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The Pertussis Serological Potency Test Collaborative Study to Evaluate Replacement of the Mouse Protection Test

Arno van der Ark[1]*, Ineke van Straaten-van de Kappelle[1], Rose-Marie Ölander[2], Karlheinz Enssle[3], Suresh Jadhav[4], Huib van de Donk[1] and Coenraad Hendriksen[1]

[1]*National Institute of Public Health and Environmental Protection, PO Box 1, 3720 BA Bilthoven, The Netherlands*
[2]*National Public Health Institute, National Vaccine Quality Control Laboratory, Helsinki, Finland*
[3]*Chiron-Behring, Quality Control, Marburg, Germany*
[4]*Serum Institute of India, Quality Assurance, Hadapsar, India*

Abstract. 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 pilot-phase study of the antibody detection assay, the 18323-whole cell ELISA (WCE), was included for training purposes. Significant differences in absorbance and antibody concentrations between the laboratories were found. In the Phase I study, the intra-assay, inter-assay and inter-laboratory precisions of the 18323-WCE were assessed. 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. 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 ($P>0{\cdot}05$). The PSPT and MPT correlated well in χ^2-test of homogeneity within and between the laboratories. The potencies were 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. We have demonstrated that the PSPT is a valid model to estimate the potencies of pertussis WCV's from different manufacturers. Moreover, the 18323-WCE is easy to carry out and the intra-assay precision and antibody ranking warrants a reliable potency testing of pertussis WCV's in the PSPT.

Introduction

Replacement, reduction and refinement of the use of laboratory animals testing—the three R's of Russell and Burch[1]—is a recognized worthwhile goal for reasons of animal welfare. However, there are other 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,[2–5] which can be used as a replacement for lethal challenge procedures in potency testing of Diphtheria and Tetanus Toxoid vaccines. The MPT[6] 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 *Bordetella*

*To whom correspondence should be addressed.

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

Laboratory	mouse strain	PSPT sex (*m*/*f*)	PSPT weight (*g*)	MPT sex (*m*/*f*)	MPT 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
5	did not complete Phase II study				

m male.
f female.

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 *B. pertussiss* whole cells 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 and more reproducible, allows a reduction in number of mice by at least 25%, and is less distressful to the animals. Eventually, the number of mice used might be further reduced 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. Five laboratories participated in this study: besides the RIVM, two laboratories in Europe and two laboratories from outside Europe, as is shown in Annex 1. These laboratories were selected with the help of the World Health Organization (WHO). The study was divided into three separate phases, each with its own specific objective. The pilot-phase was included for the participants to become acquainted with the 18323-WCE. Validity parameters[10] 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) $\leq$20%, $P \geq 0{\cdot}01$]. In the Phase II study four vaccines were tested in the PSPT and MPT, to demonstrate an agreement between both tests and to evaluate the reproducibility and reliability of the PSPT compared to the MPT. In addition, the influence of local conditions such as the mouse strain, housing, and animal diet, on the WCV-induced humoral antibody responses were studied. 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 from three of the participants. All data processing and statistical analysis were performed at the RIVM to avoid bias of results.

Materials and methods

Mice

For the PSPT and the MPT, equal numbers of both sexes were used apart from laboratory 4 (Table 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-Tetanus-Pertussis-polio (DPT-polio) vaccine and vaccine B is an expired batch DPT-polio from the same manufacturer. Both vaccines contain 16 OU/ml pertussis whole cells. Vaccine C and D are DPT-vaccines produced by two other manufacturers, each containing 32 OU/ml. Potencies were previously estimated in the MPT at the manufacturer's laboratories.

Reference sera

New reference sera were produced for this study by immunizing N:NIH mice (i.p.) with a protective dose of reference Kh 85/1, DPT-polio or were injected with saline for the standard reference serum (PSPTst), positive control (PSPTpc) and negative control (PSPTnc), respectively. Mice were bled on day 28, sera pooled and calibrated against the former reference serum (HIS) 4, which was in use up to this study.[8] The antibody concentrations for PSPTst were determined at 415 ELISA Units per millilitre (EU/ml), PSPTpc and PSPTnc at 360 EU/ml and 0 EU/ml, respectively.

Serum samples

For the pilot-phase, sets of 16 serum pools were made from excess PSPT-sera, which had been previously tested by the RIVM. The participating laboratories were informed on the antibody concentrations of these sera. For the Phase I study 16 other serum pools were used, each obtained from one of the vaccine dilutions used in the PSPT. Each pool was aliquoted into five samples (50 μl/sample) in such way that all the serum pools were measured randomly in each of the five ELISA-plates on the same one day. The serum pools covered the range of antibody concentrations seen in the PSPT.

18323-Whole Cell ELISA

The 18323-WCE was carried out according to the method described by van der Ark *et al.*[8] Some modifications 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. In each the standard reference PSPTst, 16 serum samples under study and two control sera (PSPTpc en PSPTnc) were serial diluted as follows: PSPTst twice in a three-fold dilution range of eight dilutions, starting at a 1/1000 dilution. PSPTpc, PSPTnc and the samples under in single five-fold dilution ranges of four dilutions, also start at a 1/1000 dilution. IgG titres were assessed using the biotin-streptavidin labeling system (Amersham, UK). Finally, binding was visualized by addition of a tetramethylbezadin (TMB; Sigma, USA) substrate solution; H_2SO_4 stopped the colouring reaction. Absorbance was measured at 450 nm and antibody concentrations were calculated by means of a four-parameter fitting analysis and expressed as ELISA Units (EU)/ml. Only serum dilutions, the corresponding ODs of which were within the linear part of the standard curve, were used to calculate the antibody concentration for each sample. To evaluate the performance of the test, the following parameters were recorded during the whole study: the minimum and maximum absorbance of the reference sera, the antibody concentrations of the control sera, the limit of detection (LOD) and the limit of quantitation (LOQ).[10] 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 acceptable precision.

Pertussis Serological Potency Test

The PSPT was performed according to detailed instructions based on the description by van der Ark *et al.*[8] 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 under anaesthesia after a 4-week interval. The serum samples were measured in 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.

Intracerebral Mouse Protection Test

The MPT was performed according to detailed instructions based on the WHO guidelines.[11] Mice (n=16) of 10–14 g 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 or killed when moribund was recorded daily. For the potency calculation, only mice dying from day 17 to 28 after immunization 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.

Collaborative study design

The pilot-phase was included for training purposes. The antibody concentrations of 16 pilot-phase serum samples were measured on five 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 16 serum pools and two controls five times on five different days. Per day, each sample was measured in five different plates—randomized per plate—to assess the intra-assay precision ($n=5$) on five different days to assess the inter-assay precision ($n=25$) by the five participating laboratories ($n=125$). 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, of two batches of DPT-polio vaccine (A and B) from one manufacturer and two batches of DPT vaccine (C and D) produced by two other manufacturers, in both tests. Raw data were processed and statistically evaluated at the RIVM to avoid bias in results.

Statistics

The precision of the 18323-WCE has been determined by the variation in antibody concentrations (CV in %) of 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. Significant differences between series of data are estimated by means of analysis of variance (ANOVA). Results are significantly different if $P<0{\cdot}05$.

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.[12] Similarity of corresponding potencies from the MPT and PSPT was estimated by means of a modified chi-squared (χ^2)-test of homogeneity.[13] The ratios of both estimates, in which the variances of individual potencies of a test are used as a weighting coefficient, were calculated and used in the χ^2-test of homogeneity. Both test systems agree if the ratio does not differ significantly from 1·00 ($P>0{\cdot}05$). 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 $P\leq 0{\cdot}05$.

Results

Pilot-phase: 1832-WCE try-out

In three of the five laboratories the pilot-phase was carried out as requested in the protocols.

Sixteen pilot-phase serum pools and two control sera were measured once on five different days. Of the two laboratories that deviated from the protocol, laboratory 3 measured the serum samples on four different days only and laboratory 5 measured the samples five times on five 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. The antibody concentrations of PSPTpc varied also per day and per laboratory (CV's from 18·6 up to 44·6%). The LOD varied between 0·5 to 5·9 EU/ml and the LOQ between 1·1 to 10·6 EU/ml. The antibody concentrations of the serum samples measured by the participants corresponded poorly with the antibody concentrations from the RIVM results (Table 2). The laboratories 1, 2, and 4 measured antibody concentrations, which were in general lower (± 90, 70, and 60%, respectively) and the antibody concentrations of laboratory 3 and 5 were higher (± 167·3, and 112·1%, respectively) compared to the mean antibody concentration of all laboratories.

Phase I: 18323-WCE validation

The Phase I study was carried out according to the detailed instructions: 16 phase I serum pools and two controls were measured five times on five different days (Table 3). The absorbance data of the laboratories 1, 2, 3 and 5 corresponded well, while the OD's of laboratory 4 remained clearly higher. (Table 3) shows the variation in antibody concentrations of PSPTpc for each of the participants (CV's of 6·9 to 20·8%). The OD's 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 pilot-phase, the antibody concentrations of laboratory 1 were comparable to the mean antibody

Table 2. Mean antibody concentrations (EU/ml) of pilot-phase samples (n=5)

Sample	given	lab.1	lab.2	lab.3	lab.4	lab.5	avg.	CV (%)
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	390	302	263	431	467	362	27·3

concentrations of all participants, whereas the antibody concentrations of laboratory 2 and 5 were approximately 20 and 30% lower, respectively (Table 3). The laboratories 3 and 4 found antibody concentrations, which were ± 20 and 10% higher, respectively.

The pooled intra-assay precision for laboratories 1, 2, and 4 was less than 14%, 13% and 15%, respectively, and for laboratory 5 less than 20% (Table 3). The pooled intra-assay precision of laboratory 3 varied per day, from 15·5 to 23·3% (Table 3). In general, 2 of the 16 serum samples were out of the 20% variation range, except for laboratory 3. The pooled inter-assay precision for laboratories 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 (Table 3) and/or the measured antibody concentrations differed significantly ($P \leq 0{\cdot}01$) varying per day and per laboratory. The inter-laboratory precision is determined at 25·1%. The antibody concentrations of fifteen of the 16 serum pools varied more than the intended 20% and the antibody concentrations of six serum samples were significantly different. Therefore, we decided to determine the ranking of the serum samples based on their antibody concentrations by means of the Kruskal–Wallis method. The ranking of the antibody concentration of serum samples corresponded well between the laboratories, as is shown in Table 4.

Phase II: comparative study of the PSPT and MPT

Four of the five laboratories participated in the comparative study of the PSPT and MPT. In Phase II 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 used (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%. The inter-laboratory precision was determined at 18·1%.

The mean antibody concentration of the serum samples per vaccine dose varied considerably between the laboratories (up to a factor of 10) and to a lesser degree between the tests within the laboratories (up to a factor of 4), as illustrated in Tables 5a and 5b. The number of non- and low-responding mice also varied considerably per vaccine dilution, per test and per laboratories. Less than 3% of the mice in laboratories 1 and 2 did not induce 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. Sera of which OD's were below the OD's of the PSPTnc (non-responders) were left

Table 3. Precision of the 18323-WCE. The antibody concentrations (EU/ml) of sixteen Phase I serum samples were measured in the 18323-WCE (five plates on five different days). The coeficients of variation (CV in %) were calculated to determine the intra-assay (n=5), inter-assay (n=25) and inter-laboratory precision (n=125)

serum pool	lab.	mean Ab.conc.	pooled intra-assay	inter-assay	inter-lab.	serum pool	lab.	mean Ab-conc.	pooled intra-assay	inter-assay	inter-lab.
1	1	252	12·1	12·5	19·9	10	1	403	11·2	16·6	25·0
	2	214	8·5	10·9			2	296	12·9	15·5	
	3	296	29·6	21·9			3	453	20·4	21·9	
	4	299	16·8	15·3			4	480	11·2	14·2	
	5	205	24·1	18·6			5	288	15·1	17·0	
2	1	128	13·1	13·1	26·2	11	1	342	11·1	15·1	27·0
	2	110	9·2	11·2			2	255	9·1	11·9	
	3	173	24·8	25·6			3	397	21·7	21·3	
	4	162	17·5	24·4			4	438	14·7	18·8	
	5	94	14·7	19·6			5	228	22·0	23·2	
3	1	79	10·6	16·6	22·4	12	1	110	9·3	14·0	28·0
	2	64	6·1	13·1			2	82	7·9	9·9	
	3	84	20·2	21·1			3	147	20·1	30·2	
	4	88	14·7	17·1			4	131	13·6	18·6	
	5	60	21·2	24·8			5	75	14·4	20·6	
4	1	45	15·1	19·5	24·7	13	1	508	10·4	15·9	26·1
	2	38	9·0	10·5			2	413	10·7	14·7	
	3	50	21·6	28·4			3	681	28·0	35·7	
	4	52	15·5	20·4			4	598	10·6	14·3	
	5	28	17·0	18·4			5	398	14·2	19·2	
5	1	433	16·0	19·0	24·6	14	1	402	9·5	15·9	21·1
	2	365	12·0	13·2			2	319	15·1	11·7	
	3	528	22·6	23·2			3	431	15·5	18·1	
	4	572	13·4	19·2			4	451	15·7	17·8	
	5	343	18·4	22·2			5	308	16·7	21·2	
6	1	390	10·3	17·7	23·9	15	1	202	15·3	18·6	35·7
	2	296	10·9	13·3			2	195	35·5	37·3	
	3	486	15·9	24·5			3	270	33·3	36·5	
	4	461	11·5	15·9			4	280	28·2	37·6	
	5	297	12·2	17·5			5	145	13·6	25·1	
7	1	144	15·8	18·6	25·6	16	1	1			
	2	118	9·2	12·1			2	3			
	3	193	26·7	24·2			3	1			
	4	181	13·3	16·8			4	0			
	5	106	11·8	13·8			5	0			
8	1	81	8·8	15·7	26·2	PSPT	1	0			
	2	62	6·6	8·6		nc	2	0			
	3	99	15·5	20·5			3	1			
	4	102	14·9	19·3			4	0			
	5	58	15·3	19·4			5	0			
9	1	641	9·4	16·6	25·4	PSPT	1	344	6·9	13·6	20·5
	2	480	9·7	11·1		pc	2	296	10·9	14·1	
	3	827	22·5	27·0			3	272	7·3	11·5	
	4	676	16·1	17·4			4	335	9·5	14·4	
	5	449	32·6	20·3			5	440	14·9	20·8	

out of the potency calculations, while low-responders were included. In practice only a few mice per test were excluded.

The MPT's were also performed according to detailed instructions. Besides the mouse strain and animal husbandry, each laboratory used its own

Table 4. Intra-laboratory ranking of the serum pools under test and reference sera based the antibody concentrations determined in Phase I study

serum sample	laboratory 1		laboratory 2		laboratory 3		laboratory 4		laboratory 5	
	rank*	range	rank*	range	rank*	range	rank*	range	rank*	range
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 concentrations (n=25).

Table 5a. Mean antibody concentration per vaccine dose (EU/ml) of vaccine A and B in the PSPT (Phase II)

vac.	dose (μl)	laboratory 1		laboratory 2		laboratory 3		laboratory 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†	601	168	26	31	90	193	172	127
	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†	509	220	33	37	210	214	107	170
	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†	528	255	28	31	164	359	190	150
	slope	1·10	0·95	0·67	0·89	1·01	1·16	1·59	1·10

* potency calculated with three vaccine doses.
† average antibody concentration of the vaccine doses.

Table 5b. Mean antibody concentration per vaccine dose (EU/ml) of vaccine C and D in the PSPT (Phase II)

vac.	dose (μl)	laboratory 1		laboratory 2		laboratory 3		laboratory 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	24	35	166	153	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	34	35	270	352	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	115	114	362	323	190	355
	slope	0·96	0·90	1·01	1·04	0·54	0·87	1·44	0·92

* potency calculated with three vaccine doses.
† average antibody concentration of the vaccine doses.

challenge culture and procedure. The survival of mice was recorded and the raw data was sent to the RIVM. The Tables 6a and 6b show clearly the differences in the survival of mice per laboratory, as can be seen in the ED50's (effective dose), slopes, and LD50's (lethal dose, control of challenge culture) of the tests. The ED50's of the reference vaccine in laboratory 1, the LD50's of laboratory 3,

Table 6a. Survival (total/survived) of mice after i.c. challenge for vaccine A and B in the MPT (Phase II)

vac.	dose (μl)	laboratory 1		laboratory 2		laboratory 3		laboratory 4	
		exp. 1	exp. 2	exp. 1	exp. 2*	exp. 1	exp. 2	exp. 1	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
	ED50†	7·20	5·94	17·60	19·20	36·26	22·00	21·15	13·47
	slope	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
	ED50†	21·95	27·40	31·90	21·21	22·70	15·30	18·82	23·63
	slope	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
	ED50†	13·01	11·51	22·65	13·52	17·45	31·11	16·08	23·60
	slope	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 three vaccine doses.
† effective dose.
nc not calculable.
‡ lethal doses.

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 significantly in the PSPT nor in the MPT, within or between the laboratories (Table 7). As is shown in Table 8, 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 7). The pooled variance of the MPT is about twice the pooled variance of the PSPT, indicating a better reproducibility of the PSPT.

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 out of the five laboratories had no previous experience with the 18323-WCE at the beginning of the study. For this reason, the 18323-WCE try-out as pilot-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.[14] In a solid-phase enzyme immuno assay such as the 18323-WCE, the antibody binding capacity is measured rather than its concentration[15] and is influenced by for example the temperature, humidity and time of incubation, resulting in consequently lower or higher antibody concentrations.

In Phase I, the intra-assay, inter-assay, and inter-laboratory precision of the 18323-WCE has been

Table 6b. Survival (total/survived) of mice after i.c. challenge for vaccine C and D in the MPT (Phase II)

vac.	dose (μl)	laboratory 1		laboratory 2		laboratory 3		laboratory 4	
		exp. 3	exp. 4	exp. 3*	exp. 4*	exp. 3	exp. 4*	exp. 3	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
	ED50†	6·80	4·94	16·90	12·22	31·07	24·40	23·79	16·28
	slope	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
	ED50†	2·30	2·00	13·52	10·28	7·32	10·28	8·71	5·73
	slope	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
	ED50†	4·23	2·30	6·96	3·86	8·34	8·71	5·20	4·23
	slope	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 three vaccine doses.
† effective dose.
nc not calculable.
‡ lethal doses.

studied. Tijssen[15] 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. 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 ($CV \leq 20\%$, $P \geq 0{\cdot}01$) 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 3, the intra-assay and inter-assay precision varied per laboratory (from 6·1 to 35·5% and 8·6 to 37·6%, respectively). The pooled inter-laboratory precision has been determined at 25·1% (19·9 to 35·7%). Except for laboratory 3, the pooled intra- and inter-assay precision but not the inter-laboratory precision of the 18323-WCE was within the intended range ($CV \leq 20\%$). 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 ($P<0{\cdot}01$) in the antibody concentrations for the serum pools were found throughout the Phase I study. 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 4). The ranking of the serum pools corresponded well between laboratories. 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.

Table 7. Results comparative study of the PSPT and MPT (Phase II)

vac.	Lab.	test	PSPT potency (IU/ml)	lower limit	upper limit	mean var.	MPT potency (IU/ml)	lower limit	upper limit	mean var.
A	1	1	3·6*	2·7	5·0		1·9*	0·9*	4·3	
		2	5·3	4·0	7·1		1·4*	0·4*	3·8	
		wgm†	4·4	3·6	5·5	0·066	1·7*	0·9*	3·3	0·211
	2	1	4·8	3·4	7·0		3·9*	0·9*	16·2	
		2	4·5	3·1	6·9		5·6	1·6*	20·7	
		wgm†	4·7	3·6	6·1	0·084	4·8	1·8*	12·4	0·302
	3	1	9·2	6·0	16·1		6·4	2·2	14·0	
		2	5·6	2·7	14·3		11·0	5·5	22·1	
		wgm†	8·1	5·3	12·4	0·147	9·1	5·2	15·8	0·180
	4	1	1·6*	0·0*	4·7		3·4	1·3	8·2	
		2	5·9	4·1	9·1		6·8	3·0	15·6	
		wgm†	5·8	3·9	8·6	0·084	5·5	2·8	10·9	0·196
	interlab.mean		5·0	4·3	5·7	0·171	4·6	3·3	6·4	0·222
B	1	1	3·0*	1·9*	4·5		3·3*	1·4*	7·7	
		2	5·6	4·0	7·9		3·1*	1·2*	7·7	
		wgm†	4·4	3·3	5·8	0·086	3·2*	1·7*	6·0	0·198
	2	1	4·0	2·8	6·0		4·2	1·3*	14·5	
		2	4·3	2·5	7·6		7·1	2·5	22·3	
		wgm†	4·1	3·0	5·6	0·104	5·6	2·5	12·6	0·255
	3	1	5·3	3·6	8·5		8·2	2·7	18·3	
		2	5·4	2·7	14·3		5·5	2·6	11·9	
		wgm†	5·3	3·6	7·8	0·140	6·4	3·6	11·4	0·138
	4	1	1·9*	1·0*	4·2		3·2*	1·3*	8·2	
		2	4·1	3·0	5·6		8·8	3·3	22·9	
		wgm†	3·6*	2·7	4·8	0·114	5·2	2·7	10·1	0·210
	interlab.mean		4·3	3·8	5·0	0·111	4·9	3·5	6·8	0·212
C	1	1	9·1	6·3	13·8		17·9	7·5	44·8	
		2	6·9*	4·3	11·1		10·4	2·4*	17·9	
		wgm†	8·1	6·0	11·0	0·096	14·1	7·2	27·5	·210
	2	1	7·2*	1·9*	13·8		5·3*	1·4*	20·3	
		2	5·4*	4·1	7·8		7·0*	2·0*	24·2	
		wgm†	5·5*	4·2	7·3	0·142	6·2*	2·5*	15·3	·286
	3	1	19·2	14·2	27·7		27·9	15·2	52·1	
		2	18·0	11·6	32·5		13·9	7·0	37·4	
		wgm†	18·8	14·2	24·9	0·114	20·6	13·0	32·8	0·146
	4	1	8·6	6·0	12·8		17·1	8·0	37·4	
		2	7·8*	5·6	11·3		16·3	8·4	31·3	
		wgm†	8·2	6·3	10·6	0·081	16·6	10·1	27·4	0·159
	interlab.mean		9·1	7·9	10·4	0·103	15·9	11·9	21·2	0·200
D	1	1	18·8	12·8	31·4		9·6	3·9*	23·9	
		2	14·1	9·4	24·8		13·0	5·5	30·5	
		wgm†	16·5	11·8	22·9	0·103	11·3	6·1	21·0	0·172
	2	1	22·7	3·5*	43·5		10·9	3·3*	43·3	
		2	17·3	4·7	30·0		17·9	6·3	56·9	
		wgm†	19·0	9·0	40·1	0·242	14·5	6·3	33·3	0·264
	3	1	32·1	19·8	64·8		22·5	11·3	45·7	
		2	13·4	9·3	22·4		49·7	8·6	56·9	
		wgm†	18·3	12·8	26·0	0·114	21·5	12·2	37·6	0·172
	4	1	10·6	6·7	18·6		23·0	10·9	51·0	
		2	13·9	10·2	20·2		28·4	11·9	61·4	
		wgm†	12·8	9·6	17·0	0·095	25·4	14·5	44·6	0·177
	interlab.mean		15·4	12·9	18·4	0·139	18·2	13·4	24·9	0·204

* does not meet the W.H.O. requirements. For vaccine A and B a potency of 4·0 IU/ml with a lower limit of 2·0 IU/ml and for vaccine C and D a potency of 8·0 IU/ml with a lower limit of 4·0 IU/ml.

† weighted geometric mean (wgm) of test 1 and 2.

Table 8. Correlation PSPT and MPT in Chi-squared test of homogeneity

laboratory	vaccine	ratio	confidence	interval	*P*
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
	Pooled	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
	Pooled	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
	Pooled	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
	Pooled	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
	Pooled	0·887	0·738	1·065	0·571

In the Phase II study, four batches of pertussis WCV's were tested in duplicate, but not always simultaneously, by both test methods according to detailed instructions by four out of the five 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 mouse strain within the laboratories was not clear, while the differences in immunity found between the laboratories may be due to different mouse-strains[16] used, diet[17] and other aspects of animal husbandry. The number of non- and low-responding mice also varied considerably per test and laboratory, which might be partly due to an inconsistent i.p. injection procedure.[18] We have noticed in our own laboratory that the number of non- and low-responders was reduced after a period of time, because the number 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, despite the number of low-responders and the large differences in antibody concentrations, the potencies of the vaccines in the PSPT's did not differ significantly ($P \geq 0{\cdot}05$) as is shown in Table 8.

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 ($P \geq 0{\cdot}05$).

The PSPT and MPT correlated well within and between the laboratories ($P \geq 0{\cdot}05$ in a χ^2-test of

homogeneity). The pooled results of all potency determinations in the PSPT and MPT ($n=32$) were similar, as is indicated by a PSPT/MPT ratio of 0·877 (0·738–1·065). Within the laboratories, the ratios 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 Van Straaten-van de Kappelle *et al.*[7] 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 did not meet the required potency but also eight of the 32 PSPT potency determinations failed the requirements. However, when looking at the lower limit of 2·0 IU/human dose it can be seen that in the MPT four more tests did not pass this limit while in the PSPT only one test failed. Taking into account the average results per laboratory in the MPT, the number of vaccines passing the requirements in the PSPT agreed with the MPT.

In conclusion, the small-scale collaborative study of the PSPT in four laboratories demonstrated that the PSPT is a valid model to estimate the potencies of pertussis WCV's of different manufacturers by means of serology. The overall similarity between corresponding potencies in the PSPT and MPT was demonstrated by means of a χ^2-test of homogeneity: $P>0{\cdot}05$ and a PSPT/MPT ratio of 0·877 (0·738–1·065). Moreover, the PSPT is more reproducible, and 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 was due to the smaller 95% confidence intervals rather than to the invalidity of the test. There are a few cases in which the vaccine meets the potency requirements in one test but not the other. However, the number of vaccines passing the requirements correlates well, when the average results per laboratory were taken into account. The 18323-WCE is easy to perform and the intra-laboratory precision and the inter-laboratory antibody ranking should warrant the expectation of a reliable potency testing of pertussis WCV's in the PSPT.

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Annex 1. Participants collaborative study on the PSPT*

Dr. Maria Luisa Brero,
Instituto Nacional de Biologica,
Servicio Vacunas Bacterianas,
Buenos Aires, Argentina.
Dr. Rose-Marie Ölander,
National Public Health Institute,
National Vaccine Quality
Control Laboratory,
Helsinki, Finland.
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.
Dr. Suresh S. Jadhav,
Serum Institute of India,
Quality Assurance,
Hadapsar, India.
Dr. Karlheinz Enssle,
Chiron – Behring,
Quality Control,
Marburg, Germany.

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