	56.4
	day 5	66	33	46	48	39	46	25.4		day 5	122	121	95	112	168	657	17.5
5	day 1	392	598	733	522	484	546	17.6	14	day 1	470	601	608	443	541	449	14.6
	day 2	456	605	196	375	505	427	26.6		day 2	340	364	375	296	380	351	7.5
	day 3	530	460	532	380	421	467	11.5		day 3	666	316	380	628	317	461	32.3
	day 4	755	401	982	419	547	621	31.9		day 4	482	371	419	634	445	470	15.0
	day 5	644	449	890	411	517	582	25.4		day 5	503	372	411	425	398	422	8.0
6	day 1	495	388	529	318	365	419	17.8	15	day 1	189	159	227	168	541	257	44.3
	day 2	372	430	382	320	396	380	7.2		day 2	192	156	99	240	286	200	35.9
	day 3	818	809	337	768	554	657	25.7		day 3	240	203	240	295	317	259	14.6
	day 4	374	562	450	413	460	452	10.5		day 4	599	211	286	188	445	346	40.9
	day 5	576	422	538	385	703	525	18.5		day 5	397	226	207	204	398	286	31.0
7	day 1	142	152	203	136	155	158	11.6	16	day 1	0	0	0	0	0	0	0
	day 2	141	169	110	118	152	138	13.9		day 2	0	0	0	0	0	0	0
	day 3	191	194	205	207	186	197	3.9		day 3	0	3	2	1	0	1	1
	day 4	316	184	395	297	178	247	27.1		day 4	5	3	0	0	3	2	2
	day 5	255	139	222	157	218	198	20.3		day 5	0	0	0	0	0	0	0
8	day 1	104	84	98	77	89	88	15.7	PSPT nc	day 1	2	0	0	0	0	0	0
	day 2	74	103	61	69	75	76	14.0		day 2	0	0	0	0	0	0	0
	day 3	122	118	104	124	94	113	9.6		day 3	2	0	2	0	0	1	1
	day 4	130	138	133	93	106	120	13.7		day 4	2	0	2	2	2	2	2
	day 5	90	136	116	68	73	97	24.3		day 5	0	0	0	0	0	0	0
9	day 1	807	695	749	538	653	688	10.8	PSPT pc	day 1	328	294	264	285	315	297	6.5
	day 2	661	719	726	547	676	666	7.4		day 2	280	281	238	250	267	263	5.9
	day 3	1195	657	604	1097	699	850	27.8		day 3	311	293	239	300	270	283	8.0
	day 4	1612	1113	671	833	703	986	30.5		day 4	331	261	293	300	270	278	9.9
	day 5	1630	1102	550	791	645	943	35.8		day 5	257	222	239	217	250	237	6.0

Table 7. Intra-assay precision of the 18323-WCE by laboratory 4. Antibody concentrations (EU/ml) of Phase I serum samples were measured in 5 plates on 5 different days.

serum sample	plate					c.v. (%)	serum sample	plate					c.v. (%)				
	1	2	3	4	5			1	2	3	4	5					
1	day 1	321	303	255	270	245	164	13.1	10	day 1	419	500	422	396	427	433	6.2
	day 2	279	313	312	314	288	222	11.0		day 2	436	345	480	468	427	431	8.4
	day 3	280	245	248	247	222	203	11.5		day 3	612	491	475	413	383	475	13.0
	day 4	369	323	454	319	495	192	23.8		day 4	497	554	703	406	767	585	20.4
	day 5	332	269	258	253	250	243	24.6		day 5	518	456	439	436	530	476	8.1
2	day 1	119	149	181	116	146	142	13.9	11	day 1	236	427	392	309	330	339	16.7
	day 2	105	125	132	157	148	133	11.3		day 2	419	464	475	531	578	494	9.9
	day 3	171	147	114	107	113	130	17.5		day 3	538	397	328	377	287	383	17.7
	day 4	180	193	269	161	447	250	34.6		day 4	509	375	639	425	684	526	20.5
	day 5	177	134	136	156	173	155	10.4		day 5	504	406	421	427	492	450	8.5
3	day 1	55	92	77	68	75	73	13.2	12	day 1	88	127	136	115	119	117	10.5
	day 2	75	90	99	82	87	90	4.4		day 2	100	104	139	166	128	127	16.0
	day 3	98	85	81	75	49	78	16.2		day 3	142	101	105	100	98	109	12.1
	day 4	99	89	134	58	166	109	29.7		day 4	139	170	220	127	225	176	20.9
	day 5	85	97	82	79	96	88	7.9		day 5	140	123	118	110	138	126	8.3
4	day 1	50	54	39	42	44	46	11.0	13	day 1	641	584	485	435	535	535	11.5
	day 2	52	53	47	38	57	49	11.1		day 2	583	546	462	450	673	543	12.8
	day 3	61	46	44	43	28	44	16.5		day 3	612	600	646	394	511	552	14.5
	day 4	63	47	87	61	111	74	27.5		day 4	669	631	854	591	889	727	15.9
	day 5	85	97	82	79	96	48	11.2		day 5	712	697	585	542	618	631	9.3
5	day 1	602	572	483	453	503	523	9.9	14	day 1	351	359	453	369	445	395	10.8
	day 2	726	510	493	467	561	551	13.4		day 2	350	454	467	472	497	448	8.7
	day 3	687	497	518	407	401	502	16.0		day 3	472	487	409	290	264	384	22.3
	day 4	632	665	964	643	1030	787	21.4		day 4	523	299	643	517	720	540	20.9
	day 5	480	501	485	455	574	499	6.1		day 5	485	452	455	371	673	487	15.2
6	day 1	399	363	425	363	379	386	5.4	15	day 1	211	227	277	156	445	263	34.1
	day 2	396	387	452	526	465	445	9.7		day 2	237	191	165	203	497	258	12.5
	day 3	577	497	442	374	347	447	16.0		day 3	254	236	180	173	264	221	16.3
	day 4	483	563	676	493	665	576	13.2		day 4	247	239	382	232	720	364	25.8
	day 5	428	474	453	325	565	449	13.0		day 5	222	158	192	214	673	292	52.2
7	day 1	228	177	166	135	120	165	18.4	16	day 1	1	0	0	0	0	0	0
	day 2	184	186	147	176	166	171	7.1		day 2	0	0	0	0	0	0	0
	day 3	175	158	166	155	124	156	8.2		day 3	0	0	0	0	0	0	0
	day 4	210	194	306	175	295	236	21.9		day 4	0	0	0	0	0	0	0
	day 5	176	156	160	161	221	179	10.7		day 5	0	0	0	0	0	0	0
8	day 1	133	85	98	77	89	96	15.7	PSPT nc	day 1	0	0	0	0	0	0	0
	day 2	106	85	77	92	110	94	12.2		day 2	0	0	0	0	0	0	0
	day 3	90	103	87	79	55	83	15.1		day 3	0	0	0	0	0	0	0
	day 4	129	109	159	110	176	137	18.2		day 4	2	0	0	0	0	0	0
	day 5	117	84	102	86	121	102	13.5		day 5	0	0	0	0	0	0	0
9	day 1	327	465	806	673	674	589	26.2	PSPT pc	day 1	376	370	317	316	345	6.6	
	day 2	486	728	686	509	688	619	15.8		day 2	321	301	353	356	333	5.2	
	day 3	852	778	593	571	510	661	18.7		day 3	314	316	303	283	284	300	4.4
	day 4	795	834	875	714	987	841	8.6		day 4	332	326	455	300	442	371	16.7
	day 5	705	570	754	584	735	669	11.1		day 5	345	324	230	337	397	326	12.1

Table 8. Intra-assay precision of the 18323-WCE by laboratory 5. Antibody concentrations (EU/ml) of Phase I serum samples were measured in 5 plates on 5 different days.

serum sample	plate					c.v. (%)	serum sample	plate					c.v. (%)				
	1	2	3	4	5			1	2	3	4	5					
1	day 1	154	149	160	217	139	164	23.6	10	day 1	209	185	353	319	269	267	6.2
	day 2	238	200	193	268		225	36.2		day 2	377	275	215	371		310	8.4
	day 3	166	255	206	182	208	203	16.0		day 3	200	317	290	318	302	285	13.0
	day 4	229	114	155	233	227	192	27.5		day 4	240	212	247	261	243	241	20.4
	day 5	229	212	164	393	219	243	17.0		day 5	103	89	102	114	99	336	8.1
2	day 1	77	82	89	100	99	89	9.4	11	day 1	242	232	24	59	158	143	16.7
	day 2	117	93	74	152		109	18.7		day 2	259	237	228	168		223	9.9
	day 3	67	89	89	84	103	87	10.1		day 3	209	228	294	204	108	253	17.7
	day 4	75	59	97	43	69	69	20.3		day 4	198	187	200	241	219	209	20.5
	day 5	126	108	97	99	149	116	15.1		day 5	100	96	108	118	250	312	8.5
3	day 1	52	33	59	53	34	46	22.3	12	day 1	54	57	55	70	58	59	10.5
	day 2	64	49	40	97		59	26.0		day 2	97	82	58	93		83	16.0
	day 3	43	66	82	57	129	75	31.8		day 3	60	68	71	112	81	78	12.1
	day 4	52	66	47	44	83	51	11.7		day 4	74	51	62	45	80	62	20.9
	day 5	66	58	83	78	56	68	14.4		day 5	105	97	105	120	104	93	8.3
4	day 1	22	18	27	33	21	24	20.1	13	day 1	386	259	368	341	244	320	11.5
	day 2	38	28	25	31		31	10.8		day 2	437	408	349	367		390	12.8
	day 3	26	28	30	32	36	31	9.6		day 3	273	430	331	416	648	420	14.5
	day 4	34	35	24	22	36	27	20.8		day 4	335	408	295	279	419	347	15.9
	day 5	38	30	21	36	20	29	23.7		day 5	598	466	511	525	464	513	9.3
5	day 1	282	343	259	301	296	7.0		14	day 1	252	270	192	291	256	252	10.8
	day 2	446	318	475	250		372	19.0		day 2	417	245	250	317		307	8.7
	day 3	259	430	345	217	412	333	22.8		day 3	231	255	217	341	282	265	22.3
	day 4	284	240	341	341	520	326	25.8		day 4	249	439	520	261	247	343	20.9
	day 5	423	452	223		462	390	17.2		day 5	427	345	390	326	374	372	15.8
6	day 1	252	248	252	318	217	258	9.4	15	day 1	107	83	122	121	256	138	34.1
	day 2	334	314	238	387		318	10.6		day 2	152	107	111	147		129	12.5
	day 3	228	266	289	265	263	262	5.1		day 3	144	119	97	86	282	146	16.3
	day 4	374	254	388	201	271	298	22.4		day 4	136	145	103	119	247	150	25.8
	day 5	370	366	268	316	432	351	13.3		day 5	198	137	143	171		162	52.2
7	day 1	77	93	122	111	87	98	15.1	16	day 1	0	0	0	0	0	0	0
	day 2	123	94	94	115		107	11.8		day 2	0	0	0	0		0	0
	day 3	91	116	121	137	114	116	9.3		day 3	0	0	0	0	0	0	0
	day 4	107	82	96	109	99	99	7.7		day 4	0	0	0	0	0	0	0
	day 5	100	96	108	118	100	110	15.0		day 5	0	0	0	0	0	0	0
8	day 1	49	43	58	65	31	49	19.7	PSPT	day 1	0	0	0	0	0	0	0
	day 2	55	51	55	102		66	22.2	nc	day 2	1	1	0	0		0	0
	day 3	51	57	49	55	77	58	13.6		day 3	1	0	0	1	1	0	0
	day 4	48	58	50	46	39	48	9.4		day 4	0	0	0	0	0	0	0
	day 5	105	97	105	120	104	71	11.5		day 5	0	0	0	0	0	0	0
9	day 1	450	526	440	542	446	356	22.8	PSPT	day 1	292	378	340	444	372	365	10.7
	day 2	426	564	562		498	547	12.1	pc	day 2	473	495	346	411		431	9.8
	day 3	485	481	452	606	485	378	5.9		day 3	411	590	661	623	818	621	15.5
	day 4	436	492	529	450	554	412	5.6		day 4	356	357	361	405	297	355	6.6
	day 5	355	335	438	537	392	552	7.8		day 5	427	343	494	488	401	431	11.2

Table 9. Inter-assay and Inter-laboratory precision of the 18323-WCE

	serum sample	Inter-assay (n=25)					Inter-laboratory (n=125)
		lab.1	lab.2	lab.3	lab.4	lab.5	
Mean antibody concentration in EU/ml	1	252	214	296	299	205	253
	2	128	110	173	162	94	134
	3	79	64	84	88	60	75
	4	45	38	50	52	28	43
	5	433	365	528	572	343	448
	6	390	296	486	461	297	386
	7	144	118	193	181	106	149
	8	81	62	99	102	58	81
	9	641	480	827	676	449	615
	10	403	296	453	480	288	384
	11	342	255	397	438	228	332
	12	110	81	147	131	75	109
	13	508	413	681	598	398	519
	14	402	319	431	451	308	382
	15	202	195	270	280	145	218
	16	1	3	1	0	0	1
PSPTnc	0	0	1	0	0	0	0
	PSPTpc	389	301	271	335	440	347
Coefficient of Variation in %	1	12.5	10.9	21.9	15.3	18.6	19.9
	2	13.1	11.2	25.6	24.4	19.6	26.2
	3	16.6	13.1	21.1	17.1	24.8	22.4
	4	19.5	10.5	28.4	20.4	18.4	24.7
	5	19.0	13.2	23.2	19.2	22.2	24.6
	6	17.7	13.3	24.5	15.9	17.5	23.9
	7	18.6	12.1	24.2	16.8	13.8	25.6
	8	15.7	8.6	20.5	19.3	19.4	26.2
	9	16.6	11.1	27.0	17.4	20.3	25.4
	10	16.6	15.5	21.9	14.2	17.0	25.0
	11	15.1	11.9	21.3	18.8	23.2	27.0
	12	14.0	9.9	30.2	18.6	20.6	28.0
	13	15.9	14.7	35.7	14.3	19.2	26.1
	14	15.9	11.7	18.1	17.8	21.2	21.1
	15	18.6	37.3	36.5	37.6	25.1	35.7
	16						
PSPTnc	PSPTpc	19.8	14.2	9.6	10.2	20.4	20.5
ANOVA p-value	1	0.789	0.096	0.091	0.039	0.216	0.056
	2	0.415	0.113	0.449	0.220	0.182	0.018
	3	0.008	0.001	0.528	0.122	0.182	0.066
	4	0.115	0.593	0.269	0.015	0.500	0.105
	5	0.344	0.110	0.368	0.004	0.668	0.063
	6	0.010	0.089	0.015	0.012	0.088	0.000
	7	0.282	0.036	0.004	0.020	0.472	0.000
	8	0.005	0.089	0.016	0.007	0.073	0.000
	9	0.001	0.099	0.333	0.059	0.002	0.025
	10	0.015	0.484	0.297	0.059	0.145	0.048
	11	0.041	0.209	0.946	0.018	0.007	0.165
	12	0.042	0.780	0.016	0.007	0.009	0.000
	13	0.005	0.055	0.197	0.028	0.015	0.128
	14	0.007	0.080	0.350	0.178	0.094	0.012
	15	0.190	0.873	0.499	0.679	0.941	0.946
	16						
PSPTnc	PSPTpc	0.000	0.033	0.017	0.263	0.001	0.000

Table 10. Precision of the 18323-WCE during Phase I based on the antibody concentrations of 16 different serum pools measured in 5 different plates on 5 different days

precision	lab.	day	C.V. (%)	number of sera of which C.V. > 20%	p < 0.01
intra-assay (n = 5)	1	1	11.3	2	-
		2	13.3	3	-
		3	11.3	2	-
		4	11.1	2	-
		5	10.8	1	-
	2	1	12.0	1	-
		2	10.5	1	-
		3	13.1	1	-
		4	10.5	1	-
		5	8.8	1	-
	3	1	15.3	2	-
		2	17.1	5	-
		3	27.9	11	-
		4	21.7	8	-
		5	19.2	5	-
	4	1	14.0	2	-
		2	10.6	0	-
		3	14.8	1	-
		4	21.3	11	-
		5	13.9	2	-
	5	1	19.3	7	-
		2	14.5	2	-
		3	16.6	4	-
		4	16.2	5	-
		5	13.3	1	-
inter-assay (n=25)	1		16.6	0	6
	2		13.7	1	1
	3		24.5	14	1
	4		18.6	3	3
	5		20.1	8	3
inter-laboratory (n=125)			25.1	15	6

Table 11. Mean ranking based on antibody concentration per day for each laboratory according Kruskal-Wallis

	serum sample	day	lab.1	lab.2	lab.3	lab.4	lab.5		serum sample	day	lab.1	lab.2	lab.3	lab.4	lab.5
1	1		10.6	10.2	10.7	10.7	10.4	10	1		12.6	13.7	14.2	14.2	13.6
	2		10.0	10.3	11.4	11.4	10.4		2		12.6	13.3	12.1	12.1	12.8
	3		10.0	11.0	11.0	11.0	10.4		3		14.2	13.8	12.8	12.8	12.8
	4		10.0	10.0	10.6	10.6	10.4		4		14.0	12.0	14.8	15.0	12.3
	5		10.2	10.0	10.2	10.2	10.8		5		14.4	12.2	13.4	13.4	12.0
2	1		7.2	7.4	7.4	7.4	7.8	11	1		11.7	11.2	12.6	12.6	8.8
	2		6.4	7.3	10.2	10.2	7.5		2		12.2	11.5	13.8	13.8	11.0
	3		6.8	7.4	6.2	6.2	6.6		3		13.4	12.2	13.2	13.2	12.2
	4		7.2	7.5	6.8	6.8	6.2		4		12.0	11.8	13.6	11.8	10.8
	5		6.8	7.4	6.6	6.6	7.6		5		12.0	11.2	12.0	12.0	11.8
3	1		4.4	4.5	4.4	4.4	5.0	12	1		5.8	6.0	5.7	5.7	6.0
	2		5.3	5.3	4.8	4.8	4.5		2		6.5	5.0	6.2	6.2	5.5
	3		4.4	4.4	4.4	4.4	5.6		3		6.2	5.0	7.8	7.8	6.0
	4		4.0	4.3	3.8	3.8	5.2		4		6.6	6.0	7.0	6.6	5.8
	5		4.6	4.4	4.6	4.6	4.6		5		6.2	6.0	5.8	5.8	6.2
4	1		3.4	3.0	3.0	3.0	3.2	13	1		15.6	15.7	16.4	16.4	15.8
	2		3.0	3.0	3.0	3.0	3.0		2		16.2	16.8	14.8	14.8	15.0
	3		3.0	3.0	3.4	3.4	3.0		3		16.6	16.2	16.2	16.2	16.4
	4		3.4	3.0	3.2	3.2	3.0		4		15.6	17.0	15.0	16.8	15.4
	5		3.0	3.0	3.0	3.0	3.0		5		17.0	17.2	16.6	16.6	17.4
5	1		16.0	16.2	15.8	15.8	15.2	14	1		12.5	14.4	14.2	14.2	13.4
	2		14.8	16.1	15.2	15.1	16.3		2		14.6	14.3	12.7	12.7	13.0
	3		16.0	16.0	15.0	15.0	15.0		3		13.6	13.6	13.4	13.4	12.6
	4		15.8	16.0	15.8	15.7	13.9		4		13.6	14.6	13.4	13.0	15.2
	5		14.6	16.2	16.4	15.2	14.4		5		13.2	13.4	13.6	13.6	13.6
6	1		12.6	13.8	13.7	13.7	13.4	15	1		9.0	8.8	9.0	9.0	9.0
	2		15.2	13.8	14.6	14.6	13.5		2		9.2	9.0	8.2	8.2	8.8
	3		13.4	13.4	15.6	15.6	12.2		3		9.0	9.0	9.4	9.4	8.4
	4		14.2	13.6	13.2	13.2	14.4		4		9.2	9.0	10.6	9.8	9.2
	5		13.2	13.2	15.2	15.2	13.4		5		8.6	8.9	9.8	9.7	9.0
7	1		8.0	7.4	7.4	7.4	7.8	16	1		1.7	1.6	1.5	1.5	1.5
	2		7.6	7.4	7.4	7.4	7.8		2		1.6	1.5	1.5	1.5	1.1
	3		8.0	7.4	7.4	7.4	7.8		3		1.5	1.3	1.6	1.6	1.0
	4		7.2	7.4	7.4	7.4	7.8		4		1.4	1.8	3.0	1.6	1.4
	5		8.1	7.4	7.4	7.4	7.8		5		1.5	1.8	1.5	1.5	1.8
8	1		4.4	4.5	4.5	4.9	5.0	PSPTnc	1		1.3	1.4	1.5	1.5	1.5
	2		4.4	4.3	4.4	4.4	5.0		2		1.4	1.5	1.5	1.5	1.9
	3		4.6	4.6	4.8	4.8	4.2		3		1.5	1.7	1.4	1.4	2.0
	4		4.6	4.7	6.4	5.0	5.0		4		1.6	1.2	1.4	1.4	1.6
	5		4.6	4.6	5.2	5.2	4.8		5		1.5	1.2	1.5	1.5	1.2
9	1		16.4	18.0	17.8	17.8	16.4	PSPTpc	1		17.8	12.8	10.6	10.4	16.6
	2		18.0	17.6	18.0	18.0	17.8		2		12.0	12.5	10.8	10.8	16.5
	3		17.8	18.0	17.4	17.4	16.4		3		11.0	12.0	10.2	10.2	18.0
	4		17.8	17.8	15.2	17.4	17.4		4		13.0	13.2	9.6	9.6	16.0
	5		18.0	17.2	17.4	17.4	17.6		5		13.6	15.2	9.8	9.6	15.0

Table 12. Intra-laboratory ranking of the serum pools under test and reference sera based the antibody concentrations determined in Phase 1 study

serum sample	laboratory 1*	laboratory 2*	laboratory 3*	laboratory 4*	laboratory 5*
1	10.2 (8-14)	10.3 (10-12)	10.8 (8-16)	10.8 (8-16)	10.5 (9-14)
2	6.9 (5-8)	7.4 (7-8)	7.4 (4-17)	7.4 (4-17)	7.1 (4-9)
3	4.5 (3-8)	4.6 (4-6)	4.4 (3-6)	4.6 (3-6)	5.0 (4-8)
4	3.2 (3-5)	3.0 (3-3)	3.1 (3-4)	3.1 (3-4)	3.0 (3-4)
5	15.4 (12-18)	16.1 (14-17)	15.6 (9-18)	15.6 (9-18)	15.0 (11-17)
6	13.7 (11-17)	13.6 (11-17)	14.5 (10-18)	14.5 (10-18)	13.4 (11-17)
7	7.8 (5-9)	7.7 (7-9)	8.2 (5-14)	8.2 (5-12)	7.8 (5-10)
8	4.5 (4-6)	4.5 (4-5)	5.1 (4-12)	5.1 (4-7)	4.8 (4-7)
9	17.6 (15-18)	17.7 (16-18)	17.2 (5-18)	17.2 (16-18)	17.1 (12-18)
10	13.6 (11-16)	13.0 (11-17)	13.5 (10-17)	13.5 (7-17)	12.7 (11-15)
11	12.3 (10-15)	11.6 (10-15)	13.0 (11-17)	12.7 (8-16)	10.9 (3-14)
12	6.3 (5-7)	5.9 (4-6)	6.5 (5-11)	6.4 (5-11)	5.9 (4-6)
13	16.2 (10-18)	16.6 (15-18)	15.8 (6-18)	16.2 (10-18)	16.0 (13-18)
14	13.5 (11-17)	14.1 (12-16)	13.5 (12-16)	13.4 (12-16)	13.6 (10-18)
15	9.0 (7-10)	8.9 (8-9)	9.4 (6-15)	9.2 (6-15)	8.9 (7-10)
16	1.5 (1-2)	1.6 (1-2)	1.8 (1-2)	1.5 (1-2)	1.4 (1-2)
PSPTnc	1.5 (1-2)	1.4 (1-2)	1.5 (1-2)	1.5 (1-2)	1.6 (1-2)
PSPTpc	13.5 (10-18)	13.1 (10-15)	10.2 (8-11)	10.2 (8-11)	16.4 (13-18)

* mean ranking of antibody concentration with range

Table 13a. Mean antibody concentration per vaccine dose (EU/ml) in the PSPT (Phase II)

	vac.	dose (μ l)	lab.1		lab.2		lab.3		lab.4	
			exp.1*	exp.2	exp.1	exp.2*	exp.1	exp.2	exp.1*	exp.2*
ref.	50.0	1235	354		54	59	209	403	150	311
	25.0	717	213		21	30	90	180	297	120
	12.5	333	69		17	16	34	149	134	47
	6.25	119	34		12	17	26	40	106	30
	yG	750	203		39	47	119	260	301	33
	slope	1.12	1.18		0.69	0.94	1.03	1.02	0.73	1.56
A	80.0	791	411		74	76	393	318	146	309
	40.0	730	299		32	33	281	283	153	179
	20.0	364	116		16	22	113	134	82	151
	10.0	151	55		11	15	51	122	44	42
	yG	675	231		40	49	205	374	269	313
	slope	0.82	1.01		0.91	0.89	1.01	0.52	0.91	1.40
B	80.0	1447	473		41	59	283	800	390	327
	40.0	312	259		39	35	168	289	258	161
	20.0	223	141		19	17	62	134	41	98
	10.0	128	65		11	14	27	71	69	14
	yG	706	255		40	46	164	359	331	259
	slope	1.10	0.95		0.67	0.89	1.01	1.16	1.59	1.10

* potency calculated with 3 vaccine doses

yG average antibody concentration of the vaccine doses used to calculate the potency

Table 13b. Mean antibody concentration per vaccine dose (EU/ml) in the PSPT (Phase II)

	vac.	dose (μ l)	lab.1		lab.2		lab.3		lab.4	
			exp.3	exp.4	exp.3*	exp.4*	exp.3	exp.4*	exp.3	exp.4
ref.	50.0	581	933		21	57	339	334	325	477
	25.0	296	707		48	45	151	163	274	152
	12.5	142	285		16	24	67	71	53	89
	6.25	82	205		11	10	38	43	16	34
	yG	367	673		28	42	166	232	170	250
	slope	0.95	0.81		1.07	1.12	1.10	1.19	1.16	1.23
C	50.0	1016	945		65	65	646	432	517	427
	25.0	444	553		29	33	335	620	298	195
	12.5	327	489		23	24	216	243	171	176
	6.25	78	221		19	18	158	111	24	47
	yG	483	686		36	45	270	328	214	264
	slope	1.16	0.64		0.77	0.70	0.66	0.73	0.89	0.97
D	50.0	1364	1794		242	229	693	560	654	840
	25.0	1049	1403		104	110	475	471	311	339
	12.5	663	1048		89	90	355	164	104	247
	10.0	172	231		25	26	220	97	76	113
	yG	613	998		54	64	362	340	190	355
	slope	0.96	0.90		1.01	1.04	0.54	0.87	1.44	0.92

* potency calculated with 3 vaccine doses

yG average antibody concentration of the vaccine doses used to calculate the potency

Table 14a. Survival (total/survived) of mice after i.c. challenge in the MPT (Phase II)

vac.	dose (μ l)	lab.1 exp.1	lab.1 exp.2	lab.2 exp.1	lab.2 exp.2*	lab.3 exp.1	lab.3 exp.2	lab.4 exp.1*	lab.4 exp.2*
ref.	62.5	16/16	16/15	16/11	16/11	16/15	16/14	16/16	16/14
	12.5	16/ 9	16/11	16/ 7	16/ 7	16/ 0	16/ 2	16/ 5	15/ 6
	2.5	16/ 3	16/ 2	16/ 4	16/ 3	16/ 0	16/ 0	16/ 1	16/ 1
	0.5	16/ 1	16/ 3	16/ 1	16/ 1	16/ 0	16/ 0	16/ 0	16/ 1
	<i>ED50</i>	7.20	5.94	17.60	19.20	36.26	22.00	21.15	13.47
	<i>slope</i>	1.68	1.28	0.89	1.01	6.49	3.27	1.83	1.39
A	100.0	16/15	16/12	16/11	16/12	16/15	15/14	15/13	16/13
	20.0	16/ 7	15/ 6	16/ 6	16/ 8	16/ 8	16/11	16/ 9	16/ 8
	4.0	16/ 1	16/ 3	16/ 4	16/ 3	15/ 0	16/ 1	16/ 2	16/ 2
	0.8	16/ 0	16/ 1	16/ 2	16/ 2	15/ 0	16/ 0	15/ 0	16/ 0
	<i>ED50</i>	21.95	27.40	31.90	21.21	22.70	15.30	18.82	23.63
	<i>slope</i>	2.24	1.09	0.79	0.92	2.86	2.59	1.66	1.57
B	100.0	16/13	16/14	16/12	16/16	16/12	16/13	12/11	16/14
	20.0	16/13	16/12	16/ 8	16/ 7	16/ 5	16/ 7	16/ 7	15/ 7
	4.0	16/ 3	16/ 4	16/ 3	16/ 2	16/ 1	16/ 0	16/ 4	15/ 4
	0.8	16/ 0	16/ 0	16/ 1	16/ 2	15/ 0	16/ 0	16/ 1	15/ 0
	<i>ED50</i>	13.01	11.51	22.65	13.52	17.45	31.11	16.08	23.60
	<i>slope</i>	1.53	1.59	1.41	1.43	2.63	2.07	1.27	1.93
LD50		722	1339	1030	575	56	116	205	nc

* potency calculated with 3 vaccine doses
 nc not calculable

ED50
LD50 effective dose
lethal doses

Table 14b. Survival (total/survived) of mice after i.c. challenge in the MPT (Phase II)

vac.	dose (μ l)	lab.1 exp.3	lab.1 exp.4	lab.2 exp.3*	lab.2 exp.4*	lab.3 exp.3	lab.3 exp.4*	lab.4 exp.3	lab.4 exp.4
ref.	62.5	16/15	16/16	16/10	16/12	16/13	16/14	16/15	16/15
	12.5	16/ 9	16/10	15/ 8	15/ 8	16/ 2	16/ 2	13/ 2	15/ 4
	2.5	16/ 5	16/ 6	16/ 4	16/ 3	16/ 0	14/ 1	16/ 0	16/ 2
	0.5	16/ 1	16/ 4	16/ 2	16/ 2	16/ 0	16/ 0	16/ 0	16/ 0
	<i>ED50</i>	6.80	4.94	16.90	12.22	31.07	24.40	23.79	16.28
	<i>slope</i>	1.53	1.59	0.67	0.88	2.92	2.63	3.69	1.85
C	50.0	16/16	16/16	16/12	16/12	16/16	16/15	16/15	16/16
	10.0	16/14	16/14	16/ 8	16/ 9	16/11	16/ 9	16/11	16/10
	2.0	16/ 5	16/ 5	16/ 1	16/ 2	16/ 1	16/ 0	16/ 0	16/ 2
	0.4	16/ 3	16/ 4	16/ 1	16/ 2	16/ 0	15/ 0	16/ 0	16/ 1
	<i>ED50</i>	2.30	2.00	13.52	10.28	7.32	10.28	8.71	5.73
	<i>slope</i>	1.62	1.44	1.24	0.96	2.88	2.74	2.74	1.83
D	50.0	16/16	16/16	16/16	16/16	16/15	16/13	16/15	16/16
	20.0	16/14	16/16	16/ 8	16/10	16/10	16/10	16/11	16/13
	4.0	16/ 1	16/ 5	16/ 2	16/ 5	16/ 1	16/ 0	16/ 4	16/ 1
	0.8	16/ 1	16/ 1	16/ 1	16/ 2	16/ 0	14/ 0	16/ 2	16/ 1
	<i>ED50</i>	4.23	2.30	6.96	3.86	8.34	8.71	5.20	4.23
	<i>slope</i>	2.32	2.69	1.72	1.41	2.24	5.38	1.98	2.06
LD50		nc	455	388	1030	89	101	496	272

* potency calculated with 3 vaccine doses
 nc not calculable

ED50
LD50 effective dose
lethal doses

Table 15. Results comparative study of the PSPT and MPT (Phase II)

vac.	lab.	PSPT potency*	lower limit	upper limit	MPT potency*	lower limit	upper limit
A	1	<u>3.6</u> 5.3	2.7 4.0	5.0 7.1	<u>1.9</u> <u>1.4</u>	<u>0.9</u> <u>0.4</u>	4.3 3.8
	2	4.8 4.5	3.4 3.1	7.0 6.9	<u>3.9</u> <u>5.6</u>	<u>0.9</u> <u>1.6</u>	16.2 20.7
	3	9.2 5.6	6.0 2.7	16.1 14.3	6.4 11.0	2.2 5.5	14.0 22.1
	4	<u>1.6</u> 5.9	<u>0.0</u> 4.1	4.7 9.1	<u>3.4</u> 6.8	<u>1.3</u> 3.0	8.2 15.6
	1	<u>3.0</u> 5.6	<u>1.9</u> 4.0	4.5 7.9	<u>3.3</u> <u>3.1</u>	<u>1.4</u> <u>1.2</u>	7.7 7.7
	2	4.0 4.3	2.8 2.5	6.0 7.6	4.2 7.1	<u>1.3</u> 2.5	14.5 22.3
	3	5.3 5.4	3.6 2.7	8.5 14.3	8.2 5.5	2.7 2.6	18.3 11.9
	4	<u>1.9</u> 4.1	<u>1.0</u> 3.0	4.2 5.6	<u>3.2</u> 8.8	<u>1.3</u> 3.3	8.2 22.9
	1	9.1 <u>6.9</u>	6.3 4.3	13.8 11.1	17.9 10.4	7.5 <u>2.4</u>	44.8 17.9
	2	<u>7.2</u> <u>5.4</u>	<u>1.9</u> 4.1	13.8 7.3	<u>5.3</u> <u>7.0</u>	<u>1.4</u> <u>2.0</u>	20.3 24.2
C	3	19.2 18.0	14.2 11.6	27.7 32.5	27.9 13.9	15.2 7.0	52.1 37.4
	4	8.6 <u>7.8</u>	6.0 5.6	12.8 11.3	17.1 16.3	8.0 8.4	37.4 31.3
	1	18.8 14.1	12.8 9.4	31.4 24.8	9.6 13.0	3.9 5.5	23.9 30.5
	2	22.7 17.3	<u>3.5</u> 4.7	43.5 30.0	10.9 17.9	<u>3.3</u> 6.3	43.3 56.9
	3	32.1 13.4	19.8 9.3	64.8 22.4	22.5 49.7	11.3 8.6	45.7 56.9
D	4	10.6 13.9	6.7 10.2	18.6 20.2	23.0 28.4	10.9 11.9	51.0 61.4

* potencies in IU/ml

— does not meet the W.H.O. requirements

Table 16. Reproducibility PSPT and MPT

lab.	vac.	PSPT potency* (IU/ml)	lower limit	upper limit	p	mean var.
1	A	4.4	3.6	5.5	-	0.066
	B	4.4	3.3	5.8	-	0.086
	C	8.1	6.0	11.0	-	0.096
	D	16.5	11.8	22.9	-	0.103
	<i>pooled</i>					0.088
2	A	4.7	3.6	6.1	-	0.084
	B	4.1	3.0	5.6	-	0.104
	C	5.5	4.2	7.3	-	0.142
	D	19.0	9.0	40.1	-	0.242
	<i>pooled</i>					0.143
3	A	8.1	5.3	12.4	-	0.147
	B	5.3	3.6	7.8	-	0.140
	C	18.8	14.2	24.9	-	0.114
	D	18.3	12.8	26.0	-	0.114
	<i>pooled</i>					0.124
4	A	5.8	3.9	8.6	-	0.084
	B	3.6	2.7	4.8	-	0.114
	C	8.2	6.3	10.6	-	0.081
	D	12.8	9.6	17.0	-	0.095
	<i>pooled</i>					0.094
inter-laboratory	A	5.0	4.3	5.7	0.919	0.171
	B	4.3	3.8	5.0	0.911	0.111
	C	9.1	7.9	10.4	0.999	0.103
	D	15.4	12.9	18.4	0.788	0.139
	<i>pooled</i>					0.123
lab.	vac.	MPT potency* (IU/ml)	lower limit	upper limit	p	mean var.
1	A	1.7	0.9	3.3	-	0.211
	B	3.2	1.7	6.0	-	0.198
	C	14.1	7.2	27.5	-	0.210
	D	11.3	6.1	21.0	-	0.172
	<i>pooled</i>					0.198
2	A	4.8	1.8	12.4	-	0.302
	B	5.6	2.5	12.6	-	0.255
	C	6.2	2.5	15.3	-	0.286
	D	14.5	6.3	33.3	-	0.264
	<i>pooled</i>					0.277
3	A	9.1	5.2	15.8	-	0.180
	B	6.4	3.6	11.4	-	0.138
	C	20.6	13.0	32.8	-	0.146
	D	21.5	12.2	37.6	-	0.172
	<i>pooled</i>					0.159
4	A	5.5	2.8	10.9	-	0.196
	B	5.2	2.7	10.1	-	0.210
	C	16.6	10.1	27.4	-	0.159
	D	25.4	14.5	44.6	-	0.177
	<i>pooled</i>					0.186
inter-laboratory	A	4.6	3.3	6.4	0.981	0.222
	B	4.9	3.5	6.8	0.352	0.212
	C	15.9	11.9	21.2	0.618	0.200
	D	18.2	13.4	24.9	0.302	0.204
	<i>pooled</i>					0.210

* geometric mean of potencies

— does not meet the W.H.O. requirements

Table 17. Correlation PSPT and MPT

laboratory	vaccine	Chi-square test of homogeneity			p
		ratio	confidence interval		
1	A	2.714	1.378	5.346	0.911
	B	1.322	0.667	2.618	0.424
	C	0.455	0.202	1.024	0.348
	D	1.351	0.714	2.555	0.854
	<i>Pooled</i>	1.330	0.947	1.870	0.179
2	A	0.977	0.361	2.643	0.771
	B	0.729	0.305	1.746	0.506
	C	1.023	0.362	2.888	0.993
	D	1.428	0.453	4.498	0.848
	<i>Pooled</i>	0.964	0.585	1.589	0.985
3	A	0.849	0.420	1.715	0.953
	B	0.856	0.422	1.736	0.625
	C	0.882	0.511	1.523	0.268
	D	1.031	0.519	2.048	0.294
	<i>Pooled</i>	0.900	0.651	1.245	0.905
4	A	0.829	0.344	1.999	0.381
	B	0.517	0.240	1.114	0.759
	C	0.489	0.278	0.858	0.932
	D	0.476	0.215	0.903	0.294
	<i>Pooled</i>	0.531	0.377	0.747	0.985
inter-laboratory	A	1.274	0.861	1.887	0.381
	B	0.840	0.578	1.221	0.692
	C	0.672	0.484	0.932	0.738
	D	0.907	0.627	1.312	0.377
	<i>Pooled</i>	0.887	0.738	1.065	0.571

References

1. Russell WMS. and Burch RL. The Principles of Humane Experimental Technique. London: Methuen & CO LTD,1959.
2. Kreeftenberg JG, van der Gun JW, Marsman FR, Sekhuis VM, Bhandari SK and Maheswari SC. An investigation of a mouse model to estimate the potency of the diphtheria component in combined vaccines. *J Biol Stan* 1985; 13:229-234.
3. Hendriksen CFM, van der Gun JW, Marsman FR and Kreeftenberg JG. The use of the *in vitro* toxin binding inhibition (ToBI) test for the estimation of the potency of tetanus toxoid. *Biologicals* 1991; 19:23-29.
4. Huet M, Relyveld E and Camps S. Methode simple de controle de l'activite des anatoxines tetaniques adsorbees. *Biologicals* 1990; 18:61-67.
5. Maheshwari SC, Sharma SB, Ahuja S and Saxena SN. Development of a mouse model to estimate the potency of the diphtheria toxoid component of diphtheria-tetanus and diphtheria-tetanus-pertussis vaccines. *J Biol Stand* 1988; 16:139-146.
6. Kendrick PL, Eldering G, Dixson MK and Misne, J. Mouse protection tests in the study of pertussis vaccines: a comparative series using intracerebral route of challenge. *Am J Public Health* 1947; 37:803-810.
7. Van Straaten-van de Kappelle I, van der Gun JW, Marsman FR, Hendriksen CFM and van de Donk HJM. Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. *Biologicals* 1997; 25:41-57.
8. Van der Ark AAJ, van Straaten-van de Kappelle I, Akkermans AM, Hendriksen CFM and van de Donk HJM. Development of Pertussis Serological Potency Test: Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model. *Biologicals* 1994; 22:233-242.
9. Van der Ark AAJ, van Straaten-van de Kappelle I, van de Donk HJM and Hendriksen CFM. Evaluation of Whole Cell Vaccine-induced humoral antibody responses in the Pertussis Serological Potency Test in relation to the Mouse Protection Test. Bilthoven: National Institute of Public Health and the Environment; 1997 July Report no. 623860 004.
10. Appendix: Methods currently used in some countries for quality control of acellular pertussis vaccines. In: WHO Expert Committee on Biological Standardization. Forty Seventh Report. WHO Tech. Rep. Series. 1998;878:74-76.
11. Requirements for pertussis vaccine. WHO Expert Committee on Biological Standardization. Fortieth Report. WHO Tech. Rep. Series. 1990;800:136-138.
12. Finney DJ. Chapter 14: The Combination of Estimates. In: Statistical Methods in Biological Assay. London: Charles Griffin and Company Limited, 1964.
13. *Ph.Eur.* Collaborative study on alternative methods for potency testing of tetanus toxoid vaccines for human use: Phase I study (in press).
14. Tijsen P. Practice and Theory of Enzyme Immunoassays in Laboratory Techniques. In: Biochemistry and Molecular Biology. Amsterdam: Elsevier Science Publishers BV, 1993; 15: 418-421
15. Hardegree MC, Pittman M and Maloney CJ. Influence of mouse strain on the assayed potency (unitage) of tetanus toxoid. *Applied Microbiology* 1972; 24:120-126.
16. Knight PA and Lucken RN. The effects of laboratory animal diets on the potency tests of bacterial vaccines. *Developments in Biological Standardization* 1980; 45:143-149.
17. Walvoort HC. Chapter 19: Assessment of distress through pathological examination. In: Hendriksen CFM and Koeter HBWM, editors. *Animals in Biomedical Research*. Amsterdam: Elsevier Science Publishers BV, 1991.

Appendix 3 Mailing list

- 1 Inspectie Gezondheidsbescherming, Waren en Veterinaire Zaken, drs. H. Verburg
- 2 Inspectie Gezondheidsbescherming Waren en Veterinaire Zaken, dr. F. Schuring
- 3 Inspectie Gezondheidsbescherming Waren en Veterinaire Zaken, mr. J. de Haan
- 4 Inspectie Gezondheidsbescherming, Waren en Veterinaire Zaken, drs. P. de Greeve
- 5 De Directeur-Generaal Volksgezondheid, dr. H.J. Schneider
- 6 Voorzitter Gezondheidsraad, Prof.dr. J.J. Sixma
- 7 Secretariaat Platform Alternatieven voor Dierproeven, drs. P. de Greeve
- 8 Zorg Onderzoek Nederland, Dr. A.A.J. van Iersel
- 9 Dr. Jan van der Valk, Nederlands Centrum Alternatieven voor Dierproeven
- 10 - 11 CAD – Coördinatiepunt Alternatieven voor Dierproeven
- 12 Dr. Maria Luisa Brero, Instituto Nacional de Biologica, Servicio Vacunas Bacterianas, Buenos Aires, Argentina
- 13 Dr. Rose-Marie Ölander, National Public Health Institute, National Vaccine Quality Control Laboratory, Helsinki, Finland
- 14 Dr. Suresh S. Jadhav, Serum Institute of India, Quality Assurance, Hadapsar, India
- 15 Dr. Karlheinz Enssle, Chiron - Behring, Quality Control, Marburg, Germany
- 16 Dr. Julie Milstien, World Health Organisation
- 17 Dr. Nora Dellepiani, World Health Organisation
- 18 Dr. Elwin Griffiths, World Health Organisation
- 19 Dr. Jean-Marc Spieser, European Pharmacopeia
- 20 Dr. Peter Castle, European Pharmacopeia
- 21 Depot Nederlandse Publikaties en Nederlandse Bibliografie
- 22 Directie RIVM
- 23 – 26 Sectordirecteuren RIVM
- 27 – 32 Laboratoriumhoofden sector 1
- 33 Head LGM, Dr. J.F. van Sonderen
- 34 Dr. P.M.J.M. Jongen, LGM
- 35 A.M. Gommer, LGM
- 36 Dr. J.G. Kreeftenberg, BIS
- 37 J. Hendriks, BIS
- 38 F.R. Marsman, SB I
- 39 R.W.M. van Kinderen, CDL
- 40 J.F. Visser, CDL
- 41 – 47 Auteurs
- 48 SBD/Voorlichting & Public Relations
- 49 Bureau Rapportenregistratie
- 50 Bibliotheek RIVM
- 51 – 65 Bureau Rapportenbeheer
- 66 – 90 Reserve exemplaren