# Pertussis Serological Potency Test as an Alternative to the Intracerebral Mouse Protection Test: Development, Evaluation and Validation

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Summary
*We have developed the Pertussis Serological Potency Test (PSPT) as an alternative to potency testing of pertussis Whole Cell Vaccines (WCV) in the intracerebral Mouse Protection Test (MPT). The PSPT is based on the humoral antibody response against the whole range of B. pertussis surface-antigens per vaccine dose and correlates well with the MPT. WCV-induced-antibody responses against „protective" antigens; or the biological activitv of pertussis antibodies poorly correlated or did not correlate at all with mouse protection. Compared to the MPT the PSPT is more reproducible, reduces the animal distress and the number of animals in use. Moreover, the number of animals in use 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.*

*Zusammenfassung: Serologischer Pertussis Wirksamkeitstest als Alternative zum intrazerebralen Maus Protektionstest: Entwicklung, Prüfung und Validierung*
*Als Ersatz für den Wirksamkeitstest für Pertussis Ganzzell Vaccinen (Whole Cell Vaccines, WCV) im intrazerebralen Maus-Protektionstest (MPT) wurde ein serologischer Pertussis-Wirksamkeitstest (Pertussis Serological Potency Test, PSPT) entwickelt. Der PSPT basiert auf der humoralen Antikörper-Antwort gegen das gesamte Spektrum der B. pertussis-Oberflächenantigene der Impfdosis und korreliert mit dem MPT. WCV induzierte Antikörper gegen „protektive" Antigene oder die biologische Aktivität der Pertussis-Antikörper korrelieren nur gering, wenn überhaupt, mit dem MPT. Verglichen mit dem MPT ist der PSPT besser reproduzierbar, er reduziert sowohl die Belastung der Tiere als auch die Tierzahlen. Darüberhinaus kann die Zahl der verwendeten Tiere noch weiter reduziert werden, wenn der Dreipunkt-Assay durch einen Einpunkt-Assay ersetzt wird und die in vitro Tests für die Wirksamkeitsprüfungen von Tetanus, Diphtherie und Pertussis in einem einzigen Tiermodell kombiniert werden.*

*Keywords: B. pertussis, antibody, potency, whole cell vaccine*

## 1 Introduction

The only potency assay for pertussis whole cell vaccines (WCV's) that has shown correlation with protection in children is the intracerebral (i.c.) mouse protection test (MPT), developed by Kendrick and co-workers (1947; Medical Research Council, 1956). The MPT, however, is also an inhumane and imprecise test (van Straaten-van de Kappelle et al., 1997), requiring large numbers of animals, and a lethal challenge procedure. Recently, we have developed the Pertussis Serological Potency Test (van der Ark et al., 1994) as an alternative to the MPT with the ultimate goal of refining and reducing the use of animals. The PSPT is based on *in vitro* assessment of the humoral immune response against the whole range of surface-antigens of *Bordetella pertussis* in mice after immunisation with Whole Cell Vaccines (WCV). Potency is estimated by means of a parallel line assay, based on the vaccine-dose dependent antibody concentration measured in the 18323-Whole cell ELISA (18323-WCE).

## 2 Animals, materials and methods.

### 2.1 Mice
N:NIH/RIVM outbred mice weighing 10-14 g or 20-24 g were used. Animals were obtained from the breeding facilities of the RIVM.

### 2.2 Vaccines
Lyophilised whole cell in-house reference Kh 85/1(40 OU/ml) with a potency of 6.0 IU/ml and pertussis WCV's obtained from different manufacturers were used.

### 2.3 Mouse Protection Test
The MPT was performed according to European Pharmacopoeia requirements (Ph.Eur., Monograph 1997:0160).

### 2.4 Pertussis Serological Potency Test
The PSPT was performed as described earlier (van der Ark et al., 1994).

### 2.5 Serology
Mouse IgG antibodies against the whole range of *B. pertussis* surface-antigens were measured in an ELISA using a whole cell coating of the international challenge strain 18323 (van der Ark et al., 1994). The ELISA measuring anti-PT antibodies was described by Sato et al. (1984). Antibodies against filamentous heamagglutinin (FHA), pertactin, and anti 92-kDa outer membrane protein (OMP) were determined by an indirect ELISA technique. The 18323-complement activation ELISA (18323-CAE), 18323-bactericidal antibody assay (18323-BAA) are described in detail in RIVM report no. 623860 004 (1997). PT-neutralising antibodies were measured by CHO-neutralisation assay (CNA) according to Gillenius et al. (1985).

## 3 Results and discussion

### 3.1 Evaluation of the humoral immune responses in the i.c. MPT.
Mice (10-14 g) were immunised (i.p.) with a protective dose of the reference or Diphtheria-Pertussis-Tetanus-polio (DPT-p) vaccine. Blood samples from the tail vein were taken during the 4 weeks of the test. Half

the group of immunised mice was challenged (i.c.) with virulent *B. pertussis* (strain 18323) at day 14, while the other group received no challenge. Serum antibody concentrations were measured in the 1 8323-WCE, antigen specific ELISAs (PT and FHA) and *in vitro* functional test systems like the CNA for PT-neutralising antibodies, the 1 8323-CAE to measure the antibody-dependent activation of the classical complement pathway, and 18323-BAA to measure bactericidal killing.

The humoral immune response of the MPT shows a normal primary and secondary antibody response after intraperitoneal (i.p.) immunisation. The IgM response is probably a T-cell independent humoral response to lipopolysaccharides, whereas the IgG response is a T-cell dependent response to antigens like OMP's, fimbriae, PT and FHA (Wiertz et al., 1990). We have demonstrated that the i.c. challenge has a booster-effect on the WCVinduced IgG-antibody response, which differs per vaccine and per test, and may contribute to the poor reproducibility of the MPT. The i.c. challenge does not only potentiate, but also modulates antibody responses, as is shown in table 1. The booster-effects of the i.c. challenge also differ per antigen and clearly enhance the capacity of the pertussis antibodies to activate the classical pathway of the complement system, but not the bactericidal capacity.

Furthermore, we have demonstrated a correlation between the mean pertussis antibody concentration at the day of challenge and the proportion of surviving mice at each vaccine dose in the MPT ($r = 0.910$). This enabled us to predict the actual survival of each mouse and estimated the potency of the vaccine (under test) based on the individual antibody concentration of each mouse at day 14. In a series of five experiments homogeneity between potencies based on the predicted and actual survival in the MPT was proven in a $\chi^2$-test. A ratio of 0.995 (0.566-1.750) with a p-value of 0.990 was found.

**Table 1: Influence of the i.c. challenge on the antibody responses during the MPT**

| assay | day | Kh 85/1 - chal.* | reference + chal.† | DPT-polio - chal.* | + chal.† |
|---|---|---|---|---|---|
| **18323-WCE‡** | 17 | 171 | 208 | 251 | 303 |
| | 20 | 247 | 438 | 498 | 648 |
| | 24 | 475 | 1043 | 861 | 990 |
| | 28 | 455 | 1117 | 1111 | 1466 |
| **PT-ELISA‡** | 17 | 0 | 5 | 4 | 6 |
| | 20 | 12 | 4 | 4 | 5 |
| | 24 | 4 | 4 | 7 | 16 |
| | 28 | 5 | 124 | 7 | 404 |
| **FHA-ELISA‡** | 17 | 7 | 4 | 32 | 5 |
| | 20 | 5 | 17 | 40 | 37 |
| | 24 | 6 | 16 | 59 | 66 |
| | 28 | 9 | 8 | 78 | 83 |
| **18323-CAE§** | 17 | 335 | 395 | 680 | 675 |
| | 20 | 460 | 1083 | 1203 | 1569 |
| | 24 | 783 | 1918 | 2043 | 2451 |
| | 28 | 808 | 2103 | 2488 | 3546 |
| **18323-BAA°** | 17 | 200 | 150 | 100 | 100 |
| | 20 | 200 | 200 | 200 | 200 |
| | 24 | 400 | 200 | 200 | 400 |
| | 28 | 200 | 400 | 400 | 400 |
| **CHO-neutr.** | 17 | 0 | 0 | 0 | 0 |
| | 20 | 0 | 0 | 0 | 0 |
| | 24 | 0 | 0 | 0 | 0 |
| | 28 | 0 | 0 | 0 | 0 |

*** Sera of non-challenged mice** **‡ ELIS Units/ml** **°Bactericidal Units/ml**
**† Sera of challenged mice** **§Comp. Activation Units/ml**

### 3.2 Development of the PSPT (Fig. 1)

Fourteen days after immunisation, antibody concentrations are low and hardly discriminate between the last two vaccine doses of the vaccine dilution range. We have examined the IgG antibody response per vaccine dose up to 6 weeks after immunisation (i.p.). Mice (10-14 g) were immunised with graded doses of the reference or DPT-p vaccine, blood samples were taken every week, and the antibody concentrations were measured in the 18323-WCE. To induce antibody concentrations within the linear part of the sigmoid curve, the vaccine doses were fixed at 4.0, 2.0, 1.0, and 0.5 opacity units (OU) per ml for reference and at 2.5, 1.25, 0.625, and 0.313 OU/ml for DPT-polio. Mice were immunised (i.p.) with 0.5 ml vaccine dilution. Potencies are calculated by means of a parallel line assay with log transformation. Log transformation is chosen to obtain a normal distribution of the antibody concentration per vaccine dose, by which the influence of hyper- and non-responders was limited. The mean antibody concentrations of the graded vaccine doses were significantly different. Potencies based on the antibody concentrations at day 21, 28 and 35 corresponded well with the MPT. We have chosen to bleed the mice at day 28 for practical reasons. Statistical evaluation of the preliminary results fixed the number of mice at 12 animals per vaccine dose, which resulted in a reduction of 25% of animals in use.

The Optimum route of immunisation was investigated. Antibody concentrations are significantly lower in mice immunised subcutaneously (s.c.) compared to intraperitoneal immunisation, and s.c. immunisation resulted in more hyper- and non-responders. The effect of body weight of mice on the range of induced antibody concentrations per vaccine

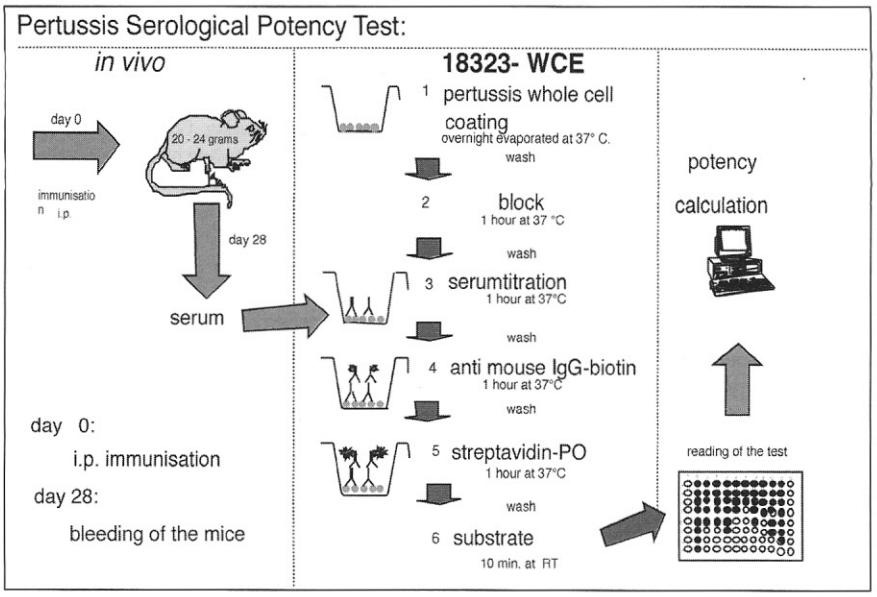


**Figure 1: Course of the Pertussis Serological Potency Test (PSPT)**

dose was also examined. Mice of 20-24 g in weight have a more mature immune system and induce higher antibody levels with significant lower variation compared to mice of 10-14 g. Mean coefficients of variation (c.v.) for mice of 10-14 g and 20-24 g were 73% and 44%, respectively.

The reproducibility of the PSPT and MPT were compared by means of analysis of variance. In a series of six experiments the potency of one DPT-p vaccine was examined in the MPT and PSPT, and the mean variances were 0.098 and 0.009, respectively. The lower mean variance of the PSPT indicates a better reproducibility, which resulted in clearly smaller 95% confidence intervals, probably due to the fact that the booster-effect of the i.c. challenge is excluded and because of the use of more mature mice.

### 3.3 18323-WCE validation

The 18323-WCE is validated at our institute and in an international collaborative study on the PSPT (manuscript in preparation). We have looked into the repeatability and reproducibility of the assay and have determined intra- and inter-assay variation (repeatability), as well as, intra- and inter-laboratory variation (reproducibility). To assess the intra-assay variation 3 serum samples were titrated 16 times in 3 different plates on one day at our own laboratory. The inter-assay, intra- and inter-laboratory variation was assessed by measuring the antibody concentrations of 16 serum pools (with different levels of antibodies) on 5 different days at 5 different international laboratories. The serum pools were distributed at random per individual plate. We have set our goals to assess a variation in performance within 20% (coefficient of variation) of the mean antibody concentration per vaccine dose with a 99%-confidence interval ($p > 0.01$).

The intra- and inter-assay variation as well as the intra-laboratory variation are within the 20% range for 4 out of 5 laboratories. The inter-laboratory variation is above the set 20% range. Furthermore, we have found significant differences in antibody concentrations for several serum pools which varied per plate, per day, and per laboratory, whether the variation was within or above the 20% range. Equal distribution of the serum samples per vaccine dose over the plates and, if necessary, per day may reduce the variation in repeatability and intra-laboratory variation. Moreover, the antibody concentrations are used to estimate a relative potency by means of a parallel line assay with log transformation and, therefore, the proportion of the antibody concentrations per vaccine dose may be of more importance than the absolute antibody concentrations of the serum samples.

### 3.4 PSPT in-house validation and evaluation of humoral immune responses

Thirteen WCVs (four DPT-p vaccines produced at our own institute and nine DPT vaccines made by other manufacturers, prepared in different ways and widely differing in potency) were tested in a comparative in-house validation study of PSPT and MPT. Potencies obtained by the PSPT and based on the antibody response against the whole range of surface antigens correlate well with the corresponding potencies of the MPT. Both tests are similar with respect to homogeneity according the $\chi^2$-test, which do not differ significantly per production procedure. We have found a ratio of 1.042 (0.831 - 1.306) with a p-value of 0.950.

Additionally, we have studied immunogenicity of WCVs in *B. pertussis*-antigen specific ELISAs and *in vitro* functional test systems to assess correlation with mouse protection. We have chosen to measure the antibody responses against PT, FHA, pertactin, and 92-kDa OMP, because active immunisation with purified pertactin, FHA and detoxified PT (PTx) protected mice against a lethal respiratory or aerosol challenge and PTx was also protective against an i.c. challenge (Zealey et al., 1992). It is noticeable that for a MPT a small amount of active PT is needed in the vaccine under test. The presence of OMPs like the 92-kDa OMP greatly enhances the protective capacity of outer membrane vesicles (Poolman et al., 1990).

In general, all vaccines induced a dose-dependent antibody response against 92-kDa OMP, most of the vaccines could induce an antibody response against pertactin and some against FHA. Only one vaccine induced a reliable antibody response against PT (van der Ark et al., 1996). Potencies based on the concentration of specific antibodies against PT, FHA and pertactin could not be calculated due to low antibody responses or in case of anti 92-kDa OMP antibodies do not correlate with protection in MPT, due to scattering (a ratio of 1.523 (1.192-1.946) and $p < 0.001$ is found in a $\chi^2$-test of homogeneity; van der Ark et al., 1997).

The functional activity of WCV-induced antibodies may be of more importance. Sera of the in-house validation study were pooled per vaccine dose and tested in CNA, 18323-CAE and 18323-BAA. WCVs hardly induced antibodies against PT and consequently not one of the vaccines could induce antibodies which were able to neutralise *in vitro* PT-toxicity. The low amounts of PT present in WCVs (Ibsen et al.,1993) is probably insufficient to induce protection by PT-neutralising antibodies. Roberts et al. (1990) presumed that the effector mechanisms of antibodies against pertactin may be of minor importance in preventing adherence of *B. pertussis* to the respiratory tract epithelium but could be involved in complement mediated lysis or opsonisation of *B. pertussis*. Therefore, we have focused on

complement-mediated immune responses. The capacity of sera to activate the complement classical pathway is determined by measuring the $C_3$-depositions on the 18323-whole cell coat in an ELISA (18323-CAE), and bactericidal killing of virulent *B. pertussis* is examined in an *in vitro* assay (18323-BAA). All serum pools were capable of activating the complement system *in vitro*, and the amount of $C_3$-depositions on 18323-whole cells is in proportion to the concentration of pertussis antibodies (R = 0.845). The capacity of pertussis antibodies to activate the complement system indicates that antibody-dependent cell-mediated cytotoxicity e.g. phagocytosis may be involved in mouse protection against i.c. challenge. We have also demonstrated a correlation between the bactericidal capacity and the concentration of pertussis antibodies (R = 0.821). Bactericidal antibodies are regarded as non-protective and correlation between *in vitro* activity of pertussis antibodies and mouse protection to be fortuitous (Ackers and Dolby, 1972). On the other hand, „protective" antigens such as pertactin and FRA administrated as a single component also failed to pass the MPT, but administrated in combination with other „protective" antigens enhanced protection. We assume that the induction of bactericidal antibodies may enhance protection in the MPT. We have estimated potencies based on the limited data from both tests, to get an indication whether the biological activity of pertussis antibodies may be a parameter to estimate potencies. Potencies were statistically invalid and showed poor correlation with the MPT. Moreover, the 18323-CAE and -BAA are laborious and quite difficult to reproduce and therefore not suitable as test method for potency testing.

In conclusion: Protection against i.c. challenge in the MPT is not related to an antibody response against a single „protective" antigen, nor restricted to a single immune mechanism, but may be related to a synergistic effect of humoral immune responses against a wide range of „protective" and „non-protective" surface-antigens.

## 4 Advantages of the PSPT

Correlation between MPT and PSPT confirms that the mean concentration of pertussis IgG-antibodies per vaccine dose is a promising substitute measuring potencies of WCVs, without the variable effects of an i.c. challenge. The PSPT is more reproducible, as is shown by the smaller confidence intervals of the potencies.

Furthermore, the use of the PSPT also leads to a reduction in animal distress (refinement) and the number of animals in use (reduction). By simplifying the multiple-dose design to a single-dose assay (after consistency in manufacturing and testing have been proven) the number of animals could be reduced even more. Moreover, the stability of final products or controlling the manufacture procedure could be monitored easier and cheaper when less animals are used. An other option is combining *in vitro* serological assay's for potency testing of tetanus-, diphtheria-, and pertussis components in one animal model.

To promote the replacement of the MPT by the PSPT, we have started a collaborative study which includes a validation of the 18323-WCE (Phase I) and a smallscale validation of the PSPT (Phase II). Assessment of intra- and inter-laboratory variation in potency and reproducibility in a small-scale pre-validation could provide more information about e.g. the influence of the mouse strain in use and the practicability of the PSPT at the local facilities.

## References


Ackers, J.P. and Dolby, J.M. (1972). The antigen of *Bordetella pertussis* that induces bactericidal antibody and its relationship to protection in mice. *J. Gen. Microbiol. 70*, 371-382.

Ark, van der, A. A. J., van Straaten-van de Kappelle, I., Akkermans, A. M., Hendriksen, C. F. M. and van de Donk, H. J. M. (1994). 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 22*, 233-242.

Ark, van der, A. A. J., van Straaten-van de Kappelle, I., Hendriksen, C. F. M. and van de Donk, H. J. M. (1996). Pertussis serological potency test as an alternative to the intracerebral mouse protection test. In F. Brown, K. Cußler, C. F. M. Hendriksen, (eds), *Replacement, reduction and refinement of animal experiments in the development and control of biological products,* (271-281) Basel: Karger Verlag.

Ark, van der, A. A. J., van Straaten-van de Kappelle, I., van de Donk, H. J. M. and Hendriksen, C. F. M. (1997). Evaluation of whole cell vaccine-induced humoral antibody responses in the pertussis serological potency test in relation to the mouse protection test. *RIVM report no. 623860 004,* 1-29.

Gillenius, P., Jäätmaa, E., Aselöf, P., Tiru, M. and Granström, M. (1985). The standardization of an assay for pertussis toxin and antitoxin in micro plates culture of chinese hamster ovary cells. *Biol. Stand. 13*, 61-66.

Kendrick, P. L., Eldering, G., Dixon, M. K. and Misner, J. (1947). Mouse protection tests in the study of pertussis vaccines: a comparative series using intracerebral route of challenge. *Am. J. Public Health 37*, 803-810.

Medical Research Concil (1956). Vaccination against whooping-cough: relation between protection in children and results of laboratory tests. *Br. Med. J. 2*, 454-462.

Poolman, J. T., Hamstra, H. J., Barlow, A. K., Kuipers, B., Loggen, H. and Nagel, J. (1990). Outer membrane vesicles of *Bordetella pertussis* are protective antigens in the mouse intracerebral challenge model. In C. R. Manclark, (ed), *Proceedings of the sixth international symposium on pertussis*, 148-255. D.H.H.S. Publications (FDA) 90-1164

Roberts, M., Tite, I. J., Fairweather, N. F., Dougan, G. and Charles, J. G. (1992). Recombinant P.69/pertactin: Immunogenicity and protection of mice against *Bordetella pertussis* infection. *Vaccine 10*, 43-48.

Sato, H. and Sato, Y. (1984). *Bordetella pertussis* infections in mice: Correlation of specific antibodies against two antigens, pertussis toxin, and filamentous heamagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. *Infect. Immun. 46*, 411-415.

Straaten-van de Kappelle, van, I., van der Gun, J. W., Marsman, F. R., Hendriksen, C. F. M. and van de Donk, H. J. M. (1997). Collaborative study on test systems to assess toxicity of whole cell pertussis vaccines. *Biologicals 25*, 41-57.

Wierts, E. J. H. J., Walvoort, H. C., van Loveren, H., van Straaten-van de Kappelle, I., van der Gun, J. W. and Kreeftenberg, J. G. (1990). A-cellular and whole cell pertussis vaccines protect against the lethal effects of intracerebral challenge by different T-cell dependent humoral routes. *Biologicals 18*, 173-180.

Zealy, G., Lossmore, S., Yacoob, R. and Klein, M. (1992). Modern pertussis vaccines. *Vaccine Research 1*, 413-427.



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