Evaluation of Two Serological Methods for Potency Testing of Whole Cell Pertussis Vaccines

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ABSTRACT

The European Pharmacopoeia (Ph. Eur.) and the World Health Organization (WHO) require the performance of extensive quality control testing including a potency test before a vaccine batch is released for human use. Whole cell pertussis (wP) vaccine potency is assessed by a mouse protection test (MPT) based on the Kendrick test. This test compares the vaccine dose necessary to protect 50% of mice against the effect of a lethal intracerebral dose of Bordetella pertussis and the dose of a suitable reference vaccine needed to give the same protection level. Due to the large variability in the results of this test and the severe distress which is inflicted on the many animals involved, its replacement by an alternative method is highly desirable.

At the initiative of the European Directorate for the Quality of Medicines and HealthCare (EDQM) of the Council of Europe, in collaboration with the WHO and the In-vitro toxicology Unit/European Centre for the Validation of Alternative Methods (ECVAM) of the European Commission (EC) Joint Research Centre-Institute for Health and Consumer Protection (JRC-IHCP), wP vaccine specialists from all over the world were invited to present an overview of candidate alternatives at a symposium organised in Geneva (Switzerland) in March 2005.

Although no alternative method was found suitable for immediate implementation of batch potency control, the Pertussis Serological Potency Test (PSPT), initially developed in mice and recently transferred to guinea pigs (gps), was identified as a model of interest. Using the PSPT in gps to test several components of combined vaccines such as Diphtheria-Tetanus-wP vaccines in the same animal series would allow further implementation of the European 3Rs policy to batch potency control, by additional method refinement and reduction of animal use.

The present study evaluated 2 features of the serological response to wP vaccination: 1) the overall antibody response as measured by a "whole cell" ELISA (PSPT-wC-ELISA) which uses the B. pertussis 18323 challenge strain prescribed for the MPT to coat the assay plates and 2) the functional neutralising antibodies to pertussis toxin (PT, one of the main virulence factors of B. pertussis), as measured by the Chinese Hamster Ovary (CHO) cell assay.

The results showed that 1) the gp model can be used for wP vaccine potency testing; 2) despite good repeatability and precision, the CHO cell assay did not generate results comparable to the MPT. Moreover, the CHO cell assay showed significant differences in the ability of wP vaccines to induce neutralising anti-PT antibodies, which did not correlate to the overall antibody response evaluated by PSPT-wC-ELISA; 3) comparable potencies were obtained in the MPT and the PSPT-wC-ELISA.

This study, supported by the previous ones correlating the PSPT-wC-ELISA in mice with the MPT, confirms that PSPT-wC-ELISA in gps is a promising approach for batch release potency testing of wP vaccines for which consistency in production has already been demonstrated by the MPT. However, a large scale validation study is required prior to the adoption of PSPT-wC-ELISA as a compendial reference method for wP vaccines batch release control.

KEYWORDS

Whole cell pertussis vaccines, combined vaccines, ELISA, CHO cell assay, serology, pertussis toxin.

AIM

The aim of the present study was to investigate whether serological potency testing in gps, using either the functional CHO cell assay or an indirect ELISA based on plates coated with wP bacteria, strain 18323 (PSPT-wC-ELISA), is a possible alternative to the current MPT stipulated by the Ph. Eur. and WHO for batch release of the wP component of combined vaccines. This approach would reduce the overall number of animals used for potency testing of combined vaccines and refine the potency test by avoiding the challenge with virulent wP bacteria.

A meeting organised in Geneva on 16 March 2005 at the initiative of the EDQM, Council of Europe and in collaboration with the WHO and the EC JRC-IHCP-In-vitro toxicology Unit/ECVAM (proceedings available at http://www.edqm.eu/site/Download-527.html), concluded that such alternative methods for testing consistency of production are envisaged acceptable for manufacturers and Official Medicines Control Laboratories (OMCLs) and have the potential to become reference methods for the Ph. Eur., the WHO and the US-FDA (United States Food and Drug Administration).

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1. INTRODUCTION

The potency of wP vaccines for human use needs to be checked by the manufacturers and, in most countries, by an OMCL prior to commercialisation. To comply with the requirements of the Ph. Eur. [1] and the WHO [2], wP vaccines need to have a potency of at least 4 IU per dose, (with a lower limit of the 95 % confidence interval of at least 2 IU) as determined in the Kendrick test [3]. These potency specifications are based on the correlation between the protection in children and the results of the MPT [4]. In this test, the dose necessary to protect 50 % of mice (effective dose 50 or ED-50) against the effect of a lethal dose of *B. pertussis* administered intracerebrally is compared with the dose of a reference vaccine needed to give the same protection. The test inflicts severe pain and distress to the animals, requires highly experienced operators, is sensitive to small technical changes resulting in a high variability and requires frequent repeats due to test invalidity [5,6].

Although acellular pertussis combined vaccines are more frequently used in Europe and USA, wP vaccines are still produced and used worldwide in immunisation programmes. Furthermore, in order to provide vaccines with wide disease coverage, wP may be combined not only with diphtheria (D) and tetanus (T) toxoids, but also with inactivated polio viruses (IPV), *Haemophilus influenzae* glycoconjugate (Hib) and/or hepatitis B vaccine.

As up to 200 mice per test are required and both manufacturers and OMCLs perform the assay, a very high number of animals are used to release 1 batch of wP vaccine. It is extremely important that the wP potency assay continues to be performed to guarantee the use of fully active vaccines. It is also important to search for an alternative assay that can provide the same information on the quality of a vaccine, whilst refining the animal test, reducing the number of animals used and showing a better reproducibility than the MPT.

Potential alternatives to the MPT have already been described, e.g. the respiratory challenge test (aerosol challenge) [7] and the nitric oxide test [8], but they require special or custom-made equipment/facilities and cannot be performed in “standard” medicines control laboratories. A promising model is the PSPT in which the challenge test is replaced by the *in vitro* assessment of mouse humoral response to the wide range of surface antigens of *B. pertussis* [9]. The method was extensively evaluated in-house (Nederlands Vaccin Instituut, NVI, Bilthoven, The Netherlands) as well as in a small collaborative study, with quite satisfactory results [10].

Recently, it was shown that potency for D and T can be determined in the same set of gps by *in vitro* serological assays [11,12], thus saving animals as well as reducing their pain and distress. These new tests have been validated and incorporated into the Ph. Eur. [13,14] as alternatives to the challenge tests for T and D potency assay. As wP is generally combined with D and T toxoids, using 1 set of animals for the serological testing of all 3 components would be the ideal situation. In this way, reduction and refinement of the use of animals would be met for the control of DTwP vaccines, as requested by the “European Convention on the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes” [15] and the Council Directive 86/609/EEC [16].

Whole cell pertussis vaccine is not a well characterised active substance. Though the mechanisms underlying protection are not yet fully understood, it is known that both serological and cell-mediated immune responses are involved [17,18]. In this small inter-laboratory study involving the ISS and NVI, 2 alternative serological assays in gps for the determination of wP potency were evaluated.

The selected methods reflect 2 features of the gp serological response to wP vaccines: 1) the CHO cell assay measures the functional neutralising antibodies to pertussis toxin (PT), one of the bacteria main virulence factors [19]; 2) the wC-PSPT-ELISA measures the overall antibody response to the *B. pertussis* strain 18323 (used as challenge strain in the MPT) [9].

The study was designed to obtain results using the minimal number of animals. For this purpose, gps were immunised in the 2 laboratories following a common schedule, using vaccines of different compositions and exchanging the sera between the 2 laboratories to assay them by the proposed *in vitro* methods, i.e. CHO cell assay and PSPT-wC-ELISA.

The outcome of the collaboration is of particular interest as it demonstrates the applicability of the serological potency test in gps to wP vaccines, the ability of the PSPT to discriminate between wP vaccines of different potencies and the correlation of the wP potencies estimated by the wC-ELISA and the MPT.

2. MATERIALS AND ANIMALS

2.1 Test vaccines

The potency of 4 licensed lots of combined vaccines (coded A, B, C and D), obtained from different manufacturers, was tested. These included final lots of DTwP and DTwP-Hib combinations with provided estimated potency for wP determined by the current regulatory required MPT (Table 1) [1, 2]. Vaccine C was selected due to its relatively high potency in the MPT. Vaccine D was an expired vaccine with a known low potency around the minimum requirement of 4 IU/dose. Vaccines A and B were products available on the market. Both vaccines A and B were tested in the MPT by the respective manufacturers, but not as part of this study, while vaccines C and D were re-tested in the MPT at the same time as the PSPT in gps.

2.2 Reference standards and reagents

- *Reference vaccine*: WHO 3rd International Standard (IS) for Pertussis vaccine (lyophilised inactivated *Bordetella pertussis*, 46 IU/ampoule, code 66/303) [20].
- *Reference sera*:
  - Mouse partially purified anti-PT IgG (NIBSC, batch code JNIH-12) with a defined activity of 200 anti-PT ELISA units (ELU) per ampoule. The titre determined by CHO cell assay in this study corresponded to 1:200 - 1:400.
  - *B. pertussis* mouse antiserum BRP batch 1 (EDQM Catalogue Number B1142000) with an assigned anti-PT activity of 19.5 ELU per vial.
- *PT*: Pertussis toxin BRP batch 1 (EDQM Catalogue Number Y0000021) with a defined concentration of 50 µg/vial. It was shown to have a Minimal Clustering Concentration (MCC) of 156 pg/ml in the CHO cell assay.

2.3 Animals

Equal numbers of male and female gps weighing 250-400 g were used for each vaccine dilution group. Specifications of gps used at NVI and ISS are reported in Table 2. All animals were SPF and certified by the breeder for the absence of *B. bronchiseptica*. The health status of the animals was recorded at arrival and monitored during the experiments by comparison with sentinel animals. The sentinels were from the same batch of animals and were housed during the experiment in the same room as the study animals. The 6 sentinel animals at NVI and ISS were checked pre- and post-immunisation for the presence of antibodies against

B. bronchiseptica and *B. parapertussis* by qualitative ELISA, at day 0 and at day 35 (bleeding day).

2.4 Positive gp control serum

Gps were immunised with the highest dose (9.2 IU) of the reference vaccine for the production of a positive anti-PT/anti *B. pertussis* gp control serum (coded wP gp Pos). The positive gp control serum, given an arbitrary titre of 100 ELU/ml, was used in all assays.

2.5 Negative gp control serum

A wP negative control gp serum (coded wP gp Neg) was obtained from the sentinel animals, after confirmation of the absence of antibodies against *B. bronchiseptica* and *B. parapertussis*. The negative gp control serum was used as negative control in all serological assays.

3. METHODS

3.1. Immunisation protocol

In each laboratory, a series of 3-fold dilutions of the test vaccines and the WHO reference vaccine was prepared in 0.9 % sterile saline solution. For each vaccine, random groups of 12 gps were immunised with the respective vaccine dilution (Table 3; 5 groups at ISS and 4 groups at NVI). At NVI, the WHO reference vaccine was reconstituted in 0.5 times the volume used at ISS. As a result, the same vaccine dose, in terms of IU, was injected in a volume of 0.5 ml (NVI) instead of 1 ml (ISS), subcutaneously lateral to the linea alba (ISS) or to the groin fold (NVI) of the gps. Animals were housed for further 35 days under SPF conditions in cages (ISS) or ground pens (NVI) in rooms with controlled light (12:12 light/dark cycle), constant temperature (20-24°C) and constant relative humidity (55 %). Animals had *ad libitum* access to a commercial diet and tap water. In each laboratory, 6 additional gps were used as sentinel animals.

3.2. Blood and serum collection

At NVI, all animals were bled by heart puncture at day 35. At ISS, 50 % of the animals, 6 of each vaccine dose group, were bled at day 34 and the rest at day 35. Before bleeding, animals were anaesthetised with an intramuscular injection of an atropine/xylazine/ketamine mixture (0.5:1.25:4 A/X/K, approximately 0.05 ml/100 g body weight). Blood samples were collected in numbered vials, incubated at 37°C for 1h and stored overnight at +4°C. Blood samples were further centrifuged at 800 *g* for 20 min, serum was separated from cells, aliquoted and stored below -20°C. Serum samples were shipped under temperature controlled conditions between the 2 institutes.

3.3 Serological assays

All serum samples were checked for the presence of anti−PT antibodies by both the CHO cell assay and anti-PT ELISA, and for *B. pertussis* antibodies by the PSPT-wC−ELISA.

All assays were validated according to the ICH guideline for repeatability and precision [21].

3.3.1 CHO cell assay

The ability of antibodies to neutralize the clustering activity of PT was determined by the CHO cell assay, according to good cell culture practice [22] and following the procedure of Gillenius [19].

Briefly, CHO-K1 cells (ATCC CCL-61, batch no. 3639694) were grown in polystyrene flasks (BD Falcon) in a complete Ham's medium containing Ham's F12K medium (EuroClone) supplemented with 10 % FBS (EuroClone), 4 mM L-Glutamine (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin (Penicillin/Streptomicin solution, EuroClone) and 1.5 g/l sodium bicarbonate (EuroClone). For neutralisation studies, experiments were carried out in 96-well microtitre plates (BD Falcon) in which 100 µl of 2-fold serial dilutions of gp sera in complete Ham's medium were mixed with 25 µl of PT (final concentration: 7.5 ng/ml) and incubated at 37 °C for 1 h. A volume of 75 µl of a CHO cell suspension containing 1.33 x 10^5 cells/ml were added to each well (final concentration: 10^4 cells/well). The presence of CHO cell clusters was determined after 2 days of incubation at 37 °C in a 5 % CO_2 atmosphere. The titre of a serum was set at the dilution where complete neutralisation of the PT cluster activity was obtained. In all experiments, each plate included 2 positive controls (JNIH-12 mouse IgG anti-PT serum and wP gp Pos serum) and a negative control serum (wP gp Neg).

Cell cultures were screened every 4-5 passages for mycoplasma contamination using a PCR kit (EZ-PCR Mycoplasma Test Kit, Biological Industries, Israel Beit Haemek LTD).

3.3.2 PT-ELISA

The PT preparation used in the CHO cell assay was also used to coat the PT-ELISA plates. The purity of the PT preparation was checked by western blot. Two sera, one PT-specific (JNIH-12, mouse IgG anti-PT) and one *B. pertussis* specific (wP gp Pos, giving high anti-PT titres in CHO cell assay), were used for this purpose. The profiles of the mouse anti-PT and the positive gp serum (wP gp Pos) were almost identical, differing only by the presence of a low molecular weight band visible with the mouse serum IgG (data not shown).

Polystyrene flat-bottomed microtitre plates (Nunc Maxisorp, cat. code 442404) were coated with 100 µl of PT (1 µg/ml) in carbonate buffer (0.1 M, pH 9.6) overnight at 4 °C. Plates were washed with phosphate buffered saline (pH 7.4) - 0.05 % Tween 80 (PBST) and blocked with 5 % skimmed milk diluted in PBST (200 µl/well) at 37 °C for 1 h. The plates were further washed 3 times with PBST. Two-fold dilution series of test sera in PBST containing 1 % skimmed milk were added to the wells (100 µl/well) at 37 °C for 2 h. After washing, 100 µl of goat anti-gp horseradish peroxidase conjugate (Sigma) were added. Plates were incubated at 37 °C for 2 h. After washing, 100 µl of substrate (ABTS, Sigma A-9941) were added. Plates were incubated at room temperature for 30 min. Absorbance was read at 450 nm by an automated microtitre plate reader (Perkin Elmer - Wallac). All plates included a positive control (wP gp Pos) and a negative control (wP gp Neg) serum.

3.3.3 Whole cell ELISA (PSPT-wC- ELISA)

The original mouse-wC-ELISA protocol as described by van den Ark *et al.* [9] was adapted to a gp version. Flat-bottomed Polysorb microtitre plates (Nunc, cat. code 475094) were coated with a suspension of whole cell *B. pertussis* strain 18323 (ATCC, code 9797; 0.25 Opacity Units/ml PBS, 100 µl/well) and incubated at 37°C overnight without lid to allow the liquid phase to evaporate. Plates were blocked with a 1 % solution of BSA in PBS (120 µl/well) at 37 °C for 1 h and washed with a solution of 0.1 % Tween 80 in tap water. Sera diluted in PBS with 0.5 % w/v BSA and 0.05 % Tween 80 was added (100 µl/well) and incubated at 37 °C for 1 h. After washing, 100 µl of biotin-conjugated anti-gp antibody (Biotin-SP-AffiniPure F(ab')$_2$ Fragment Goat anti-gp IgG (H+L), Jackson ImmunoResearch, cat. code 106-066-003) were added and incubated at 37°C for 1 h. Plates were washed and incubated at 37 °C for 1 h with 100 µl of peroxidase-conjugated streptavidin (Jackson ImmunoResearch, cat. code 016-030-084). After washing, 100 µl of substrate (TMB, Sigma) were added. The plates were incubated at room temperature for 10 min. Detection reaction was stopped by addition of 100 µl of 2M H_2SO_4. Absorbance at 450 nm was measured with an automated

microtitre plate reader (Biotek or Perkin Elmer −Wallac). All plates of all experiments included a positive control (wP gp Pos) and a negative control (wP gp Neg).

3.4 ELISA detection of antibodies against *Bordetella* species other than *B. pertussis.*

Antibodies to *B. bronchiseptica* and *B. parapertussis* were detected by ELISA according to Boot *et al* [23]. Briefly, microtitre plates were coated with merthiolate inactivated *B. bronchiseptica* or *B. parapertussis* organisms at a protein concentration of 7.5 µg/ml. Gp test samples were titrated using a peroxidase-based detection system (goat-anti-gp peroxidase conjugate, Sigma, cat. code A7289, used at 1:15-20.000). After incubation and washing, 100 µl of substrate were added to each well. The detection substrate consisted of 1.67 ml tetramethyl-benzidine (TMB) in dimethylsulphoxide (6 mg/ml) in 100 ml of 0.1 M sodium acetate buffer pH 5.5, and 10 µl of a 30 % solution of H_2O_2 (Perhydrol, Merk, Darmstadt). The reaction was stopped after 10 min by adding 100 µl of 2M H_2SO_4 and absorbances were read at 450 nm as described in Boot *et al* [24].

Positive and negative control sera were run in duplicate in each assay. Serum samples were considered positive if the optical density (OD) of a serum dilution exceeded a value defined as the mean OD +/- 3 SD of 3 negative control sera wells.

3.5 Statistical analyses

3.5.1 CHO cell assay - titres and scores calculations

The titre of a serum sample was determined as the dilution at which a total inhibition of the clustering effect was seen. Each serum titre was further transformed into a score (score 1= 1:8, score 2=1:16, score 3=1:32, etc.) used individually or to calculate a mean score for each treatment group.

3.5.2 PT–ELISA - titres and scores calculations

Sera titres were calculated using a cut-off value equal to 10 % of the difference between the maximal and minimal OD values of each plate (i.e. the OD difference between wP gp Pos and wP gp Neg). Each serum titre was further transformed into a score (as above, item 3.5.1.) that was used individually or used to calculate a mean score for each treatment group.

3.5.3 PSPT-wC-ELISA - titres calculations

The antibody titres were expressed in ELISA units per millilitre (ELU/ml). For each plate, the 25-75 % absorbance range, reflecting the linear part of the titration curve, was determined as 25 % and 75 % of the sum of the maximal and minimal absorbance values.

For each serum, OD values within this 25-75 % range were used to calculate the antibody titre relative to the positive control serum (titre arbitrarily set at 100 ELU/ml), using the 4-parameter fit of the KCjunior software (BioTek Instruments). A cut-off value of 5 ELU/ml was defined, based on the titre of the negative control sera. Titres below this value were considered non-specific and unreliable, and were arbitrarily assigned a value of 2.5 ELU/ml.

3.5.4 Vaccine potency calculations

The potencies of the test vaccines were calculated by parallel lines [25] using the WHO IS as the reference vaccine, with the CombiStats software (http://www.combistats.eu).

Mean potencies were calculated as the weighted geometric mean of 3 or 6 values of potency estimates.

4. RESULTS

4.1. CHO cell assay and PT-ELISA

The CHO cell assay was optimised and validated according to the ICH guideline [21] for measurements of gp anti-PT antibodies. Repeatability (intra-assay precision) and intermediate precision (intra-laboratory variation) were calculated on the basis of the titres of negative serum (wP gp Neg) and 2 positive control sera (anti-PT JNIH-12 mouse serum and wP gp Pos), as well as of the MCC of the PT BRP batch 1.

The test gave consistent results, showing good repeatability and precision (Table 4). However, gp serum was found to affect the morphology of CHO cells, causing cell elongation and aggregation. This made the interpretation of the test at sera dilutions lower than 1:8 difficult (Figure 1). This effect was not visible at any dilution of the anti-PT positive JNH-12 mouse control serum. In contrast, *B. pertussis* mouse antiserum BRP batch 1 was extremely toxic for CHO cells, up to the dilution of 1:32. No explanation could be found for this effect.

It was suggested that gp serum could alter the cell morphology due to the presence of traces of anaesthetics. Therefore, a set of experiments was performed both at ISS and NVI using gp sera from animals anaesthetised with different products (isofluorane, CO_2/O_2, ketamine/xylazine). All gp sera affected the morphology but not the viability of CHO cells, independently of the anaesthetics used (data not shown).

Since it was not possible to discriminate the PT-specific clustering effect from the morphological changes due to the presence of gp serum at a dilution of 1:4, all titrations started from a dilution of 1:8.

Neutralising anti-PT antibodies induced by immunisation with wP vaccines could be detected by the CHO cell assay. Sentinels' serum samples were negative for anti-PT antibodies in the CHO cell assay and were thus used as negative control in all experiments.

A very low number of gp responders was obtained in all groups immunised at ISS, including the group immunised with the 3rd WHO IS reference vaccine (Table 5). By contrast, the group of gps immunised at the NVI with the 3rd WHO IS reference vaccine showed a higher response, both in terms of number of responders and antibody titres (Table 6). A dose-response curve was found for the gps immunised with vaccines C and D, as well as with the reference vaccine at NVI (Table 6).

To determine whether the low number of responders obtained at ISS was due to a problem of sensitivity of the CHO assay or reflected a real lack of immunological response, all sera were re-tested using a more sensitive PT-ELISA test.

The precision of the method was calculated using the wP gp positive and negative control sera. The assay had a good repeatability and intermediate precision and was considered reliable (data not shown).

Sera from non-immunised gps were negative for the presence of anti-PT antibodies also in PT-ELISA.

In the PT-ELISA assay a dose response was seen in all vaccinated groups from both ISS and NVI (Tables 5 and 6). It was found that the anti-PT response (expressed as a score) of the gps immunised with the reference vaccine was higher at NVI than at ISS. Animals having neutralising anti-PT antibodies in the CHO cell assay also showed the highest titres in the PT-ELISA.

The source of the coating antigen strongly affected the results of the PT-ELISA test, as shown by parallel testing of sera from gps immunised with vaccine C using either the NVI in house preparation of purified PT or the PT BRP batch 1 as coating antigen. Anti-PT responses were notably higher when using the NVI PT preparation (Table 7).

On the basis of the CHO cell assay results, wP potency could be calculated only for vaccines C and D (Table 8). On the

basis of the PT-ELISA titres, a wP potency was calculated for all vaccines (Table 8). However, no correlation was found between the potencies estimated by PT-ELISA and by the MPT.

4.2. PSPT-wC-ELISA

Sera of all animals were assessed by PSPT-wC-ELISA at ISS and NVI, in 3 independent experiments. The mean coefficient of variation for all triplicate tests was 6 % at NVI and 8 % at ISS (Table 9). For immunological assays, e.g. ELISA, a variation coefficient of less than 10 % can be considered satisfactory.

Pre- and post-experiment serum samples obtained from sentinel animals in both laboratories were tested for the presence of *B. bronchiseptica* and *B. parapertussis* antibodies as recommended by the Working Group on Health Monitoring of Rodent and Rabbit Colonies of the Federation of European Laboratory Animal Science Associations (FELASA) [26]. All sera were found negative for *B. parapertussis*, while 2 NVI post-experiment sera and one ISS pre-experiment serum were found positive for *B. bronchiseptica*. The ISS pre-experiment serum had a bordeline absorbance (i.e. positive in the 1st assay but negative in the repeat assay). Nevertheless, the positivity of the 2 NVI sera did not bias the results of this study because only serum results above the cut-off value (based on the negative control sera) were taken into account.

The humoral response of gps measured by PSPT-wC-ELISA showed that in all cases a good dose response curve was obtained, both for gps immunised with the reference vaccines and for those immunised with the various wP vaccines (Table 9).

When comparing the antibody response of the gps immunised with the reference vaccine in the 2 laboratories, it is evident that higher levels of antibodies against the wP bacteria antigens were obtained in the gps vaccinated at ISS.

Potencies estimated with PSPT-wC-ELISA correlated very well with those obtained in the MPT for vaccines A, C and D (Table 8) while a higher potency was obtained by PSPT for vaccine B. However, these results should be seen in the context of the limited amount of *in vivo* data available and the poor reproducibility of the Kendrick test [6].

5. DISCUSSION

The results show that gps immunised with wP vaccines are able to develop an antibody response both against PT and against the other antigens present on the surface of *B. pertussis* strain 18323.

PT is the most virulent antigen characterised for *B. pertussis* [27]. Anti-PT antibodies are protective as demonstrated by the protection conferred by acellular pertussis vaccines which contain PT as the only pertussis antigen [28].

Because wP vaccines were shown to induce anti-PT antibodies in humans and in animals [18, 19, 28], the CHO cell culture assay developed and standardised in 1985 by Gillenius *et al.* [19] appeared to be a good approach for wP vaccine serological potency assay. This *in vitro* assay was already used in clinical trials to determine the titre of anti-PT neutralising antibodies in human sera [29, 30]. It was also found suitable to measure anti-PT antibodies in animal sera [19].

This study showed that gp sera decreased the sensitivity of the assay by affecting the morphology and inducing the elongation and aggregation of the CHO cells. Although the original description of the method includes the use of gp serum, neither a reference to such effects nor an explanation could be found in the literature or during this study. Other interfering activity in human serum samples in a CHO cells pertussis serology assay has been reported by Østergaard *et al.* [31].

The CHO cell assay gave consistent results with low intra-assay and inter-assay variability. The assay sensitivity was however poor. This could be one of the reasons explaining the very low number of responder gps detected by this method.

The different combined vaccines used in this study induced a neutralising anti-PT response. However, the intensity of the responses varied and did not necessarily correlate with the potency estimated by the MPT (example: vaccines D and C). This could be explained by the ability of the MPT to detect a global serological response to the vaccine strain, while the CHO cell assay only detects the response against the PT antigen. Differences in either the vaccine strains used or the manufacturing process might account for the differences in the anti-PT response observed with the CHO cell assay.

When the presence of anti-PT antibodies was checked by PT-ELISA, it was found that the number of responder gps was higher than observed with the CHO cell assay. The results of PT-ELISA need however to be interpreted carefully. First, because PT-ELISA, unlike the CHO cell assay, does not only detect neutralising antibodies. Second, because the results obtained by PT-ELISA strongly depend on the PT preparation used as coating antigen.

Interestingly, when the same toxin was used as coating antigen in the CHO cell assay and in the PT-ELISA, the potency estimates of the 2 methods correlated well.

However, both the CHO cell assay and PT-ELISA showed that the anti-PT response is strongly influenced by several factors. The anti-PT antibody response induced by the different wP vaccines did not always correlate with the potency measured by the MPT.

In contrast, a good correlation was found between the potencies estimated by the MPT and the PSPT-wC-ELISA. The results obtained in the gp model confirmed those of previous studies on PSPT-wC-ELISA in mice [10]. The differences between the immunisation protocols of the 2 laboratories did not impact on the outcome of the PSPT-wC-ELISA but had a strong influence on the anti-PT response estimated by either the CHO cell assay or the PT-ELISA. This may be related to the use of the same *B. pertussis* strain specified in the Ph. Eur. for the MPT [1] as coating antigen for the PSPT-wC-ELISA plates.

A good intra- and inter-laboratory reproducibility in the 2 participant laboratories was found with the PSPT-wC-ELISA. Even if the anti *B. pertussis* immune response induced by the WHO 3rd IS reference wP vaccine was much higher in gps from ISS than from NVI, the overall potency estimates of the vaccine were not influenced.

It is important to note that the animals used in the present study were of SPF quality and checked by the breeder to be negative for *B. bronchiseptica*. The use of such animals for PSPT testing should be prescribed in future studies because cross reactivity between species of *Bordetella* (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*) has been shown [32, 33]. According to Kloos *et al.* [34], the DNA homology between *B. pertussis* and *B. parapertussis* is about 88-94 % and is about 72-93 % between *B. pertussis* and *B. bronchiseptica*. Additionally, anti-*B. parapertussis* and *B. bronchiseptica* antibodies could bind to a wP coat antigen and consequently bias the results. Therefore, sentinel animals have to be housed in the same animal room to monitor the microbiological status both during and at the end of the experiment. The data from the present study indicate the prevalence of low levels of non-vaccine induced antibodies that are able to bind to antigens of *Bordetella*. Nevertheless, they did not bias the results because only sera results above the cut-off value based on the negative control sera were elaborated.

6. CONCLUSIONS

From the results of this study, it is evident that wP vaccines induce a dose-dependent production of anti-PT antibodies in gps. It is difficult to measure the neutralising anti-PT antibodies by the CHO cell assay because gp sera at low dilutions induce elongation and aggregation of the cells. An overall anti-PT response to wP vaccines can be determined by PT-ELISA. However, no correlation could be found between the wP vaccine potency determined by the MPT and the anti-PT antibody response.

In contrast, wP vaccines induce a dose-dependent antibody response in gps, that is detectable by PSPT-wC-ELISA. This assay has been shown to be easily transferable, provided that the same batch of the critical reagent, i.e. the wP coating antigen, is accessible to laboratories. Intra- and inter-laboratory assay variability resulted within CVs of 20 %. PSPT-wC-ELISA discriminates between vaccine batches of different potencies, and correlates well with the MPT.

PSPT based on the wC-ELISA is a promising alternative method for the batch release potency testing of wP vaccines for which production consistency has already been demonstrated by the classical challenge test.

However, additional validation data are needed to support the establishment of PSPT-wC-ELISA in gps as a compendial alternative method to the MPT. It is expected that such data will be generated in a future international collaborative study involving worldwide vaccine quality control laboratories both from the private (manufacturers) and the public (OMCLs) sectors. The study will be run under the aegis of the Biological Standardisation Programme (BSP), a Council of Europe-EDQM and European Commission co-sponsored research programme, possibly with additional support from other Organisations.

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8. ABBREVIATIONS

AVG: average; BRP: Biological Reference Preparation; BSP: Biological Standardisation Programme; CHO: Chinese Hamster Ovary; CV: coefficient of variation; D: Diphtheria; EC: European Commission; ECVAM: European Centre for the Validation of Alternative Methods; EDQM: European Directorate for the Quality of Medicines and HealthCare; ELU: ELISA Units; FELASA: Federation of European Laboratory Animal Science Associations; gp: guinea pig; Hib: *Haemophilus influenzae* type b; IHCP: Institute for Health and Consumer Protection; IPV: Inactivated Polio Virus; IS: International Standard; ISS: Istituto Superiore di Sanità; IU: International Units; JRC Joint Research Centre; MCC: Minimal Clustering Concentration; MPT: Mouse Protection Test; NIBSC: National Institute for Biological Standards & Control; NVI: Nederlands Vaccin Instituut; OD: optical density; OMCL: Official Medicines Control Laboratories; PBST: Phosphate buffered saline pH 7.4, 0.05% Tween; PCR: polymerase chain reaction; Ph. Eur.: Pharmacopée Européenne/European Pharmacopoeia; PSPT: Pertussis Serological Potency Test; PT: Pertussis Toxin; SD: Standard Deviation; SPF: Specific Pathogen Free; T: Tetanus; US-FDA: United States Food and Drug Administration; wC-ELISA: whole cell ELISA; WHO: World Health Organization; wP: whole cell Pertussis.

9. REFERENCES

[1] Assay of pertussis vaccine, general chapter 2.7.7. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe; 2008.

[2] Recommendations for whole-cell pertussis vaccine; Technical Report Series No. 941, Annex 6. WHO Expert Committee on Biological Standardization; 2007.

[3] Kendrick PL, Eldering G, Dixon MK, Misner J. Mouse protection tests in the study of pertussis vaccine. *Am J Public Health Nations Health* 1947;**37**(7):803-10.

[4] Vaccination against whooping-cough. Final Report to the Medical Research Council. *Brit Med J* 1959;**1**(5128):994-1000.

[5] Xing D, Das RG, O’Neill T *et al.* Laboratory testing of whole cell pertussis vaccine: a WHO proficiency study using the Kendrick test. *Vaccine* 2001;**20**(3-4):342-51.

[6] van Straaten-van de Kappelle I, van der Gun JW, Marsman FR *et al.* Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. *Biologicals* 1997;**25**(1):41-57.

[7] Canthaboo C, Xing D, Douglas A *et al.* Investigation of an aerosol challenge model as alternative to the intracerebral mouse protection test for potency assay of whole cell pertussis vaccines. *Biologicals* 2000;**28**(4):241-6.

[8] Xing D, Canthaboo C, Corbel MJ. Development of a nitric oxide induction assay as a potential replacement for the intracerebral mouse protection test for potency assay of pertussis whole cell vaccines. *Dev Biol Stand* 1999;**101**:95-103.

[9] van der Ark A, van Straaten-van de Kappelle I, Akkermans A *et al.* 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**(3):233-42.

[10] van der Ark A, van Straaten-van de Kappelle I, Ölander RM *et al.* The Pertussis Serological Potency Test. Collaborative study to evaluate replacement of the Mouse Protection Test. *Biologicals* 2000;**28**(2):105-18.

[11] Sesardic D, Winsnes R, Rigsby P *et al.* Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines - extended study: correlation of serology with in vivo toxin neutralisation. *Pharmaeuropa Bio* 2004;2003;(2):69-76.

[12] Winsnes R, Hendriksen C. Collaborative study for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use. Part 1. *Pharmeuropa Bio* 2000;(1):83-124.

[13] Assay of diphtheria vaccine (adsorbed), general chapter 2.7.6. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe; 2008.

[14] Assay of tetanus vaccine (adsorbed), general chapter 2.7.8. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe; 2008.

[15] European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Strasbourg, France: Council of Europe; ETS No. 123.18.III.1986.

[16] European Commission Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes 86/609/EEC. *Official Journal of the European Communities* 1986:L358, 1-29.

[17] Ausiello CM, Lande R, Urbani F *et al.* Cell-mediated immunity and antibody responses to *B. pertussis* antigens in children with a history of pertussis infection and in recipients of an acellular pertussis vaccine. *J Infect Dis* 2000;**181**(6):1989-95.

[18] Canthaboo C, Williams L, Xing D *et al.* Investigation of cellular and humoral immune responses to whole cell and acellular pertussis vaccines. *Vaccine* 2000;**19**(6):637-43.

[19] Gillenius P, Jaatmaa E, Askelof P *et al.* The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. *J Biol Stand* 1985;**13**(1):61-6.

[20] Xing D, Gaines Das R, Newland P *et al.* Third International Standard for pertussis vaccine: international confirmation study of activity of British Standard for pertussis vaccine, coded 66/303. *Biologicals* 2001;**29**(2):133-6.

[21] Validation of analytical procedures: Text and Methodology. ICH; Q2 (R1). June 1997 [guideline].

[22] Coecke S, Balls M, Bowe G *et al.* Guidance on good cell culture practice. A Report of the second ECVAM task force on good cell culture practice. *Altern Lab Anim* 2005;**33**(3):261–87.

[23] Boot R, Bakker RHG, Thuis H *et al.* An enzyme-linked immunosorbent assay (ELISA) for monitoring guinea pigs and rabbits for *Bordetella bronchiseptica* antibodies. *Lab Anim* 1993a;**27**(4):342-9.

[24] Boot R, Bakker RHG, Thuis H *et al.* An enzyme-linked immunosorbent assay (ELISA) for monitoring rodent colonies for *Streptobacillus monoliformis* antibodies. *Lab Anim* 1993b;**27**(4):350-7.

[25] Statistical analysis of results of biological assays and tests, general text 5.3. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe; 2008.

[26] Nicklas W, Baneux P, Boot R *et al.* Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. *Lab Anim* 2002;**36**(1):20-42.

[27] Carbonetti NH, Artamonova GV, Andreasen C *et al.* Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of *Bordetella pertussis* infection of the respiratory tract. *Infect Immun* 2005;**73**(5):2698-703.

[28] Taranger J, Trollfors B, Bergfors E *et al.* Immunologic and epidemiologic experience of vaccination with a monocomponent pertussis toxoid vaccine. *Pediatrics* 2001;**108**(6):E115.

[29] Meade BD, Lynn F, Reed GF *et al.* Relationships between functional assays and enzyme immunoassays as measurements of responses to acellular and whole-cell pertussis vaccines. *Pediatrics* 1995;**96**(3 Pt 2):595-600.

[30] Greco D, Salmaso S, Mastrantonio P *et al.* A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. *N Eng J Med* 1996;**334**(6):341-8.

[31] Østergaard E, Sørensen C, Nielsen L *et al.* Elimination of interfering activity in serum samples in the Chinese hamster ovary pertussis serology assay. *Clin Vaccine Immunol* 2008;**15**(8):1244-7.

[32] Musser JM, Hewlett EL. Peppler MS et al. Genetic diversity and relationships in populations of Bordetella spp. *J Bacteriol* 1986;**166**(1):230-7.

[33] Holby N, Hertz JB, Andersen V. Cross-reactions between *Bordetella pertussis* and twenty-eight other bacterial species. *Acta Pathol Microbiol Scand* [B]1976;**84B**(6):395-400.

[34] Kloos WE, Dobrogosz WJ, Ezzell JW *et al.* DNA-DNA hybridization, plasmids and genetic exchange in the genus *Bordetella*. In: Manclark CR, Hill JC, editors. Third International Symposium on Pertussis; U.S. Department of Health, Education and Welfare Publication no. 79-1830. U.S. Government Printing Office; Washington DC: 1979:70-80.

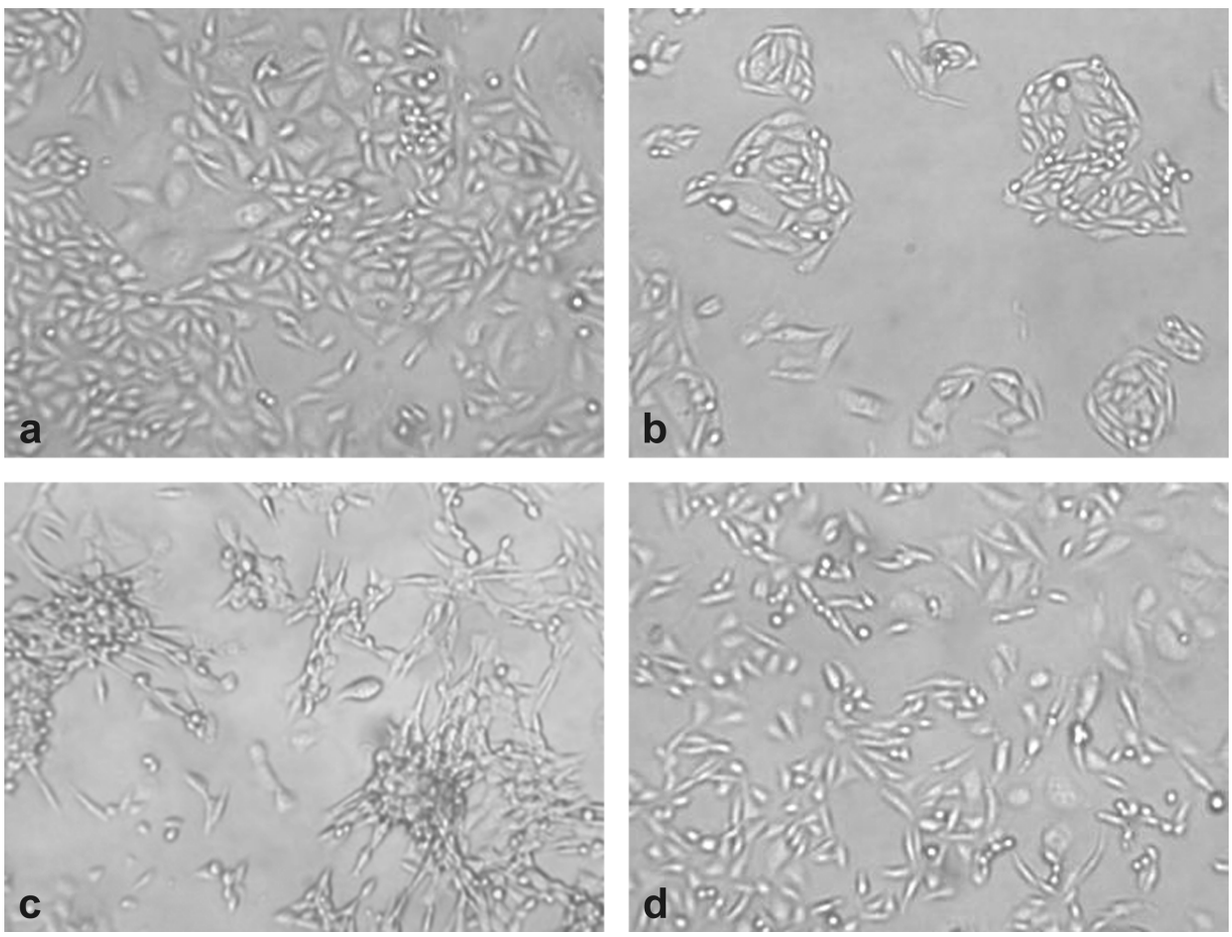

Figure 1. - *CHO cell clusters in the presence of PT and altered morphology in the presence of low dilution of gp serum. a: untreated CHO cells b: CHO + PT c: CHO + gp serum (1:4 dilution) d: CHO + gp serum (1:64 dilution)*

Table 1 - *Summary of information on vaccines used for immunisation of gps at ISS and NVI*

Vaccine code	Composition	Estimated wP potency[1]
A	DTwP	8 IU /0.5 ml[2] (not provided)
B	DTwP-Hib	4 IU /0.5 ml[2] (2 - 9)
C	DTwP	13 IU / 0.5 ml[3] (7 - 26)
D	DTwP-IPV (expired)	4 IU /1 ml[3] (1-13)
Reference Vaccine	wP	46 IU / ampoule

[1] IU/single human dose with 95% CL in parentheses
[2] Estimated by the manufacturer
[3] Estimated by NVI
D = Diphtheria
T = Tetanus
Hib = *Haemophilus influenzae* type b
IPV = inactivated poliomyelitis vaccine
wP = whole cell pertussis

Table 2 - *Specifications of the gps*

Specifications	ISS	NVI
Breeder	Charles River	Harlan Netherland BV
Guinea pig strain	Dunkin Hartley Crl:(HA)BR	HsdPoc.doc
Sex	Female	Female / male (50/50)
Weight at start	255-305 g	248-419 g
Injection site	Lateral to linea alba	Groin fold
Volume of injection	1 ml	0.5 ml

Table 3 - *Immunisation information: doses of vaccine expressed as volume and in IU (injection volume corresponded to 1 ml at ISS and 0.5 ml at NVI)*

ISS			NVI		
Vaccine	Dose		Vaccine	Dose	
	µl[1]	IU		µl[2]	IU
Reference	1000	9.2	Reference	500	9.2
	666	6.1		-	-
	333	3.1		167	3.1
	111	1.0		56	1.0
	37	0.3		19	0.3
Vaccines A and B	1000		Vaccines C and D	500	
	666			-	
	333			167	
	111			56	
	37			19	

[1] Injection volume adjusted to 1 ml by addition of saline
[2] Injection volume adjusted to 0.5 ml by addition of saline

Table 4 - *CHO cell assay precision*

Titration	Repeatability[1] (intra-assay precision)	Intermediate precision[2] (intra-laboratory precision)
JNH-12	1:200 – 1:400	1:200 – 1:400
Anti – wP gps pool serum	1:128 - 1:256	1:128 - 1:256
Negative gps serum	0	0
BRP batch 1 PT MCC (pg/ml)	1:78 – 1:156	1:78 – 1:156

[1] Results from 6 plates

[2] Results from 19 assays (30 assays for PT titration)

Table 5 - *Anti–PT serological responses in terms of number of gp responders and scores of titres determined by CHO cell assay or PT ELISA (IgG). Data for vaccines used at ISS*

Vaccine	dose		CHO cell assay		ELISA	
	µl	IU	N of Responders/total N	Mean score	N of Responders/total N	Mean score
Reference	1000	9.2	1/12	0.2	7/12	2.2
	333	3.1	3/12	0.4	5/12	2.9
	111	1.0	1/12	0.1	2/12	0.5
	37	0.3	0/12	0	NT	-
A	1000		4/12	1.1	12/12	5.4
	333		2/12	0.5	12/12	4.3
	111		0/12	0	7/12	1.0
	37		0/12	0	0/12	0
B	1000		1/12	0.3	12/12	3.0
	333		0/12	0	9/12	0.9
	111		1/12	0.2	1/12	0.5
	37		0/12	0	NT	-

NT = not tested

Table 6 - *Anti–PT serological response in terms of number of gp responders and scores of titres determined by CHO cell assay or PT ELISA (IgG). Data for vaccines used at NVI*

Vaccine	dose		CHO cell assay		ELISA	
	µl	IU	N of Responders/total N	Mean score	N of Responders/total N	Mean score
Reference	500	9.2	9/12	2.5	10/12	6.1
	167	3.1	11/12	2.0	10/12	5.9
	56	1.0	2/12	0.4	4/12	1.6
	19	0.3	NT	-	1/12	0.1
C	500		3/12	0.4	8/12	3.5
	167		0/12	0	2/12	0.5
	56		0/12	0	0/12	0
	19		0/12	0	NT	-
D	500		6/12	1.2	10/12	6.2
	167		1/12	0.3	7/12	2.3
	56		0/12	0	2/12	0.1
	19		0/12	0	NT	-

NT = not tested

Table 7 - *PT-ELISA results of vaccine C using 2 different PT preparations as coating antigen*

Dose (µl)	PT BRP Batch 1		NVI in house PT	
	N of Responders/total N	Mean score	N of Responders/total N	Mean score
500	9/10	2.7	9/10	5.0
167	2/11	0.4	10/11	3.0
56	2/12	0.5	8/11	1.2

Table 8 - *PSPT of wP vaccines based on the anti-PT (CHO cell assay or anti-PT ELISA) or anti-whole B. pertussis (PSPT-wC-ELISA) antibody responses*

Vaccine	Composition	Potency IU/dose (95% CL)			
		MPT	CHO cell assay	PT- ELISA*	wC-ELISA*
A	DTwP	8	Not calculable	32 (13 -216)	8 (6 – 10)
B	DTwP-Hib	4 (2 - 9)	Not calculable	6 (2 – 16)	13 (10 - 15)
C	DTwP	13 (7 – 26)	1 (0.1 – 2)	1.5 (0.4 – 3)	9 (5 – 17)
D	DTwP-IPV	4 (1 -13)	2 (1 – 8)	9 (4 – 16)	4 (2 – 6)

*Weighted mean

Table 9 – *Anti-wP humoral response (IgG) by the PSPT-wC-ELISA: results for vaccines used at ISS (A) and at NVI (B)*

(ISS)

		ELISA concentrations in ELU/ml							
		Vaccine A			Vaccine B			Reference Vaccine	
Test code	Dose	1000 µl	333 µl	111 µl	1000 µl	333 µl	111 µl	1000 µl	333 µl	111 µl
ISS-1		316	236	117	768	343	143	357	141	67
ISS-2		316	215	109	628	300	122	324	128	65
ISS-3		410	268	124	856	340	147	428	157	71
ISS	AVG	347	240	117	751	328	137	370	142	69
	CV	16%	11%	6%	15%	7%	10%	14%	10%	5%

(NVI)

		ELISA concentrations in ELU/ml								
		Vaccine C			Vaccine D			Reference Vaccine		
Test code	Dose	500 µl	167 µl	56 µl	500 µl	167 µl	56 µl	500 µl	167 µl	56 µl
NVI-1		212[2]	47[1]	34	45	28	10[2]	97	46	31
NVI-2		210[2]	47[1]	35	49	29	8[2]	103	52	31
NVI-3		193[2]	45[1]	31	45	26	8[2]	91	45	29
ISS-1		297[1]	48	31	54	32	9[1]	89	41	25
ISS-2		291	53	35	54	29	11	81	41	28
ISS-3		277	51	29	57	30	14	72	37	24
Overall	AVG	247	48	32	51	29	10	89	43	28
	CV	19%	6%	8%	10%	7%	26%	13%	12%	11%
NVI	AVG	205	46	33	46	28	8	97	47	30
	CV	5%	3%	7%	5%	6%	15%	6%	8%	4%
ISS	AVG	289	51	32	55	30	11	80	40	26
	CV	3%	5%	9%	3%	6%	24%	10%	6%	8%

[1] One serum sample missing
[2] Two serum samples missing
AVG = average, CV = coefficient of variation