Collaborative Study for the Standardisation of the Histamine Sensitizing Test in Mice and the CHO Cell-based Assay for the Residual Toxicity Testing of Acellular Pertussis Vaccines

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ABSTRACT

The European Pharmacopoeia (Ph. Eur.) and the World Health Organisation (WHO) require the performance of extensive quality and safety control testing before the release on the market of vaccine products for human use. Safety testing with regard to residual pertussis toxin (PT) in acellular pertussis combination vaccines is performed through assessment of fatal sensitisation of mice to histamine challenge by the vaccine product under test.

Currently, use of different in-house procedures and no requirement for the inclusion of a standard reference in each assay render comparisons of results obtained for identical vaccine batches between different control laboratories very difficult. At the initiative of the European Directorate for the Quality of Medicines and HealthCare (EDQM), an international collaborative study was organised for the standardization of the Histamine Sensitizing Test (HIST) in mice and the Chinese Hamster Ovary (CHO)-cell-based assay (performed at the bulk product level) for the residual toxicity testing of acellular pertussis vaccines or acellular pertussis-based combination vaccines. The study was run under the aegis of the Biological Standardisation Programme, jointly supported by the Council of Europe and the European Commission under the project code BSP076.

Ten (10) laboratories participated in the study and were requested to perform 3 independent Histamine Sensitizing Tests in mice and to report results of the lethal end-point measurement as prescribed by the Ph. Eur. monographs. Some of them also reported data from an in-house validated CHO-cell-based assay. In addition, some of the laboratories reported concomitantly data obtained by measurement of the drop in temperature induced after the histamine challenge, a method currently under investigation to be added as an alternative end-point for the HIST in the Ph. Eur. monographs for acellular pertussis-based combination vaccines in order to alleviate animal suffering (in application of the 3Rs principle).

Based on the results of the collaborative study, a potency of 7500 IU/vial (International Units per vial) was assigned to the current Ph. Eur. Biological Reference Preparation (BRP) for PT. The results of the study also show that 1) intra- and inter-laboratory variations can be improved by the use of a validated standard operating procedure; 2) inclusion in each assay of a standard reference sample, calibrated in IU, can increase comparability of results among laboratories and thus help to reduce repeat testing; 3) a correlation between mortality data and temperature data was observed although, due to the limited number of data sets and the lack of a common method for the temperature end-point, further investigation of this point is required; 4) the CHO-cell-based assay did not yield comparable results and further standardisation of the assay procedure may be investigated in a follow-up project.

KEYWORDS

Histamine Sensitizing Test, acellular pertussis vaccine, pertussis toxin, biological standardisation, collaborative study, CHO-cell assay, European Pharmacopoeia.

1. INTRODUCTION

In Europe, multivalent acellular pertussis (aP) vaccines have been widely used for some time and have a known record of clinical safety. Their compositions differ in terms of aP components and with regard to their combination with other antigens. These vaccines are tested by manufacturers and are also submitted to Official Control Authority Batch Release (OCABR) which requires independent testing by an Official Medicines Control Laboratory (OMCL), notably for residual toxicity of PT and reversion to toxicity of the pertussis toxoid, on every new final bulk. The Ph. Eur. monographs for aP vaccines and for combined vaccines containing aP antigens [1] require that the residual toxicity of PT in a vaccine determined by the Histamine Sensitizing Test (HIST) does not exceed that of batches shown to be safe in clinical studies. This normally means that following histamine challenge, either no mouse should die or no more than 5% of the total number of mice die with a combined result in the case where re-testing was carried out. At the level of the purified antigenic fraction, a validated test based on the clustering effect of the toxin on CHO cells may be used instead of the HIST in mice.

Currently, a reference toxin and/or reference vaccine is not required to be included in the HIST in parallel with the vaccine under test and therefore comparability of assay results between assays and between laboratories is very difficult. Moreover, safe levels in terms of mass units or IU of PT in marketed vaccines are unknown and therefore safety limits cannot be set.

So far, Japan is the only country where a pertussis vaccine (whole cell) is used as a reference. A limit of 0.2 histamine sensitizing units (HSU) per dose has been established, which is equivalent to 1.09 IU of PT per single human dose (SHD) of Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccines according to in-house calibration against the 1st International Standard (IS) for PT (JNIH-5). For other types of combination, limits in terms of IU content of active PT have not yet been set.

In recent years, some OMCLs and manufacturers have experienced problems with the HIST as it is performed

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currently, especially for multi-component combination vaccines. The current test was originally established for DTaP vaccines and the interactions between the various components in multi-component vaccines is not known, nor is their influence on the HIST results. Indeed, some batches of combined vaccines were reported to have passed the test at the level of the individual components but repeatedly failed the test at the level of the final lot. More details about such issues were presented at the annual OMCL meeting in Langen (Germany) in May 2004 [2]. An unexpected interaction between vaccine components and/or formulation agents seemed to have resulted in an apparent increase in reactivity. Discussions on the relevance and suitability of the HIST for aP based combination vaccines are linked to the observations that difficulties in setting up test methods and pass criteria appear to be a recurrent issue with combination vaccines. No data are available on assay sensitivity and its relation to clinical effect: sensitivity or detection limits of the HIST in individual laboratories are unknown due to the lack of appropriate controls.

It was therefore proposed to evaluate whether a better standardisation of the HIST procedure and the application of the principles of biological standardisation for relative measurements (e.g. by including a reference standard calibrated in IU in each assay) may improve sensitivity assessment, repeatability and reproducibility of the test.

Consequently, a project (BSP076) was organised by the EDQM under the aegis of the Biological Standardisation Programme (BSP). The project comprised 2 steps: the 1st step was an enquiry among OMCLs and manufacturers in order to gather information on aP and/or combination vaccines control, and the 2nd step was the organisation of an international collaborative study.

The enquiry was initiated in February 2006 and information on HIST performed between 2003 and 2005 was collected. The results of the enquiry showed that both OMCLs and manufacturers had a similar rate of repeat testing with 11.4% and 12% respectively, which indicated that standardisation of the HIST is very important.

Based on the results of the enquiry, the international collaborative study was organised for the standardisation of the HIST in mice with the lethal end-point read-out as required by the Ph. Eur.

An alternative end-point method based on the reduction in rectal temperature has been successfully used for many years in Japan and more recently a refinement of this method based on dermal temperature measurement has been developed by some European laboratories in order to further reduce animal discomfort. During the preparatory phase of the collaborative study, some participants, who were in the process of validating the method, proposed to perform both measurements in parallel, on the same groups of animals, to allow a direct comparison between both end-points. As the temperature method was not included in the original study plan, it was left to the participant's decision to report data from temperature measurements, in addition to data obtained by the lethal end-point method.

In parallel, the CHO-cell-based assay for residual toxicity testing of pertussis toxoid was also performed by some laboratories. In this case, the assay was used only for the calibration of the BRP as only the reference preparations could be included in the assays due to concerns regarding interference from adjuvants enclosed in the final products.

2. AIMS

The principal aim of the collaborative study was to calibrate the Ph. Eur. BRP batch 1 for PT (EDQM Catalogue No. Y0000021) in terms of the 1st WHO IS for PT and to express its activity in IU. Through this, it was expected to establish the assay sensitivity in each laboratory, to optimise the design of the HIST, including the use of appropriate positive controls in each test and to assess the level of reactivity in HIST for combination vaccines available on the European market with a known history of clinical safety.

Another aim of the project was to assess the sensitivity for PT of the currently used in-house CHO-cell assays and to evaluate the need for further standardisation of this assay for residual toxicity testing in purified antigenic fractions.

3. PARTICIPANTS

A total of ten (10) laboratories, including vaccine manufacturers and OMCLs participated in the study. The list of participating laboratories can be found in section 8. Throughout this report, each participant is identified by a randomly assigned code number from 1 to 10 not related to the order of listing.

All participants performed HIST assays. Among them, 6 laboratories performed the HIST by the lethal end-point method only, 1 by the temperature measurement method only and 3 performed both methods. Five (5) participants also carried out CHO-cell assays.

4. MATERIALS AND METHODS

4.1. Study design

Participants were asked to carry out 3 independent HIST assays including reference preparations and vaccine samples and, where applicable, three independent CHO-cell assays including only the reference preparations.

For HIST, participants were requested to use a standardised procedure specified in the study protocol (see also section 4.3). Each assay was to include the 1st WHO IS for PT, JNIH-5 (referred to as JNIH-5 in this report), the Ph. Eur. BRP batch 1 for PT and, where available, an in-house reference (IHR). Participants were asked to report the raw data to the EDQM for each assay so that all data could be analysed centrally using as far as possible a common method.

4.2. Study design and equipment

4.2.1. Common references and vaccine samples

Two (2) reference preparations as well as 7 commercially available vaccine samples were tested in the study:

- The 1st IS for Pertussis Toxin (JNIH-5) is a freeze-dried purified preparation of pertussis toxin prepared by the Biken Kanonji Institute in Japan in 1984, currently held at the National Institute for Biological Standards and Control (NIBSC). Each ampoule has a protein nitrogen (PN) content of 10 µg. In an international collaborative study run by the WHO in 2003, JNIH-5 has been assigned a PT activity of 10,000 IU/ampoule based on the HIST [3].
- The Ph. Eur. BRP for PT (EDQM Catalogue No. Y0000021) is a freeze-dried purified preparation of pertussis toxin currently available from the EDQM. The toxin was prepared by affinity chromatography purification. The vials were filled with a nominal volume of 0.5 ml and subsequently freeze-dried. Each vial has been assigned a PT protein content of 50 µg during an international collaborative study run by the EDQM in 2000 [4].
- Seven (7) vaccine samples that can be found on the European market were kindly donated by 2 manufacturers. Six (6) of them had passed the HIST tests and were released whereas 1 of them (sample 7) failed to pass and was not released onto the European market. One (1) of the samples (sample 2) reached its expiry date in March 2008, shortly before the collaborative study started. The participants were given this information beforehand. A detailed composition of the common study samples can be found in Table 1.

Table 1 – *Information on common study samples*

Toxin Standards/References	
1st IS for Pertussis Toxin (JNIH-5)	A freeze-dried purified preparation of pertussis toxin. Each ampoule contains 10.0 µg (protein nitrogen content) and has an assigned unitage of 10,000 IU/ampoule based on HIST.
Ph. Eur. BRP for Pertussis Toxin (Y0000021)	A freeze-dried purified preparation of pertussis toxin. Each vial has an assigned protein content of 50 µg of pertussis toxin.

Vaccine products	
Sample 1	Diphtheria-Tetanus-aP (3-components) vaccine with aluminium hydroxide as adjuvant
Sample 2 *	Diphtheria-Tetanus-aP (2-components)-IPV vaccine with aluminium hydroxide as adjuvant
Sample 3	Diphtheria-Tetanus-aP (3-components)-IPV vaccine with aluminium hydroxide as adjuvant
Sample 4	Diphtheria-Tetanus-aP (3-components)-IPV-HepB vaccine with aluminium hydroxide as adjuvant
Sample 5	Freeze – Dried Hib (only used in combination with other samples)
Sample 6	Diphtheria-Tetanus-aP (5-components)-IPV-Hib vaccine with aluminium phosphate as adjuvant
Sample 7	Diphtheria-Tetanus-aP (5-components)-IPV-Hib vaccine with aluminium phosphate as adjuvant
Sample 8 #	Sample 3 + Hib (sample 5)
Sample 9 #	Sample 4 + Hib (sample 5)
* Sample 2 has reached its expiry date in March 2008	
# Samples 8 and 9 were freshly prepared just before injection	
HepB: hepatitis B; Hib: Haemophilus influenzae type b polysaccharide vaccine; IPV: inactivated poliomyelitis vaccine	

4.2.2. In-house references

Five (5) participants included an IHR for PT. Three (3) of them had an assigned protein content, although the way in which this was determined was not generally specified. Laboratory 1 used an IHR calibrated in terms of JNIH-5 and expressed in IU. Laboratory 2 included a whole cell pertussis vaccine IHR with an assigned activity in terms of HSU and for which no conversion factor to protein content was available.

4.3. Assay methods

4.3.1. HIST lethal end-point

The HIST assays were carried out using mice from local sources. In most assays, participants used 4 doses of each preparation with 10 mice per dose. Groups of mice were injected intra-peritoneally with either 1 ml of serial dilutions of the reference preparations or 1 ml of vaccine samples corresponding to 2 single human doses (SHD), as prescribed in the corresponding Ph. Eur. monographs. Challenge was carried out intra-peritoneally, at day 5 after immunisation, with a solution of 2 mg of histamine base in a volume of 0.5 ml, except for laboratory 7 which used 5 mg (in their 1st assay) or 6 mg (in assays 2 and 3) of histamine base for challenge. The number of surviving animals was recorded at 30 min, 2 hours, and 24 hours after challenge.

4.3.2. HIST temperature end-point

The temperature measurement method was performed by 4 laboratories. The measurement was carried out on the same animals as for the lethal end-point assay except for laboratory 8 who only performed the temperature assay. One single temperature measurement was carried out, either by dermal or rectal measurement, 30 min after histamine challenge.

Laboratory 8, who only performed the temperature measurement method, adapted the challenge procedure in the route, volume and concentration of histamine used for the challenge. An aP toxoid was used as the IHR in the assays.

A summary of assay conditions reported by participants for both HIST methods is given in Table 2a.

4.3.3. CHO-cell assay

Five (5) laboratories carried out CHO-cell assays with the reference preparations, using their in-house methodology and reagents. Details on assay conditions are given in Table 2b. In brief, CHO-cells from different sources were grown in tissue culture medium containing 10% foetal calf serum (FCS) except for laboratory 3 where only 1% FCS was included. Confluent cell monolayers were trypsinized, diluted in culture medium and dispensed into 96-well microtitre plates. Seeding densities ranged from 5000 to 20000 cells/well. Toxin dose-response was determined with PT reference preparations diluted serially in culture medium. After 48h incubation at 37°C in a CO_2 incubator, cells were observed directly for clustering or washed, fixed with formalin or ethanol and stained before microscopic observation. Each well was read for toxin effect and compared to negative control wells containing medium only. The highest dilution giving 100% clustering or at which clusters were observed was recorded, depending on the laboratory's definition.

4.4. Statistical analysis

4.4.1. HIST lethal end-point

The raw data from the HIST were analysed with a parallel line probit model [5]. Samples giving only extreme responses were excluded from the analysis. For each assay, the common slope, the HSD_{50} (dose of preparation giving 50% lethal sensitisation) per preparation and the relative activity of the BRP with respect to JNIH-5 expressed in IU/vial were calculated. The assays were combined by taking the unweighted geometric mean (GM) of the individual assays within laboratory and then by taking the unweighted geometric mean of the means across laboratories.

The geometric coefficient of variation (GCV) and 95% confidence limits (CL) were calculated with the following equations:

$$GCV = \left(e^{S} - 1\right) \times 100\%$$

and

$$CL = e^{m \pm ts/\sqrt{n}}$$

s = standard deviation of the ln-transformed results,

m = the arithmetic mean,

n = the number of results,

t = Student's two-sided 95% value with n-1 degrees of freedom.

4.4.2. HIST temperature end-point

Estimates of the activity from the HIST temperature method were analysed using a parallel line logistic model with

fixed asymptotes of 23°C and 35°C for the dermal method as described previously [6] and fixed asymptotes of 25°C and 37°C for the rectal method. Since the dose range was optimized for the HIST lethal end-point assay, only the 2 lowest doses were included in the analysis except for laboratory 8, assay 1, where no regression could be obtained when using the 2 lowest doses and thus the assay was declared invalid. Responses greater than the 75th percentile of the control responses were considered to be failures of injection and subsequently excluded from the analysis for all references and samples [6].

4.4.2. CHO-cell assay

Estimates of the activity from the CHO assays (A_T) were obtained using the following equation:

$$A_T = A_S \times \frac{P_T}{P_S} \times D^{W_T - W_S}$$

A_S = the assigned activity of the standard (i.e. 10 000 IU/ml),

P_T = pre-dilution factor of the Test sample (i.e. BRP or IHR)

P_S = pre-dilution factor of the Standard,

D = the dilution step (usually 2),

W_T, W_S = the average number of the last well giving complete clustering or showing clustering depending on the laboratory's definition for Test and Standard samples.

5. RESULTS

Ten (10) laboratories submitted results from assays. They are referred to by their code numbers (1 to 10) allocated at random and not necessarily corresponding to the order of listing in the list of participants.

All laboratories carried out HIST lethal end-point assays, except laboratory 8. Laboratory 6 performed 4 assays and the other laboratories performed 3 assays as requested.

Laboratory 7 deviated substantially from the standard protocol. Therefore HIST assay results were analysed including and excluding their data.

No deaths were observed in the negative control groups for any of the assays contributed.

Twenty-three (23) out of the total 28 assays were statistically valid. Two (2) assays showed statistically significant ($P<0.05$) deviations from linearity and 3 assays showed significant ($P<0.05$) deviations from parallelism, although none of these deviations were significant at the 1% level ($P>0.01$). Results have been calculated including and excluding these assays. Since exclusion of the 2 assays from laboratory 5 leaves only 1 valid assay and the results from that assay are rather different compared to other laboratories it was decided to exclude that assay as well. The results have been calculated both including and excluding these assays. In 24 out of 28 HIST lethal end-point assays, all deaths occurred within 2 hours after the histamine challenge. In 1 assay for each of the laboratories 1, 4, 7 and 10, one (1) death was recorded at 24 hours after challenge.

Four (4) laboratories carried out HIST temperature assays (laboratory 7 only included the BRP in this assay) and 5 laboratories also carried out CHO-cell assays.

5.1. HIST lethal end-point assay

Statistical validity, common slope, HSD_{50} estimates for individual assays, the overall GM estimates of the HSD_{50} values and estimates of the relative activity of the BRP to JNIH-5 obtained in the histamine lethal end-point assays are shown in Table 3. High intra-laboratory variability was observed (up to a 4-fold difference between assays in some laboratories). The HSD_{50} estimates also differed significantly between laboratories.

For JNIH-5, HSD_{50} values range from 2.3 to 80.8 ng/mouse or from 2.3 to 40.3 ng/mouse when invalid assays and results from laboratory 7 are excluded, and is on average 12.4 ng/mouse when all results are included and 13.4 ng/mouse when invalid assays are excluded. The inter-laboratory variation is not greatly affected by exclusion of invalid assays.

For the BRP, HSD_{50} values range from 9.6 to 592.5 ng/mouse or from 39 to 207.7 ng/mouse when invalid assays and results from laboratory 7 are excluded, and is on average 78 ng/mouse when all results are included and 89 ng/mouse when invalid assays are excluded. The inter-laboratory variation is somewhat improved by the exclusion of invalid assays.

Five (5) participants also included their IHR in the assays. For laboratory 2, the IHR is a whole cell pertussis vaccine and expressed as HSU/mouse. For laboratory 1, 3, 5 and 10, where purified pertussis toxin preparations were used as IHR, the HSD_{50} geometric mean varied from 4.1 to 79.2 ng/mouse for the HIST assay.

A graphical representation of individual laboratory geometric mean estimates of activity of the BRP with respect to JNIH-5 is given in Figure 1. Values range from 2650 to 24706 IU/vial, representing almost a 10-fold difference. The average relative activity is estimated to be 7978 IU/vial when all assays are included and 7500 IU/vial when invalid assays are excluded. The inter-laboratory variation is not greatly affected by the exclusion of invalid assays.

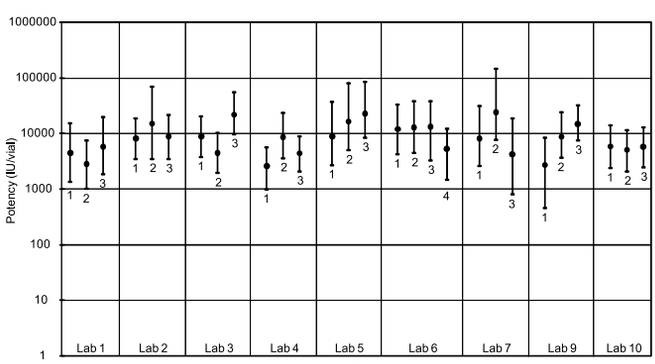

Figure 1 – *Estimated potencies and 95% confidence intervals of BRP Batch 1 with respect to JNIH-5 (IS) in IU/vial*

5.2. HIST temperature assay

Four (4) laboratories submitted results from the temperature assays. Laboratories 1, 7 and 8 performed the dermal method and laboratory 2 performed the rectal method. Laboratory 7 only provided temperature data for the BRP but no data for JNIH-5, so it was not possible to calculate a relative activity. Moreover, the data for the BRP for this laboratory showed no significant regression so the method does not seem to work very well for this laboratory. The laboratory commented that they were still in the process of developing the dermal temperature method. Assay 3 from laboratory 1 and assay 1 from laboratory 8 also showed no significant regression so no relative activities could be calculated. The results of the remaining 7 assays from laboratories 1, 2 and 8 are listed in Table 5. The individual laboratory GM of the estimated activity of the BRP with respect to JNIH-5 ranged from 2990 IU/vial in laboratory 1 to 13794 IU/vial in laboratory 8 and showed an overall average of 7924 IU/vial.

5.3. CHO-cell assay

In this collaborative study, the CHO-cell assay was used only for calibrating the BRP against JNIH-5, but not for evaluating its ability to estimate residual PT toxicity in aP products, as

final products cannot be investigated in the CHO-cell assay, adjuvants and formulation excipients being toxic to the cells.

Five (5) laboratories submitted results from the CHO-cell assays (Lab 1, 3, 4, 5 and 8), each having performed 3 assays. Results are summarized in Table 4.

The laboratory GM of relative activity of the BRP with respect to JNIH-5 varies from 1039 IU/vial in laboratory 8 to 7300 IU/vial in laboratory 1. The overall geometric mean of the laboratory means is 3221 IU/vial with 95% confidence limits of 1272 and 8156 IU/vial, which is about a factor of 2.5 above and below the estimated activity. This value is not in agreement with the results of the *in vivo* assays.

5.4. Estimation of residual toxicity in DTaP based combination vaccines

A total of 8 vaccine products were included in both HIST lethal end-point and the temperature assays. Estimates of relative activity for these products are presented in Table 3 for the lethal end-point and Table 5 for the temperature end-point assays.

In the HIST lethal end-point assays, most of these samples gave non-response values, so no quantification was possible. However, samples 6 and 7 showed the highest response in general as evidenced by the fact that 7 and 5 of the 10 laboratories recorded positive results in at least 1 assay for samples 6 and 7 respectively. The estimated activity ranged from 0.44 to 2.3 IU/SHD for sample 6 and 0.52 to 2.08 IU/SHD for sample 7, except assay 2 from laboratory 6 where an activity of 7.69 IU/SHD was observed for sample 6. For unclear reasons assay 3 in laboratory 5 showed positive responses for most samples which may suggest that mice used in this assay were more sensitive than in other assays.

Only limited data were available (3 laboratories) from the HIST temperature assays. It appeared that lower estimates for all the test samples were obtained from laboratory 1 in comparison with laboratories 2 and 8. However, the estimated relative activities of the test samples to JNIH-5 (7 of 9 assays) were generally below 1.7 IU/SHD. On average, samples 6 and 7 gave the highest activity which is consistent with the results from the HIST lethal end-point assays.

6. DISCUSSION

The study was set up to allow the calibration of the Ph. Eur. BRP for PT in IU for use in the HIST as prescribed in the Ph. Eur. monographs for aP and aP-based combination vaccines [1]. It was also designed to determine whether optimisation and standardisation of the HIST assay procedure and inclusion of appropriate positive controls in each assay could reduce inter-assay and inter-laboratory variability and increase comparability. In addition, participants also reported results for the reference preparations using the *in vitro* CHO-cell assay according to their in-house procedure. Finally, several marketed vaccines with a known history of safety were provided in order to give information on the levels of PT in both the HIST lethal end-point and the temperature assay. As an additional end-point, the results of temperature measurements in HIST were compared to those of the compendial method i.e. the lethal end-point. Results from the study suggest a possible positive relationship between the 2 methods but further confirmation is needed with more experimental data.

6.1. Calibration of the Ph. Eur. BRP in IU

The Ph. Eur. BRP batch 1 for PT was established in 2000 [4]. At that time, no IS was available for the calibration of PT activity in IU. In the establishment study, the BRP was assigned a PT content in protein mass units and its LD_{50} (dose of preparation giving 50% of lethal sensitisation) was determined. Meanwhile, the 1st IS for PT (JNIH-5) was established in 2003 and the discrepancy between mass unit content of PT and bioactivity was clearly demonstrated [3, 8, 9]. It appeared therefore essential to calibrate the BRP in terms of its bioactivity in order to improve inter-assay and inter-laboratory comparability.

Based on the results from the HIST lethal end-point assay, the Ph. Eur. BRP for PT can be assigned a potency of 7500 IU/vial for determination of residual PT by HIST in routine testing of batches of aP and aP-based combination vaccines.

Overall GM estimates of relative activity of the BRP in terms of JNIH-5 are apparently consistent between the HIST lethal end-point and the temperature methods, although the HIST temperature method gives larger variation between laboratories due to the small number of data sets contributed and also the lower value obtained by laboratory 1. However, due to the limited data available (only 3 laboratories contributed temperature data) and to the fact that the study protocol was not primarily designed for the temperature measurement method, no final conclusion can be drawn on this important point solely from the results of this study.

In CHO-cell assays the overall GM estimates for the BRP are approximately 2.5-fold lower than that from both HIST methods, contrary to what was observed in previous studies [9] where both values appeared similar. The reason for this discrepancy is unknown at present.

6.2. Standardisation of the HIST lethal end-point assay

In the present study, all participants reported mortality data except laboratory 8 who reported temperature data only. According to the assay information reported by participants, all laboratories included a negative control group in each assay. However, half of the laboratories did not include or did not report the inclusion of a reference or positive control in each assay. Therefore it is likely that no validity criteria are being set regarding the assay sensitivity in these laboratories. A careful review of the results from this study shows that only in 4 out of the 22 valid assays reported, the mouse sensitivity required by the current Ph. Eur. monographs was achieved. This observation may be linked to the fact that the intra-peritoneal immunisation route was selected for the study instead of the intra-veinous route prescribed in the monographs.

Furthermore, the results from this study suggest that assay sensitivity can largely vary between assays within the same laboratory. Therefore it would be important to define an acceptable assay sensitivity range through a validation stage in each laboratory. Control of assay sensitivity might be achieved by including, in each assay, a reference or a positive control at a suitable dose chosen from the linear region of the dose-response curve to give a reasonable positive response.

The various IHRs show even greater variability between laboratories than JNIH-5 and the BRP, thus confirming previous findings that equivalent mass does not necessarily give equivalent bioactivity [8, 9]. It is known that sensitivity of HIST can be affected by mouse strain and various environmental factors also including feeding and hydrating conditions [7]. The GM of absolute HSD_{50} values for the same reference preparation differ between laboratories by more than 11-fold for JNIH-5 and 14-fold for the BRP when all assays are taken into account. Therefore, it may be difficult to standardise the HSD_{50} values to allow direct comparison of test results on vaccine samples over different laboratories.

However, comparisons of relative activities between the same preparations are significantly more consistent between laboratories than the estimates of "absolute" response, with a reduction of GCV from >100% to approximately 50% when the activity of the BRP is expressed relative to the IS which is comparable with the results from previous similar

studies [3, 9]. Thus the results of this study indicate that "absolute" measurement carried out in each individual assay and by different laboratories for the same sample may have different meanings and the comparability of results both between assays and between laboratories could be substantially improved by using relative estimates to a common reference preparation, e.g. the BRP, included in parallel with the test sample in each assay, instead of absolute percentage of death as is the case at present.

In this study, a total of 5 different mouse strains were used by the different participants. Five (5) laboratories used a common mouse strain (CD1) and the range of HSD_{50} in these laboratories varied from 5.0 to 22.3 ng/mouse. The other mouse strains were common to maximally 2 laboratories and therefore no conclusion can be drawn from the data obtained in this study on the effect of difference in mouse strain on the assay sensitivity.

Nevertheless, the study shows that the use of a standardised procedure helps to reduce the inter-laboratory variability. Indeed, during the study for the establishment of the BRP [4], several participants deviated substantially from the standard procedure leading to large variations between laboratories: individual estimates varied from 4.5 to 153 ng/mouse, i.e. 34-fold, whereas in the present study, individual estimates of HSD_{50} vary only by 7.5-fold when all valid assays are considered. Excluding the results from laboratory 7, which used a substantially different amount of histamine for the challenge, further reduces the variation to 5.3-fold when considering the individual estimates, corresponding to a further 20% decrease in the GCV value (see Table 6), or to an approximately 2-fold variation when the GM is considered.

6.3. HIST temperature end-point assay

Overall GM estimates of relative activity of the BRP in terms of JNIH-5 using the temperature methods are apparently consistent with the results from the HIST lethal end-point assays (7924 compared to 7500 IU/vial), although the HIST temperature method gives larger variation between laboratories due to the limited data available (only 3 laboratories), with a GCV of 133% instead of 51% for the lethal end-point assay. Among the laboratories which reported data on this assay, only 1 uses it as a routine test for assessment of residual PT toxicity in vaccines. The other laboratories are still in the validation process for this test. In addition, the design of the toxin and histamine doses in this study was not optimized for the temperature measurement method which may also have contributed to the large variation observed.

Nevertheless, HIST based on measurement of the reduction in temperature (both rectal and/ or dermal) produced by histamine shock has been successfully used in Asia for some time. The test method does not necessarily require a fatal sensitization of the mice and could also provide a quantitative estimate of the activity of a test vaccine relative to the activity of a reference standard. Therefore it may provide a potential refinement model in terms of 3Rs to the current lethal end-point assay. However, further optimisation, standardisation and careful validation of this method in individual laboratories would be needed before using it in batch testing. This might provide an interim solution, pending the introduction of reliable in vitro assays. However, no decision can be made on this point based solely on the results of the present study.

6.4. CHO-cell assay

Five (5) laboratories provided data on CHO-cell assays. Participants were asked to apply their own in-house validated procedure, using their own protocols. They were requested to provide details of their in-house methodology. These details can be found in Table 2b. The procedures used differed almost for all parameters, e.g. culture medium, cell passage number, cell density in the assay, starting concentration of the reference preparations or diluents.

The GM of individual relative potency estimates for the BRP varied from 1039 to 7300 IU/vial with a GCV of 111%, the overall GM being approximately 2.5-fold lower than that from both HIST methods. This is not in agreement with the findings from a previous similar study where both relative estimates from HIST lethal end-point assay and CHO-cell assay were similar [9]. The different assay conditions and validity criteria used for this assay by individual participants may partly account for the lower estimate but this is not clear at the moment. The CHO-cell assay is in the current Ph. Eur. monographs as an alternative test for the HIST lethal method for pertussis antigen bulk. Further standardisation of this assay would be important and a collaborative study involving a larger number of participants using a standardised procedure would be desirable.

6.5. Estimation of residual PT activity in combined vaccines

Current practice in licensing of DTaP based combination vaccines is based on data compiled from the DTaP vaccine but a full understanding of the behaviour of the combination and the means to monitor it appropriately are lacking. Additionally, different detoxification methods may generate differences in the toxoid structure, activity and reversibility [10, 11] and formulation factors may also play a role in affecting both activity and/or test system. Therefore, examination of a panel of such vaccines with a known record of clinical safety for their levels of pertussis toxin activity seemed important. For most types of combinations, IU contents of active PT have not been estimated which has caused difficulties in the past to make a causal link between occasional mouse mortality in the HIST lethal test and presence of abnormal amounts of active pertussis toxin. Indeed, in some cases it was not clear whether other factors had contributed to this effect.

In this study, in the lethal end-point assays, the activities of most product samples were below the quantification limit due to non-response values except for samples 6 & 7. In some cases, intra-laboratory variations were observed for the same samples. Thus, the results may depend on the sensitivity of the particular assay and not solely on the activity of the sample tested.

The activities observed by the HIST lethal end-point method for the DTaP based combination vaccines included in the study provide an indication that the highest levels of residual PT may be in the range of about 2 IU/SHD, although the HIST temperature assay gave mean relative activities below the Japanese limit i.e. 0.2 HSU/SHD (equivalent to 1.09 IU/SHD). However it should be kept in mind that most of the assays in this study were not designed to be quantitative assays and with regard to the small number of quantitative data, it is not possible to set a common limit for PT residual activity in these combined vaccines. To this end, further studies using an adapted quantitative assay procedure would be necessary. However, in the absence of defined permissible upper limit for PT activity in vaccines, inclusion in each assay of a reference toxin, at a dose defined in the validation stage, e.g. about 2 IU/SHD, or a reference vaccine with known clinical safety may be desirable to allow the determination of relative activity. This may help to reduce the necessity of repeating tests due to abnormal mortality.

Samples 6 and 7 are the same products but from 2 different batches: sample 6 passed the HIST lethal end-point test and was released on the European market whereas sample 7 did not and was not released. In the present study, these vaccines showed the highest activities in comparison to

other products included in the study in both HIST lethal end-point and temperature assays. Taking into consideration only tests which showed positive responses in the HIST lethal end-point method, levels at 1.32 IU and 1.23 IU/SHD were observed for sample 6 and 7 respectively. With limited data from the temperature method, GM at 0.39 IU/SHD for sample 6 and 0.56 IU/SHD for sample 7 were obtained whereas activities of the other samples were equal or below 0.3 IU/SHD. However, no statistically significant difference could be demonstrated between these 2 samples in either assay. The results indicate that due to large variations, which are normal in most *in vivo* assay systems, the HIST assay may not have the ability to discriminate samples that have similar activities.

Sample 2 had reached its expiry date by the time the study was organized. From the data obtained with this sample, there seems to be no obvious link between ageing of the sample and increase in PT activity. However, it may not be reliable to draw conclusions on this important point from the observation of 1 single sample and more investigations may be needed to address this important issue.

Vaccine samples included in the study were from 3 different sources and contained different types of DTaP base. Vaccine samples 1, 3, 4, 8 and 9 were formulated with the same components of aP but in different combinations with other antigens. They were included in the study in an attempt to evaluate the effect of different antigen components on the HIST test system. When comparing sample 1, which is the DTaP base, with samples 3, 4, 8 and 9 (which are DTaP combined with Inactivated Poliomyelitis Vaccine (IPV), Hepatitis B (HepB) and/ or Haemophilus influenzae type b polysaccharide vaccine (Hib) or all components together), similar results were observed between these formulations in both the HIST lethal and temperature end-point assays. Therefore, in the present study there is no evidence that additional antigen components could affect the outcome of the assay for the type of products tested. However, this observation may need to be confirmed for other types of products marketed in Europe.

7. CONCLUSION

7.1. Calibration of the BRP

Based on the data from the HIST lethal end-point assays obtained during this study, an unitage of 7500 IU/vial was assigned to the current Ph. Eur. BRP batch 1 for PT. The BRP with the new unitage was adopted by the Ph. Eur. Commission at its meeting in December 2009 (EDQM Catalogue No. Y0000021).

7.2. Lethal challenge

In the light of the results of the study, it is clear that standardisation of the HIST assay could be improved by the use of a standard procedure, by calibrating in-house references in terms of their bioactivity and including a common reference preparation in parallel with the test sample in each assay to allow assessment of relative activities instead of absolute percentage of death as is the case at present. Therefore a recommendation was addressed to the Ph. Eur. Group of Experts 15 to amend the monographs for aP vaccines and for combined vaccines containing aP antigens [1] to include the following recommendations:

- Insertion of the Standard Operating Procedure (SOP) chosen for the study in the monographs to be used as reference for implementation of internal procedures in individual laboratories or inclusion of the reference to the SOP, i.e. reference to the report of the present study as published in *Pharmeuropa Bio & Scientific Notes*;
- Criteria for assay sensitivity should be established during a validation study, for a given application route, using multiple doses of the reference preparation and a significant dose-response should be demonstrated. A suitable dose(s) of the reference toxin, chosen in the linear region of the dose-response curve and giving a reasonable positive response, should subsequently be included in each assay. The reference preparation and test vaccine should be applied in the same route. To be considered valid each test should meet the criteria set to demonstrate assay sensitivity;
- Recommendation of the use in each assay of a toxin reference preparation or a reference vaccine, calibrated in terms of bioactivity e.g. BRP. The reference toxin at a given dose, established during the validation stage (e.g. about 2 IU/SHD), or a reference vaccine with known clinical safety included in each assay would allow the calculation of relative activity (ies) with the goal to increase inter-assay and inter-laboratory comparability. This may additionally help to reduce repeat testing due to occasional mortality observed in vaccine groups.

7.3. Temperature assay validation

The temperature assay may be considered as a potential alternative to the HIST lethal end-point assay pending the introduction of reliable *in vitro* assays. However, this decision cannot be based solely on the results of this study and careful validation and standardisation will be necessary. Assay validity criteria should also be established in each laboratory before using it in batch release testing.

7.4. CHO-cell assay

On the basis of the results obtained in this study, it is not possible to draw clear conclusions on the relation between the *in vitro* clustering activity and the *in vivo* activity in HIST of the BRP. Further standardisation of this assay appears to be necessary and a collaborative study involving a larger number of participants using a standardised procedure may be organised to this end.

7.5. Levels of PT activity in combination vaccines

In this study, levels of PT reactivity in the HIST lethal end-point assay of combination vaccines used in the European market with a known history of clinical safety suggest an approximate maximum level at about 2 IU/SHD. However, it is currently not possible to set a limit for these products due to the limited data available from the study and because most assays were not designed for quantitative purpose. If a defined limit were to be set, further data generated with an adapted operating procedure would be needed.

8. PARTICIPANTS (IN ALPHABETICAL ORDER BY COUNTRY AND INSTITUTE)

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9. ACKNOWLEDGEMENTS

The organisers wish to express their thanks to all the participants for their contributions without which this study would not have been possible. Special thanks go to Sanofi-Pasteur France, Sanofi-Pasteur Canada and GSKBio (Belgium) for having kindly donated the study samples. We also would like to thank Peter Rigsby for his help with the statistical analysis and Mrs C. Asokanathan for her kind assistance for the preparation of data tables. Ms Sally Woodward (EDQM) is acknowledged for her assistance throughout the study.

10. ABBREVIATIONS

aP: Acellular pertussis; BRP: Biological Reference Preparation; cBRP: candidate Biological Reference Preparation; BSP: Standardisation Programme; CHO: Chinese Hamster Ovary; CI: Confidence Interval; CL: Confidence Limit(s); DTaP: Diphtheria, Tetanus, acellular Pertussis; EDQM: European Directorate for the Quality of Medicines & HealthCare; EU: European Union; FCS: Foetal Calf Serum; GCV: Geometric Coefficient of Variation; GM: Geometric Mean; HepB: Hepatits B vaccine; Hib: Haemophilus influenza type b polysaccharide vaccine; HIST: Histamine Sensitizing Test; HSD: Histamine Sensitizing Dose; HSU: Histamine Sensitizing Unit; IHR: In-House Reference; IPV: Inactivated Polyomyelitis Vaccine; IS: International Standard; IU: International Unit(s); NIBSC: National Institute for Biological Standards and Control; OCABR: Official Control Authority Batch Release; OMCL: Official Medicines Control Laboratory; Ph. Eur.: European Pharmacopoeia; PN: protein nitrogen; PT: Pertussis Toxin; SHD: Single Human Dose; WHO: World Health Organization.

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Table 2a – *summary of HIST assay conditions*

Lab No.	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10
Immunisation										
Mouse strain/origin	NIH Harlan	ddY	CD1 Charles River Labs	CD1 Charles River Labs	NIH Harlan Sprague-Dawley	CD1 Charles River Labs	NMRI	C57BL/6J OlaHsd (Harlan)	CD1 Charles River Labs	CD1 Charles River Labs
Immunisation route	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female	Female
Age/Weight	9-11g	4 weeks old	24-26 g	21-29g	18-22g	19-27g	20-26 g	6-8 weeks old	5 weeks old 17-22g	24-26 g
Number/cage	5/cage	10/cage	10/cage	10/cage	10/cage	10/cage	5/cage	5/cage	5/cage	5/cage
Interval immunisation/challenge	5 days	5 days	5 days	5 days	5 days	5 days	5 days	5 days	5 days	5 days
Diluent/pH	PBSG pH 7.4	PBSG pH 7.4	PBSG pH 7.4	PBSG w/o Ca++ & Mg++ pH 7.2	PBSG pH n.r.	PBSG pH 7.1	PBSG pH 7.4	PBSG pH 7.4	PBSG pH 7.2	PBSG
Challenge										
Histamine salt	Diphosphate	Dihydrochloride	Dihydrochloride	Dihydrochloride	Dihydrochloride	Dihydrochloride	Dihydrochloride		Dihydrochloride	Dihydrochloride
Diluent	PBS	Saline	PBSG	PBSG w/o Ca++ & Mg++ pH 7.2	0.85% Saline	PBS pH 7.1	0.9% Saline		PBS w/o Ca++ & Mg++ pH 7.2	n.r.
Concentration of histamine base	2 mg/mouse	2 mg/mouse	2 mg/mouse	2 mg/mouse	2 mg/mouse	2 mg/mouse	***5 or 6 mg/mouse***		2 mg/mouse	n.r.
Volume/mouse	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml		0.5 ml	0.5 ml
Route	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.		i.p.	i.p.
Time preparation of challenge to inoculation of last mouse	80 min	80 min	45 min	~1.5h – 2.5h	90 min	~1h – 1.25h	~2h		2h	n.r.
IHR	aP	Whole cell pertussis	aP	aP	aP	aP	aP	aP toxoid	aP	aP ***0.5 ml/mouse***
HSD_{50}	n.r.	n.r.	14 ng PT	n/a	calculated for each test	n/a	n/a	n.r.	n/a	n.r.
Content/ampoule or vial	20 µg/amp. 2100 IU/amp.	45 HSU/vial	50 µg/vial	n/a	250 µg/ml	n/a	n/a	n.r.	n/a	50 µg/vial
Freeze-dried	yes	yes	yes	n/a	no	n/a	n/a	n.r.	n/a	yes
Diluent	PBSG pH 7.4	Saline	PBSG pH 7.4	n/a	PBSG pH n.r.	n/a	n/a	n.r.	n/a	PBSG
Storage	-20°C	+4°C	-20°C	n/a	-20°C before opening +4°C after	n/a	n/a	n.r.	n/a	-20°C
Temperature assays										
Rectal/Dermal	Dermal	Rectal	n/a	n/a	n/a	n/a	Dermal	Dermal	n/a	n/a
Time from injection to temperature measurement	30 min	30 min					10-30 min	30 min		
Histamine base	2 mg/mouse	2 mg/mouse					2 mg/mouse	***1.2 mg/mouse***		
Histamine salt	Diphosphate	Dihydrochloride					Dihydrochloride	Diphosphate		
Diluent	PBSG pH 7.4	Saline					0.9% Saline	PBS pH 7.4		
Volume/mouse	0.5 ml	0.5 ml					0.5 ml	***0.2 ml***		
Route	i.p.	i.p.					i.p.	***i.v.***		
Time preparation of challenge to inoculation of last mouse	80 min	80 min					~2h	***<3h each assay was run over 2 days***		

Amp.: ampoule; aP: acellular pertussis; HIST : histamine sensitizing test ; HSD : histamine sensitizing dose ; HSU: histamine sensitizing unit; IHR: in-house reference; i.p.: intraperitoneally; IU: international unit; i.v.: intravenously; Lab : laboratory; min: minute; n/a: not applicable; n.r.: not reported; PBSG: phosphate buffered saline+gelatine; w/o: without.
Deviations from standard operating procedure are indicated in bold italics.

Table 2b – *summary of CHO-cell assay conditions*

Lab No.	Lab 1	Lab 3	Lab 4	Lab 5	Lab 8
CHO cell line / origin	CHO-K1 / ECACC No. 8505/005	CHO-K1 / BioWhittaker USA	CHO / NBL	CHO-K1 / ATCC	CHO-K1 / ATCC CCL-61
Age of cells	3 days / P6	P4	P23-39	P22, P23, P24	P15, P18, P21
Culture medium	RPMI 1640 + glutamine + 10% FCS + 1% P/S	HAM F12 + 1% FCS	RPMI 1640 + 10% FCS + gentamycin	MEM-5 + 10% FCS	HAM's complete + 10% FCS + gentamycin + glutamine
Cell density in assay (cells/well)	20 000	5000	20 000	10 000	5000
Incubation time - cells & antigen	48 hours	48 hours	48 hours	48 hours	48 hours
Fixation / staining	10% formaldehyde / crystal violet	Ethanol / Giemsa	None or n.r.	None	10% glacial acetic acid / 90% ethanol / Giemsa
IHR					
Content/ampoule or vial	20 µg/amp. 2100 IU/amp.	20 µg/amp.	50 µg/ml	94 µg/ml	32 ng/ml (by ELISA)
Freeze-dried	yes	yes	no	no	no
Diluent for stock/serial dilutions	PBS / medium	sterile water / carbonate buffer + Tween 80 0.1% / medium	sterile water / medium	n.a / medium	n.a / medium
Storage	-20°C	-20°C	-20°C	-20°C before opening, +4°C after	-20°C
Positive control	IHR / JNIH-5	IHR / JNIH-5	IHR / JNIH-5	IHR / JNIH-5	IHR / JNIH-5
Negative control	culture medium	culture medium	culture medium	culture medium	culture medium

Amp.: ampoule; CHO: Chinese hamster ovary; ELISA: enzyme-linked immune-sorbent assay; FCS: foetal calf serum; IHR: in-house reference; IU: international unit; Lab: laboratory; n.a: not applicable; n.r.: not reported; PBSG: phosphate buffered saline + gelatine; P/S: penicillin/streptomycin; w/o: without
Px: passage number x

Table 3 – *Overview of HIST lethal end-point assay results*

Lab code	Assay number	Statistical validity	Common slope [1]		HSD50 (ng protein/mouse)						Relative activity BRP1 (IU/vial)		Relative activity test samples (IU/SHD)							
					JNIH-5 [2]		BRP1		IHR											
			Per assay	GM	Per assay	GM	Per assay	GM	Per assay	GM	Per assay	GM	S1	S2	S3	S4	S6	S7	S8	S9
1	1		0.55	0.62	4.0	5.1	43.9	59.1	5.4	4.1	4594	4288	<<	<<	<<	<<	0.44	1.57	<<	<<
	2		0.77		7.0		120.6		6.1		2896		<<	<<	<<	<<	0.66	1.77	<<	<<
	3		0.56		4.6		39.0		2.0		5924		<<	<<	<<	<<	1.47	0.52	<<	<<
2	1		0.83	0.70	8.7	17.6	52.1	83.6	*0.6	0.9	8360	10515	<<	<<	<<	<<	<<	<<	<<	<<
	2	NL (P=0.033)	0.46		24.4		79.3		*1.3		15398		<<	<<	<<	<<	<<	<<	<<	<<
	3		0.90		25.5		141.4		*0.8		9031		<<	<<	<<	<<	<<	<<	<<	<<
3	1		0.79	0.83	21.2	18.4	117.8	94.8	46.9	79.2	9011	9690	<<	<<	<<	<<	<<	2.08	<<	<<
	2		0.82		9.2		101.1		68.5		4549		<<	<<	<<	<<	0.97	<<	<<	<<
	3		0.88		31.8		71.5		154.5		22195		<<	<<	<<	<<	<<	<<	<<	<<
4	1		0.94	0.96	2.3	5.0	44.3	52.7	n.t.	n.t.	2650	4716	<<	<<	<<	<<	0.67	<<	<<	<<
	2		0.85		10.4		59.2		n.t.		8753		<<	<<	<<	<<	<<	<<	<<	<<
	3		1.11		5.0		55.7		n.t.		4522		<<	<<	<<	<<	<<	0.79	<<	<<
5	1	NP (P=0.040)	0.51	0.55	4.6	6.5	25.1	21.2	12.0	16.0	9139	15301	<<	<<	<<	<<	1.40	1.40	<<	<<
	2	NP (P=0.025)	0.53		13.2		39.4		21.6		16782		<<	<<	<<	<<	2.44	0.65	<<	<<
	3		0.63		4.5		9.6		15.7		23355		0.59	<<	0.59	0.29	2.24	0.59	0.29	<<
6	1	NL (P=0.049)	0.72	0.73	20.4	22.3	83.6	106.8	n.t.	n.t.	12190	10446	<<	<<	<<	<<	1.71	1.71	<<	1.71
	2		0.87		40.3		152.1		n.t.		13256		<<	<<	<<	<<	7.69	<<	<<	<<
	3		0.58		28.2		103.4		n.t.		13619		<<	<<	<<	<<	<<	<<	<<	<<
	4		0.77		10.7		98.8		n.t.		5411		<<	<<	<<	<<	<<	<<	<<	<<
7	1		0.72	0.66	49.0	58.9	292.5	304.8	n.t.	n.t.	8381	9658	<<	<<	<<	<<	<<	<<	<<	<<
	2		0.65		80.8		163.4		n.t.		24706		<<	<<	<<	<<	<<	<<	<<	<<
	3	NP (P=0.030)	0.60		51.6		592.5		n.t.		4351		<<	<<	<<	<<	<<	<<	<<	<<
9	1		0.61	0.81	11.6	15.9	207.7	109.9	n.t.	n.t.	2784	7230	<<	<<	<<	<<	0.70	<<	<<	<<
	2		0.75		14.1		78.6		n.t.		8979		<<	<<	<<	<<	2.30	<<	<<	<<
	3		1.15		24.6		81.3		n.t.		15118		<<	<<	<<	<<	<<	<<	<<	<<
10	1		0.75	0.81	7.2	6.5	60.0	56.8	75.9	67.7	6014	5694	<<	<<	<<	<<	<<	<<	<<	<<
	2		0.83		4.8		46.4		86.6		5185		<<	<<	<<	0.87	0.87	<<	<<	0.51
	3		0.86		7.8		65.8		47.2		5920		<<	<<	<<	<<	<<	<<	<<	<<
Including all assays		Geometric Mean	0.73		12.4		77.9		24.3		7978									
		GCV (%)	18.5		130.7		105.4		302		52.7									
		95% confidence limits	(0.64 - 0.83)		(6.54 - 23.6)		(44.8 - 136)		(2.66 - 222)		(5763 - 11045)									
		N (number of estimates)	9		9		9		4		9									
Excluding invalid assays**		Geometric Mean	0.78		13.4		89.2		28.0		7500									
		GCV (%)	14.9		138.4		60.6		431		51.2									
		95% confidence limits	(0.70 - 0.88)		(6.47 - 27.7)		(60.0 - 133)		(0.44 - 1766)		(5309 - 10594)									
		N (number of estimates)	8		8		8		3		8									

(1) The common slope is expressed in probit/ln(dose). Multiply by 2.3 to obtain values in probit/$\log_{10}$ (dose)

(2) For JNIH-5 1 ng/mouse is by definition 1 IU/mouse

* For laboratory 2 the IHR is expressed as HSU/mouse and is not included in the overall mean

NL = Significant non-linearity ($P<0.05$); NP = Significant non-parallelism ($P<0.05$); n.t. = not tested

GM = Geometric mean; GCV: Geometric Coefficient of Variation; IHR: In-house Reference; IU: International Unit; SHD: Single Human Dose

** Excluding assays with a p-value less than 0.05 for non-parallelism and/or non-linearity. Assay 3 from Laboratory 5 is excluded as well

Table 4 – *Overview of CHO-cell assay results*

Lab code	Assay number	Relative activity BRP1 (IU/vial)		Relative activity IHR	
		Per assay	GM	Per assay	GM
1	1	6258	7300	5003	8578 IU/vial
	2	19868		25215	
	3	3129		5003	
3	1	4961	5372	250	214 IU/vial
	2	5000		125	
	3	6250		315	
4	1	2210	3125	2806	2062 IU/ml
	2	4961		1768	
	3	2784		1768	
5	1	2000	2722	5000	6300 IU/ml
	2	3175		5000	
	3	3175		10000	
8	1	1123	1039	1.27	0.83 IU/ml
	2	1000		0.45	
	3	1000		1.01	
Geometric Mean		3221			
GCV (%)		111.3			
95% confidence limits		(1272 - 8156)			
N (number of estimates)		5			

GM = Geometric mean; GCV: Geometric Coefficient of Variation
IHR: In-house reference; IU: International Unit; Lab: Laboratory

Table 5 – *Overview of temperature assay results*

Lab code	Assay number	Relative activity BRP1 (IU/vial)		Relative activity IHR		Relative activity S1 (IU/vial)		Relative activity S2 (IU/vial)		Relative activity S3 (IU/vial)		Relative activity S4 (IU/vial)		Relative activity S6 (IU/vial)		Relative activity S7 (IU/vial)		Relative activity S8 (IU/vial)		Relative activity S9 (IU/vial)	
		Assay	GM	Assay	GM	Assay	GM	Assay	GM	Assay	GM	Assay	GM	Assay	GM	Assay	GM	Assay	GM	Assay	GM
1	1	3588		1115		0.10		0.12		0.07		0.05		0.24		0.33		0.10		0.10	
	2	2492	2990	961	1035	0.04	0.06	0.10	0.11	0.03	0.05	0.02	0.03	0.04	0.09	0.12	0.20	0.04	0.06	0.03	0.06
	3																				
2	1	16302		756		0.38		0.84		0.31		0.23		0.70		0.76		0.37		0.11	
	2	11883	12061	553	653	0.59	0.62	0.41	0.76	0.38	0.51	0.52	0.43	0.62	0.90	0.74	0.92	0.45	0.55	0.30	0.24
	3	9057		667		1.08		1.27		1.13		0.65		1.66		1.37		0.98		0.44	
8	1																				
	2	14897	13794			1.15	0.74	0.90	0.23	0.81	0.62	1.03	0.97	0.72	0.73	1.09	0.97	0.53	0.35	0.44	0.43
	3	12773				0.47		0.06		0.47		0.91		0.73		0.86		0.23		0.42	
Geometric Mean		7924				0.30		0.26		0.25		0.24		0.39		0.56		0.23		0.18	
GCV (%)		133.2				304		167		310		493		248		145		212		182	
95% confidence limits																					
N (number of estimates)		3				3		3		3		3		3		3		3		3	

GM = Geometric mean; GCV: Geometric Coefficient of Variation; IU: International Unit; Lab: Laboratory

Table 6 – *Geometric mean (GM) estimates of relative activities of BRP in terms of JNIH-5*

BRP Relative activity (IU/vial)							
Lab No.	HIST lethal end-point assay		HIST temperature assay		CHO-cell assay	
	Number of assays & validity	GM	Number of assays & validity	GM	Number of assays & validity	GM
1	3	4288	3 (3[##])	2990	3	7300
2	3 (2*)	10515	3	12061		
3	3	9690			3	5372
4	3	4716			3	3125
5	3 (1, 2[#])	15301			3	2722
6	4 (1*)	10446				
7	3 (3[#])	9658				
8			3 (1[##])	13794	3	1039
9	3	7230				
10	3	5694				
Overall results including all assays						
GM of Lab GMs	7978		7924		3221	
95% confidence limits	(5763-11045)				(1272-8156)	
GCV (%)	52,7		133		111,3	
N (number of estimates)	9		3		5	
Overall results excluding invalid assays **						
GM of Lab GMs	7500					
95% confidence limits	(5309-10594)					
GCV	51,2					
N (number of estimates)	8					
Overall results excluding data from Lab 7						
GM of Lab GMs	6833					
95% confidence limits	(4971-9393)					
GCV	41,1					
N (number of estimates)	7					

CHO: Chinese hamster Ovary; GCV: Geometric coefficient of variation; GM: Geometric mean; HIST: Histamine sensitizing test; Lab: Laboratory
*: Significant non-linearity (P<0.05), number represents assay number
#: Significant non-parallelism (P<0.05), number represents assay number
##: No regression
**: excluding assays with a P-value less than 0.05 for non-parallelism and/or non-linearity. Assay 3 from Lab 5 is excluded as well