PHARMEUROPA BIO 2000-2

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COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF

DIPHTHERIA TOXOID (ADSORBED)

THIRD INTERNATIONAL STANDARD AND EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION BATCH No. 3

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF DIPHTHERIA TOXOID (ADSORBED) THIRD INTERNATIONAL STANDARD AND EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION BATCH No. 3

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1. SUMMARY

The Second International Standard for Diphtheria Toxoid, Adsorbed (DIXA[3]) was established by the WHO ECBS in 1978. Fewer than 200 ampoules of this standard remain in stock at NIBSC. The aim of this collaborative study was to characterise and calibrate a new candidate diphtheria toxoid preparation to confirm its suitability as a replacement I.S. and as a replacement of the current European Pharmacopoeia Biological Reference Preparation (Ph. Eur. BRP, D2700000 batch No. 2).

The candidate diphtheria toxoid, adsorbed preparation was calibrated in IU with reference to DIXA, the second I.S. using the established WHO/Ph Eur methods, *in vivo* challenge in guinea pigs.

Twenty laboratories, representing 14 countries participated in the study. Six laboratories performed intradermal challenge and fourteen performed lethal challenge. Good agreement in the results was obtained by the two challenge methods. Stability was assessed within the collaborative study, and as part of the candidate characterisation studies. Results suggest that the preparation will have satisfactory stability to act as replacement I.S. and Ph Eur BRP. It was therefore recommended that the candidate preparation be established as the Third International Standard and Ph. Eur. BRP batch No. 3 for Diphtheria Toxoid, Adsorbed and assigned a unitage of 160 IU/ampoule on the basis of its calibration by *in vivo* bioassay. These recommendations were accepted by the Expert Committee of Biological Standardisation of WHO, by the Steering Committee of the Biological Standardisation Programme of EDQM and by the European Pharmacopoeia Commission.

2. INTRODUCTION

The second I.S. for Diphtheria Toxoid, Adsorbed, coded DIXA, was established in 1978 (30th Report of the WHO Expert Committee on Biological Standardisation, WHO Tech. Rep. Series, 638, 1979) and held by the Statens Serum Institute, Copenhagen, until transfer to NIBSC in 1997. This I.S. is used extensively as a primary standard for calibration of national and in-house standards and reference reagents for potency testing of adsorbed diphtheria toxoid vaccines. Fewer than 200 ampoules of DIXA remain in stock and a proposed replace-

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(3) Abbreviations: BRP: Biological Reference Preparation; cl: confidence limits; cv: coefficient of variation; DIXA: Second International Standard for diphtheria toxoid, adsorbed; ECBS: Expert Committee on Biological Standardisation; EDQM: European Directorate for the Quality of Medicines; ELISA: Enzyme-Linked Immunosorbent Assay; I.S.: International Standard; IU: International Unit; Lf: Limes flocculation; NIBSC: National Institute for Biological Standardisation and Control; Ph Eur: European Pharmacopoeia; WHO: World Health Organisation.

ment I.S. has been prepared and freeze-dried at NIBSC. Testing performed at NIBSC (moisture content, precision of fill, antigen assay, potency, immunogenicity and stability) indicated that this preparation is suitable for calibration in a collaborative study. It was intended to establish part of the same preparation as a Ph Eur BRP for use as a working standard by manufacturers and control laboratories in batch release of the current production of diphtheria vaccines.

The aim of this collaborative study was therefore to calibrate the candidate diphtheria toxoid preparation in IU with reference to DIXA, the second I.S., using the established WHO/Ph. Eur. methods i.e. *in vivo* challenge in guinea pigs. Both lethal and intradermal challenge methods were used in calibration. This report summarises the results of this calibration study in which 20 laboratories in 14 countries participated (Belgium, Croatia, Czech Republic, France, Germany, India, Italy, Norway, Russia, Switzerland, The Netherlands, United Kingdom, USA and Vietnam).

3. MATERIALS AND METHODS

3.1. Preparations used in the study

The following coded preparations were supplied to participants as testing material:

- **Preparation A. 2nd I.S. for Diphtheria Toxoid (adsorbed) - DIXA**

The 2nd I.S. for Diphtheria Toxoid (adsorbed) with a defined potency of 132 IU/ampoule (100 Lf toxoid) (DIXA). This was provided to all participating laboratories with instructions for storage and use. For the purpose of the collaborative study this preparation was coded as sample A.

- **Preparation B. Candidate replacement I.S. and Ph Eur BRP for Diphtheria Toxoid (adsorbed)**

Source of liquid bulk fill: Adsorbed, purified diphtheria toxoid (Lot No. 210597) was kindly donated from Pasteur Aventis, France as the candidate standard. Bulk liquid material was provided with full protocol confirming that formulation passed requirements for purity of diphtheria toxoid, identity, sterility (bacterial and fungal) and specific toxicity as per Ph. Eur. specifications (monograph No. 761).

Composition of liquid bulk fill: Purified diphtheria toxoid 137.4 Lf/ml (expected potency ca. 300 IU/ml), Superfose gel (2 % Superfos Biosector - Lot No. VMO461-02) at a concentration of 2.143 mg/dose (Al concentration expressed as Al_2O_3 giving the final concentration of 1mg Al per 70 IU), and phosphate buffered saline pH 6.0-6.5. Liquid bulk toxoid was stored at + 4 °C to + 8 °C at NIBSC prior to formulation for freeze-drying. Potency was determined as 548 (344-1204, mean and 95 % cl) IU/ml, against the second I.S. (DIXA) and toxoid content by in-house ELISA assay as ca. 100 Lf/ml, after desorption.

Stable freeze-dried formulation: Purified diphtheria toxoid (Lot No. 210597) was mixed in 1:1 ratio with 2% sterile trehalose dihydrate solution (Fluka catalogue No. 90210). The solution was filtered through a 0.22 µm membrane filter, prior to the aseptic fill in ampoules containing aliquots of 1.0 ml of the mixed solution, followed by freeze-drying at NIBSC's Standards Processing Division on 18th June 1998 to provide a homogenous batch, coded 98/650. The final concentration per ampoule was therefore 0.5 ml of bulk diphtheria toxoid and 1% trehalose. The entire processing was performed at + 4 °C to + 8 °C. The final fill was stored frozen at – 20ºC in the dark before use. In total, about 4,600 ampoules were produced.

Precision of fill: Precision of fill was determined at NIBSC by weighing ampoules after fill. The results showed an average value of 1.02g cv 0.13 % (n = 88). This complies with WHO specifications for International Standards.

Residual moisture content: The residual moisture content was determined, for three individual ampoules using the Karl Fischer method, as 121.1 ± 6.7 µg of water per ampoule. This represents 0.597 % water content of the total dry weight and complies with WHO specifications for International Standards. The current I.S. (DIXA) was determined to have 0.687 % water content of the total dry weight per ampoule.

Potency (% recovery): The potency of the freeze-dried formulation was determined against DIXA and compared with that of liquid bulk fill to determine % recovery. The mean potency of freeze-dried preparation was determined as 202 (149-282, 95 % cl) IU/ampoule (representing 0.5 ml of original vaccine). As the mean potency of the liquid bulk toxoid was 548 (344-1204, 95 % cl) IU/ml, recovery of biological activity after freeze-drying was approximately 75 %.

Antigen content: An in-house ELISA assay was used to determine antigen content in freeze-dried formulations. This was ca. 55 Lf/ampoule (110 Lf/ml in the original vaccine) and comparable to antigen content determined in liquid formulation by in-house ELISA.

Immunogenicity: Guinea pigs were used to determine immunogenicity of freeze-dried formulation against liquid bulk toxoid. Animals, 8 per dose, were immunised with 1/10 dilution of vaccine. Antibody responses were determined 6 weeks post immunisation by in-house ELISA and Vero cell assays. Antibody responses, determined by ELISA were significantly different for liquid and freeze-dried formulations; geometric means 2.14 and 0.51 IU/ml respectively ($p < 0.02$). However, analysis of the same samples by Vero cell assay, indicated antibody levels of 0.266 and 0.122 IU/ml respectively, which did not differ significantly ($p = 0.16$).

Stability: Representative samples (between 25-50 ampoules) were stored at each of + 4 °C, + 20 °C, + 37 °C, + 45 °C and + 56 °C for accelerated thermal degradation studies, initiated on 13th July 1998. Ampoules were taken out of degradation on 26th January 1999. Samples were tested in-house for potency and were included in the collaborative study. Results are summarised in this report (see Table 3*).

In addition to potency assays, in house antigen content and immunogenicity studies were also performed on samples from accelerated degradation. Diphtheria toxoid content of samples stored at + 4 °C, + 20 °C, + 37 °C, + 45 °C and + 56 °C for 25 weeks (from 13th July 1998 to 26th January 1999) was 97 ± 10, 72 ± 8, 74 ± 6, 68 ± 6 and 58 ± 5 Lf/ml of toxoid respectively, compared to 91 ± 10 Lf/ml for identical samples kept at – 20 °C. These results further confirm that some, albeit small loss of antigenicity may have occurred at storage temperature > 20 °C and that no detectable loss of antigenicity has occurred at 4 °C. The antibody response induced in guinea pigs, for samples stored at different temperatures were not-significantly different.

Further long term stability studies, taking samples stored at + 4 °C and + 20 °C for 18 months are in progress.

The candidate replacement I.S./Ph Eur BRP for diphtheria toxoid adsorbed was provided to all participating laboratories with instructions for storage. Potency estimates determined at NIBSC (ca. 200 IU/ampoule) were provided to participants as indicative potency, to help in preparation of dilutions. For the collaborative study this material was coded as sample B.

* Tables 1-12 are published at the end of this article.

- **Preparation C. Low potency Diphtheria Toxoid (adsorbed)**

Ampoules of diphtheria toxoid adsorbed of low potency, coded C in this study, were prepared essentially as described for the preparation coded B using the same starting bulk purified diphtheria toxoid, adsorbed. Identical concentration and filling protocol was followed but on post-filling inspection the potency was determined as ca. 50 IU/ampoule. This material was therefore not suitable as candidate replacement I.S. and was used in this study as an additional sample of low potency, provided to some participants as quality marker.

- **Preparations D, E, F. Candidate replacement I.S./Ph Eur BRP for Diphtheria Toxoid (adsorbed) - temperature degradation stability study**

Ampoules containing candidate diphtheria toxoid adsorbed stored at elevated temperatures (as described above) were provided to some participants of the study (one sample per laboratory) with instructions for storage. Potency estimate as determined at NIBSC was provided to participants as indicative potency to help in preparation of dilutions. Samples stored at + 56 °C, + 45 °C and + 37 °C were coded D, E and F respectively.

3.2. General methods and study design

Each participating laboratory was provided with three different preparations. All participants were provided with samples coded A (second I.S.) and B (candidate replacement I.S./Ph. Eur. BRP) and one of the samples coded C – F (see Table 4). Each participant was asked to perform their in-house (routinely used) assay for potency testing of diphtheria vaccine, adsorbed on two independent occasions using all three preparations.

The general outline of the method to be used was essentially as defined in WHO/Ph. Eur. monographs (European Pharmacopoeia 1997). Both lethal and intradermal challenge potency assay methods were acceptable. Participants were asked to use at least three doses per preparation. Nominal potency provided for each preparation was to be used to help with dilution of the samples for immunisation.

Participants performing lethal challenge assay were asked to use not less than fourteen animals per dilution and those performing intradermal challenge assay were asked to use not less than eight animals per dilution.

Participants were asked to use guinea pigs of the same sex (or equal numbers of males and females in each treatment group), randomly distributed and each weighing 250-350 g on arrival. Difference in body mass between the heaviest and the lightest animal was not expected to be greater than 100 g. Participants were asked to provide details of test animals and numbers used (see Table 4).

Participants were asked to reconstitute all preparations on the same day and to use sterile normal saline solution, if possible. At least three dilutions per preparation were to be used. Further, it was recommended that all injections be completed within one hour of vaccine reconstitution and dilution. For each experiment and each preparation participants were asked to provide information on dilutions used using appropriate forms.

Twenty-eight days after immunisation of guinea pigs participants were asked to perform challenge, using appropriate concentration of diphtheria toxin as per their in-house protocol. Diphtheria toxin with specific activity of > 200 Lf/mg protein (> 1500 Lf/mg protein nitrogen) was recommended as suitable for use as challenge toxin. In this collaborative study most participants used their in-house diphtheria toxin. Two laboratories were provided with diphtheria toxin from NIBSC (4500 Lf/ml).

3.3. Scoring and reporting of data

All participants were provided with appropriate assay data sheets to report data from the study. Different report forms were provided for reporting results obtained following the method of intradermal and lethal challenge. In either case participants were asked to define response or score and to indicate the time of observation (post challenge). For intradermal challenge, participants were asked to record the number of sites free from positive reactions and to indicate it by an appropriate symbol (e.g. "0" or " –").

Results of responses observed with the control unvaccinated guinea pigs, injected with diphtheria toxin to confirm that the correct dilution of challenge toxin has been used in the test, were also to be reported. Participants were asked to submit their results to both the WHO co-ordination centre and the EDQM; the statistical evaluation and analysis was carried out by the NIBSC.

3.4. Statistical analysis

Data from all assays (intradermal and lethal challenge) were analysed as described in the European Pharmacopoeia (Section 2.7.6) (1997). For lethal assays, data were analysed using a probit transformation and an in-house program which determines parameters of the dose-response line using an interactive maximum likelihood procedure. This may lead to differences with participant's results in some border line cases where other calculation methods have been used. For the intradermal challenge assays, the $(score)^2$ transformation was used. For all assays, each preparation was analysed separately against the current I.S. (sample A) and the resulting potency estimates are hence based on direct pairwise comparisons (with sample A) and expressed in IU.

4. RESULTS AND DISCUSSION

Data contributed to the study:

In total, data were received from 20 laboratories; 6 performed intradermal challenge assays (16 assays in total) and 14 performed lethal challenge assays (28 assays in total). The number of animals used per vaccine dilution ranged from 8-12 for the intradermal challenge method and 12-18 for the lethal challenge method. Only one participant used less than the recommended number of animals in the lethal challenge assay (lab. No. 24). For the lethal challenge assay, a complete summary of the data received is given in Tables 4-10. Data from intradermal challenge assays cannot be easily summarised, but can be made available if required by the project leaders.

Data excluded from the analysis:

A few doses were excluded from the lethal challenge assays before analysis. Where a maximum (100 %) response was recorded for the two largest doses included in the assay, the largest dose was omitted from the analysis as the maximum response had clearly been achieved at the smaller of the two doses. Similarly if a zero (0 %) response was recorded for the two smallest doses, the smallest dose was omitted from analysis. For similar reasons, the response for sample A at the largest dose in laboratory 9 (assay 2) and the response for sample B in laboratory 7 were also excluded from the analysis, as were responses to the two largest doses of C in the assays carried out by laboratory 2. All other data were included in the statistical analysis.

Assay validity:

In general, the assays satisfied the requirements for validity as set out in the European Pharmacopoeia.

From the lethal challenge assays, some results were classified as being invalid due to non-linearity of the response lines for certain test samples. These were preparation B in laboratory 2 (assay 1), sample C in laboratory 7 (assay 1) and samples A and D in laboratory 8 (assay 1). Non-parallelism of results obtained with samples A and B in laboratory 17 (assay 2) and samples A and E in laboratory 18 (assay 2) made these assays invalid. In the assays performed by laboratory 24, the 50 % protective dose for sample A (DIXA) did not lie between the largest and smallest doses administered to the guinea-pigs and therefore any comparisons with sample A were invalid for that participant. The results for the degradation samples from this laboratory also did not satisfy the requirements on the fiducial limits obtained in the assay, which should lie between 50 % and 200 % of the estimated potency.

For the intradermal challenge method, invalid assays due to non-linearity of the response lines for certain preparations were noted. This was the case for sample A in laboratory 12 (assay 1) and laboratory 13 (assay 1), sample B in laboratory 13 (assay 3) and sample C in laboratory 22 (assay 1) and laboratory 26 (assay 2). Non-parallelism of results observed with samples A and D in laboratory 3 (assay 2) and samples A and B in laboratory 22 (assay 1) made these assays invalid. The validity requirements on the fiducial limits obtained in the assay meant that laboratory 3 (assay 2) and laboratory 26 (assay 4) (degradation samples) were invalid.

The results from all assays have been reported in this publication, with the results from invalid assays clearly indicated.

4.1. Potency of sample coded B

The individual assay estimates of the potency of sample B are detailed in Tables 1 and 2. They are illustrated graphically in Figures 1 and 2. Table 11 gives unweighted geometric mean potency estimates (with 95 % cl) except for those cases where the log potency estimates form a homogenous set; in this case, a weighted mean has been given. The probability level of a χ^2 test for the homogeneity of the log potency estimates is also given. The results from the invalid assays have been excluded from the calculations.

Three laboratories reported data for one or two of their assays (the first two assays by laboratory 12, the first assay by laboratory 14 and the first assay by laboratory 15) but commented that in-house these assays would not have been accepted as reliable (reflecting unsuitable dose or response levels). Although being statistically valid, these assays gave the four lowest estimates obtained (see Figure 1) and mean estimates have been calculated including and excluding the results from these assays (Table 11). For sample A laboratory 24 observed responses that were unusually low, resulting in very high potencies for sample B as depicted in Figure 1 and Table 2. The reason for this in not known and mean estimates have been calculated excluding the results from this assay (Table 11).

The estimates were then examined to determine possible sources of heterogeneity. Some 46 % of the total χ^2 statistic was contributed by the single estimates from laboratories 2, 7, 12 and 15 (i.e. 4/30 estimates). Omitting these estimates gave a χ^2 statistic with $p \sim 0.18$ and a weighted geometric mean estimate of 162.1 (149.4 to 175.8, 95 % cl) IU/ampoule. The mean of laboratory means was also heterogeneous. Estimates from laboratories 2 and 15 contributed significantly to the heterogeneity (37 % of χ^2 statistic from 2/16 estimates). Omission of these estimates gave a weighted geometric mean of laboratory means of 170.7 (163.2 to 178.5, 95 % cl) IU per ampoule. The proposal of taking a value of 160 IU/ampoule

is consistent both with the unweighted geometric mean of all valid individual estimates and with the weighted geometric mean of all individual estimates after omission of questionable estimates and estimates which contributed significantly to heterogeneity.

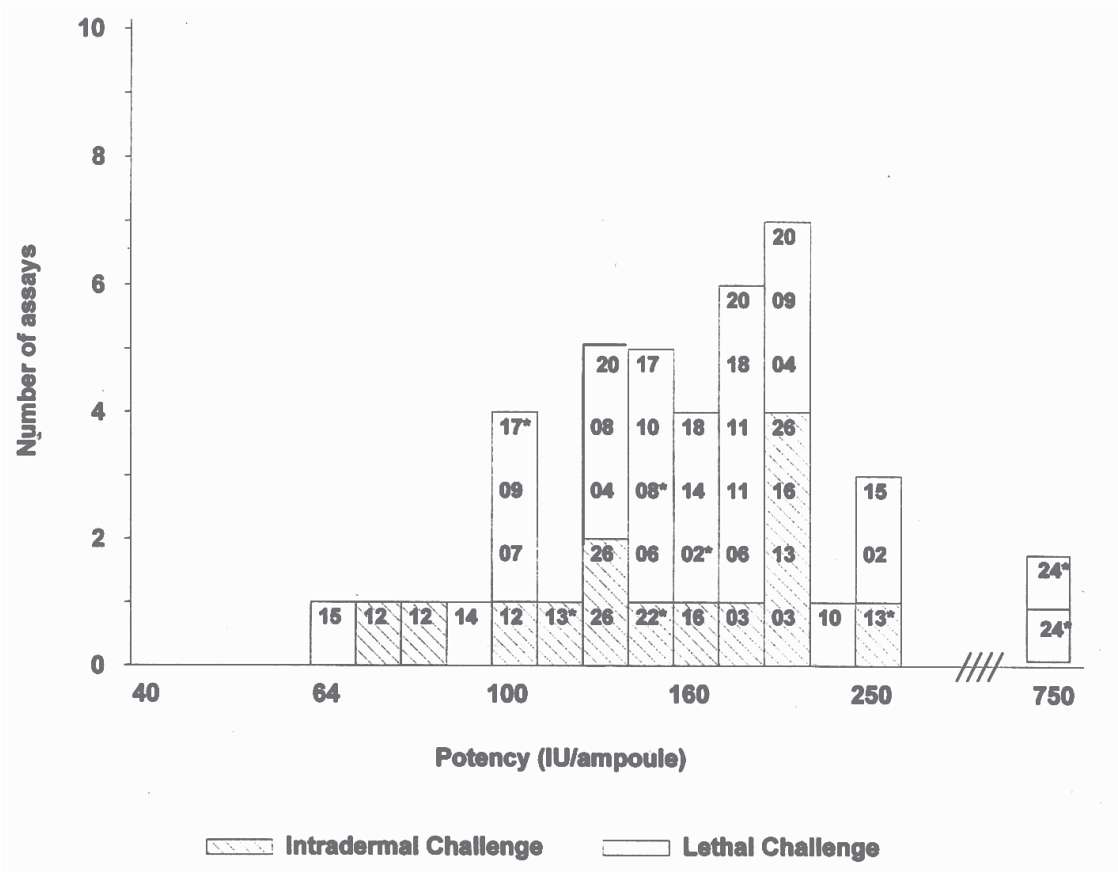

Figure 1. — *Potency of 'B' - Individual assay estimates (*denotes invalid assays)*

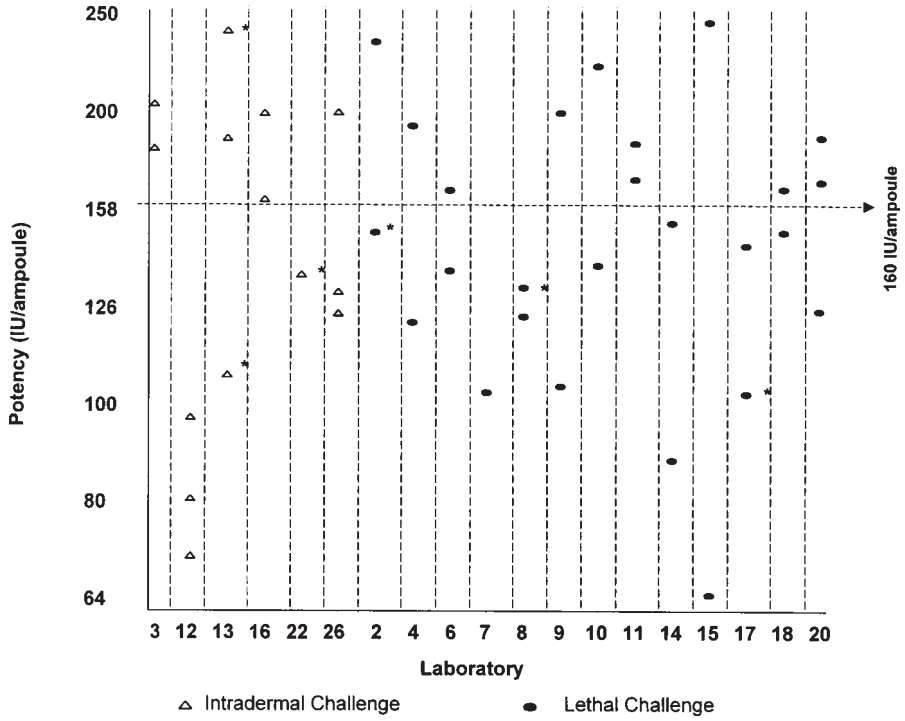

Figure 2. — *Potency of 'B' - Individual assay estimates (Lab. 24 excluded)*

4.2. Additional results of the study

Potencies of samples D, E, F (accelerated degradation of sample coded B):

The potencies of the accelerated degradation samples of the candidate standard B are given in Table 3. They have been calculated using direct pairwise comparisons of D, E or F with the candidate standard sample B from the raw data.

The data suggest that there has been some loss of activity of samples stored at these elevated temperatures. However, most individual estimates do not differ significantly from 1 and there is no clear indication of greater loss at the higher than at the lower of these temperatures (see Table 3). Thus, no reliable prediction of degradation can be made. However, the limited loss, not apparently related to temperature, at these relatively extreme temperatures suggests that the preparation will have satisfactory stability.

Potency of sample coded C (low potency diphtheria toxoid adsorbed):

The individual assay estimates of the potency of sample C are detailed in Tables 1 and 2. They are illustrated graphically in Figure 3. As for sample B, unweighted geometric mean potency estimates (with 95 % cl) are given below, together with the conclusion of a χ^2 test for the homogeneity of the log potency estimates. The results from the invalid assays have been excluded from the calculations, resulting in only two estimates being from intradermal challenge assays and so only the mean is given here.

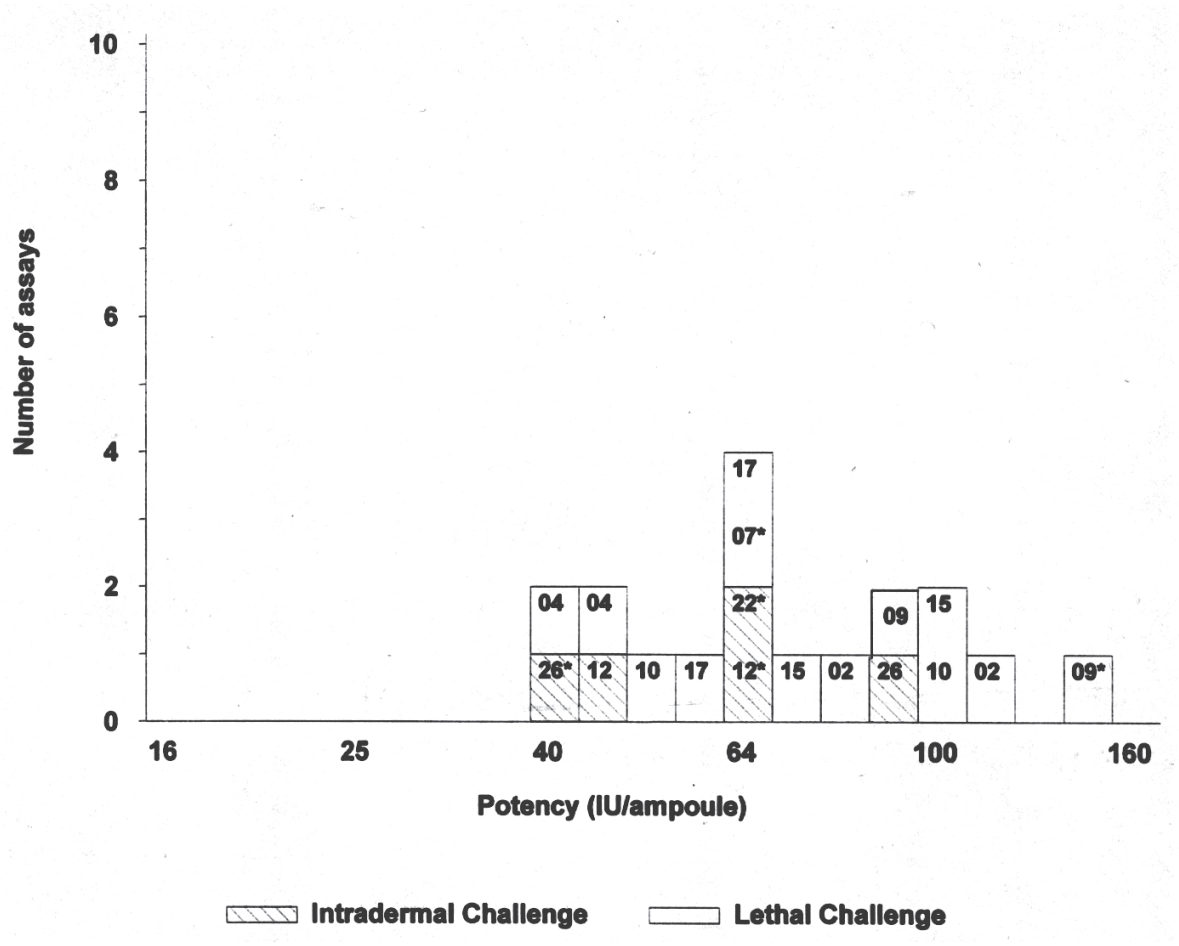

Figure 3. — *Potency of 'C' - Individual assay estimates (*denotes invalid assays)*

5. CONCLUSIONS

These data show good agreement in results obtained from lethal challenge and intradermal challenge methods. The limited loss of activity, at relatively extreme temperatures suggests that the candidate standard coded B will have satisfactory stability.

On the basis of this collaborative study and with agreement of all the participants it was recommended that the candidate standard coded B be established as the Third International Standard for Diphtheria Toxoid, Adsorbed with an assigned unitage of 160 IU/ampoule. It was also recommended that the same candidate standard (sample coded B) be established as the new European Pharmacopoeia Biological Reference Preparation working standard (batch No. 3). These recommendations were accepted by the Expert Committee on Biological Standardisation (ECBS) of WHO, by the Steering Committee of the Biological Standardisation Programme of EDQM and by the European Pharmacopoeia Commission(1).

6. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to the project leaders, Dr. Dorothea Sesardic and Dr. Randi Winsnes and to their collaborators Mr. Peter Rigsby and Dr. Rose Gaines Das as well as to all participants and donors of samples for their contributions to this study. This joint WHO/EDQM study was run under the aegis of the Biological Standardisation Programme under the internal number BSP030 with Jean-Marc Spieser acting as project coordinator.

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Table 1. — *Potency Estimates (IU/ampoule) for Intradermal Challenge Assays*

Lab	Assay	B	F (B at 37 °C)	E (B at 45 °C)	D (B at 56 °C)	C
3	1	205.6	.	.	123.8	.
	2	185.2	.	.	57.1[B,C]	.
12	1	.	.	.	.	64.2[A]
	2	70.6	.	.	.	42.6
	3	80.9	.	.	.	.
	4	98.1	.	.	.	.
13	1	244.2[A]	174.0[A]	.	.	.
	2	189.7	158.6	.	.	.
	3	108.6[A]	113.2	.	.	.
16	1	201.2	.	165.3	.	.
	2	164.4	.	148.9	.	.
22	1	137.7[B]	.	.	.	64.0[A]
26	1	125.7	.	.	.	86.0
	2	.	.	.	.	41.1[A, C]
	3	201.6	.	.	.	.
	4	132.2	.	.	89.6[C]	.

[A] denotes invalid assay due to non-linearity of one or more preparations.
[B] denotes invalid assay due to non-parallelism of preparations.
[C] denotes invalid assay due to fiducial limits of potency estimate not satisfying validity requirements.
(All sources of invalidity are shown for each estimate).

Table 2. — *Potency Estimates (IU/ampoule) for Lethal Challenge Assays*

Lab	Assay	B	F (B at 37 °C)	E (B at 45 °C)	D (B at 56 °C)	C
2	1	152.1[A]	.	.	.	75.8
	2	237.8	.	.	.	108.3
4	1	123.0	.	.	.	38.5
	2	195.3	.	.	.	45.4
6	1	138.9	.	.	128.1	.
	2	168.0	.	.	153.3	.
7	1	104.2	.	.	.	61.7[A]
8	1	133.5[A]	.	.	100.9[A]	.
	2	124.7	.	.	92.6	.
9	1	201.3	.	.	.	145.4[C]
	2	105.6	.	.	.	44.8
10	1	140.6	.	.	.	49.6
	2	224.8	.	.	.	95.8
11	1	187.4	.	133.0	.	.
	2	172.2	.	114.3	.	.
14	1	88.6	78.5	.	.	.
	2	155.4	126.9	.	.	.
15	1	64.4	.	.	.	74.2
	2	249.2	.	.	.	100.1
17	1	147.4	.	.	.	66.4
	2	103.7[B]	.	.	.	58.1
18	1	152.1	.	112.2	.	.
	2	168.4	.	125.0[B]	.	.
20	1	190.0	123.9	.	.	.
	2	171.3	137.5	.	.	.
	3	122.4	121.6[A]			
24	1	753.7[C]	626.7[C]	.	.	.
	2	722.1[C]	423.2[C]	.	.	.

For explanatory notes see footnote of Table 1.

Table 3. — *Potencies of degradation samples D, E and F relative to candidate standard sample preparation B with 95% confidence limits (shown in brackets)*

Laboratory	Assay	F (37°C)	E (45°C)	D (56°C)
Intradermal challenge assays:				
3	1	.	.	0.57 (0.29,1.08)
	2	.	.	0.20[C] (0.29,1.08)
13	1	0.69 (0.45,1.00)	.	.
	2	0.83 (0.41,1.57)	.	.
	3	1.02[A] (0.63,1.68)	.	.
16	1	.	0.83 (0.66,1.02)	.
	2	.	0.90 (0.71,1.13)	.
26	1	.	.	0.76[C] (0.33,1.43)
Lethal challenge assays:				
6	1	.	.	0.91 (0.65,1.28)
	2	.	.	0.90 (0.60,1.33)
8	1	.	.	0.74[A] (0.48,1.13)
	2	.	.	0.73 (0.44,1.15)
11	1	.	0.70 (0.47,1.00)	
	2	.	0.62 (0.43,0.87)	
14	1	0.88 (0.67,1.12)	.	.
	2	0.82 (0.63,1.03)	.	.
18	1	.	0.71 (0.44,1.06)	.
	2	.	0.75 (0.46,1.13)	.
20	1	0.64[A] (0.40,1.00)	.	.
	2	0.77 (0.50,1.15)	.	.
	3	0.99[A] (0.61,1.58)		
24	1	0.72[C] (0.00,5.49)		
	2	0.68[C] (0.32,1.21)		
Mean: (unweighted geometric mean estimate with 95% confidence limits) -excludes invalid assay estimates		0.80 (0.71, 0.89)	0.75 (0.65, 0.86)	0.76 (0.54, 1.08)

For explanatory notes see footnote of Table 1.

Table 4. — *Summary of data returned*

Laboratory	Preparations	Number of assays	Number* of animals per vaccine dilution (cage numbers)
Intradermal challenge assays:			
3	A, B, D	2	9 (4+5)
12	A, B, C	1 (A,C) 1 (A,B,C) 2 (A,B)	8
13	A, B, F	3	8 (4+4)
16	A, B, E	2	10
22	A, B, C	1	12 (6+6)
26	A, B, C, D	1 (A,C) 1 (A,B,C) 1 (A,B) 1 (A,B,D)	8 (4+4)
Lethal challenge assays:			
2	A, B, C	2	16
4	A, B, C	2	18 (6+6+6)
6	A, B, D	2	16
7	A, B, C	1	14
8	A, B, D	2	14 (7+7)
9	A, B, C	2	14 (7+7)
10	A, B, C	2	17
11	A, B, E	2	14 (7+7)
14	A, B, F	2	16 (4+4+4+4)
15	A, B, C	2	16 (4+4+4+4)
17	A, B, C	2	16 (2+2+2+2+2+2+2+2)
18	A, B, E	2	16 (8+8)
20	A, B, F	3	14 (7+7)
24	A, B, F	2	12

(*may vary for some assays).

Table 5. — *Percentage of animals responding (surviving) for lethal challenge assays (* denotes that the dose was omitted from the analysis)*

Preparation A (DIXA, I.S.)

Lab →	2		4		6		7	8		9		10		11		14		15		17		18		20			24	
Assay →	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3	1	2
Dose (IU/ml)																												
0.5								0	0											0	0							
↓					38	44								7	0													
1			57	50			0	64	36	7	71									6	31	6	0	0	0*	0	8	17
↓	13	13			100	100				36	85	18	0	36	64	25	0	50	6									
2	69	56	93	69			29	64	64							81	44			63	69	44	38	7	0	7	25	33
↓					100*	100*				57	100	29	18	71	79			80	56									
4	100	88	100*	100*			79	100	100	86	93*			100	100	100	88			88	75	88	88	64	64	50	50	42
↓												35	59					100	94									
8							100					88	77											92	100	100		

Table 6. — *Percentage of animals responding (surviving) for lethal challenge assays (* denotes that the dose was omitted from the analysis)*

Preparation B (doses assuming a potency of 200 IU/ampoule)

Lab →	2		4		6		7	8		9		10		11		14		15		17		18		20			24	
Assay →	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3	1	2
Dose (IU/ml)																												
0.5								0	0											0*	0*							
↓					19	44								14	21													
1			11	33			7*	29	7	21	14	12		29	36	0	0	0	31	0	0	6	0	0	0	0	50	42
↓	37	31			81	81				36	57																	
2	19	81	71	89			7	57	57	57	79	24	15	57	64	6	13	31	60	38	6	25	25	21	14	7	57	67
↓					94	100																						
4	69	81	100	94			57	86	71	79	100	41	50	100	93	63	75	63	100	75	81	69	75	50	50	15	79	92
↓																												
8							79					59	80											86	79	64		

Table 7. — *Percentage of animals responding (surviving) for lethal challenge assays (* denotes that the dose was omitted from the analysis)*

Preparation C (doses assuming a potency of 50 IU/ampoule)

Lab →	2		4		6		7	8		9		10		11		14		15		17		18		20		24	
Assay →	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Dose (IU/ml)																											
0.5																				6	0						
↓																											
1			11	33						79	7									25	31						
↓	75	88					50			86	58	12	24					75	25								
2			71	89																69	69						
↓	94*	88*					36			100	58	18	47					94	50								
4	94*	94*	100	94						100*	100									94	94						
↓							64					53	59					100	88								
8							100					77	88														

Table 8. — *Percentage of animals responding (surviving) for lethal challenge assays (* denotes that the dose was omitted from the analysis)*

Preparation D (doses assuming a potency of 200 IU/ampoule)

Lab →	2		4		6		7	8		9		10		11		14		15		17		18		20		24	
Assay →	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Dose (IU/ml)																											
0.5								0*	0																		
↓					13	38																					
1								0	8																		
↓					75	75																					
2								36	14																		
↓					94	100																					
4								86	64																		
↓																											
8																											

Table 9. — *Percentage of animals responding (surviving) for lethal challenge assays*

Preparation E (doses assuming potency of 200 IU/ampoule)

Lab →	2		4		6		7	8		9		10		11		14		15		17		18		20		24	
Assay →	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Dose (IU/ml)																											
0.5																											
↓																											
1														7	0							0	6				
↓														14	7												
2																						13	13				
↓														50	43												
4														71	86							50	44				
↓																											
8																											

Table 10. — *Percentage of animals responding (surviving) for lethal challenge assays (* denotes that the dose was omitted from the analysis)*

Preparation F (doses assuming a potency of 200 IU/ampoule)

Lab →	2		4		6		7	8		9		10		11		14		15		17		18		20			24	
Assay →	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3	1	2
Dose (IU/ml)																												
0.5																												
↓																												
1																0*	0*							0*	0*	0	42	25
↓																												
2																0	0							8	7	7	58	50
↓																												
4																50	56							36	21	14	67	83
↓																												
8																								57	79	64		

Table 11. — *Summary of results for potencies of sample coded B (95% confidence limits are given in brackets)*

	N	Mean (IU/ampoule)	Homogeneous estimates?
All assays:	34	147.6 (130.9 - 166.3)	No ($p < 0.001$)
	30	161.4 (147.1 - 177.1)*	No ($p = 0.001$)
Intradermal assays:	11	141.3 (108.8 - 183.6)	No ($p < 0.001$)
	9	168.9 (150.3 -189.9)*	Yes ($p = 0.09$)
Lethal assays:	23	150.7 (130.9 - 173.5)	No ($p < .001$)
	21	160.9 (144.7 -180.2)*	No ($p = 0.001$)

*Results are expressed as geometric mean IU of the second I.S. (sample A) per ampoule of sample B. Non-homogeneous ($p<0.05$) estimates are combined as unweighted geometric means. Homogeneous estimates are combined as weighted geometric means. * denotes mean excluding unreliable estimates as noted in the text. N denotes number of potency values included in the calculation of the mean.*

Table 12. — *Summary of results for potency of sample coded C (95% confidence limits are given in brackets)*

	N	Mean (IU/ampoule)	Homogeneous estimates?
All assays:	13	64.3 (51.8 - 79.7)	No ($p < 0.001$)
Intradermal assays:	2	60.5	No ($p < 0.03$)
Lethal assays:	11	65.0 (51.2 - 82.6)	No ($p < 0.001$)

Results are expressed as geometric mean IU of the second I.S. (sample A) per ampoule of sample C. Non-homogeneous ($p<0.05$) estimates are combined as unweighted geometric means. N denotes number of potency values included in the calculation of mean.

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF

A EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION FOR

BORDETELLA PERTUSSIS MOUSE ANTISERUM FOR SEROLOGICAL POTENCY TESTING OF ACELLULAR PERTUSSIS VACCINES

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF A EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION FOR *BORDETELLA PERTUSSIS* MOUSE ANTISERUM FOR SEROLOGICAL POTENCY TESTING OF ACELLULAR PERTUSSIS VACCINES

Project Leaders: F. Fuchs[(1)], R. Dobbelaer[(2)]

1. INTRODUCTION

A collaborative study was organised by the European Directorate for the Quality of Medicines (EDQM)[(3)] to assess the suitability of a candidate mouse antiserum batch specific to purified *Bordetella pertussis* antigens as a European Pharmacopoeia Biological Reference Preparation (BRP) appropriate for acellular pertussis vaccine potency test. After a preliminary phase (phase I) had been performed in three pilot laboratories, phase II was undertaken by enrolling 13 laboratories.

2. SCOPE

Following the implementation of the European Pharmacopoeia (Ph. Eur.) monograph on *Pertussis vaccine (acellular component, adsorbed*) (*1356*), the potency assay on the final lot should be performed by using an immunogenicity assay. The mouse immunogenicity assay, as described in the Ph. Eur. monograph and currently used for the approved acellular pertussis vaccines, is based on the measurement of the specific antibody responses to the different antigenic components present in the vaccine. Serum antibody levels are measured by ELISA and the immunogenicity of a vaccine being tested is compared to that of a reference vaccine.

Antibody responses to any given dose of an acellular pertussis component have been found to vary considerably between individual mice, and the ELISA part of the potency test is itself subject to some variability. Therefore, in order to allow a valid quantitation of antibody levels, a calibrated reference antiserum has to be included in every ELISA assay. This procedure allows the selection of serum dilutions within the quantitation limits of an ELISA assay and to express results in ELISA Units (ELU) that are consistent between assays.

Some manufacturers of acellular pertussis vaccines as well as some control authorities (e.g. USFDA and JNIH) have established such reference antisera. Recently, a WHO International Standard for acellular pertussis antiserum was calibrated and established against existing reference antisera (WHO/BS document 99.1901).

In Europe, various in-house reference antisera, usually calibrated against the USFDA reference antiserum [US Standard Pertussis antiserum (Mouse) Lot 1; SPAM-1] have been used by manufacturers and Official Medicines Control Laboratories (OMCLs). The availability of

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(3) Abbreviations: AFSSAPS: Agence Française de Sécurité Sanitaire des Produits de Santé, ANOVA: Analysis of Variance, BRP: European Pharmacopoeia Biological Reference Preparation, c: candidate, EDQM: European Directorate for the Quality of Medicines, ELISA: Enzyme linked immunosorbent assay, ELU: ELISA Units, FHA: Filamentous haemagglutinin, Fim 2/3: Fimbrial-2/Fimbrial-3 antigens, gCV: Geometric Coefficient of Variation, ihr: in-house reference, IS: International standard, ISSP-LP: Institut Scientifique de Santé Publique Louis Pasteur, JNIH: Japanese National Institute of Health, OMCL: Official Medicines Control Laboratory, OD: Optical density, Ph. Eur.: European Pharmacopoeia, PRN: Pertactin, PT: Pertussis toxin, s = Standard deviation on $\log_{10}$ scale, SEM: Standard error of the mean, SOP: Standard operating procedure, SPAM-1: USFDA reference antiserum, USFDA: United States Food and Drug Administration, WHO: World Health Organization.

a large batch of a European Pharmacopoeia Biological Reference Preparation (BRP) for acellular pertussis antiserum that could be used in routine release assays by both manufacturers and OMCLs should improve inter-laboratory comparability of results within Europe.

The EDQM has freeze-dried a large batch of *Bordetella pertussis* antiserum raised by immunising a large number of CD1 mice with a 5 component acellular pertussis vaccine similar in composition to that used for generating the USFDA antiserum and prepared by the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS) following a similar immunisation schedule. A preliminary characterisation of the antiserum showed that it contains quantifiable amounts of antibodies against pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (PRN) and fimbrial-2/fimbrial-3 (Fim 2/3) antigens. This preparation, still in the liquid state, was also included in the WHO study mentioned above.

The statistical analysis of this study, undertaken by AFSSAPS, demonstrates that the cBRP is suitable for use as a reference antiserum in the serological potency assays for acellular pertussis vaccines.

2.1. Pre-qualification phase (phase I)

Following a characterisation study by western blot analysis, a pre-qualification phase, referred to as phase I, has been run between June and October 1999. This study was performed in order to assess the suitability of the cBRP and to document the impact of differences in ELISA methodologies on the estimation of its antibody content. It consisted of a complete crossover study between different methodologies (different coating antigens, reagents and ELISA Standard Operating Procedures) and antisera generated with different vaccines (in-house reference sera and control sera).

The three laboratories involved in phase I received all in-house references and control sera as well as all reagents and standard operating procedures (SOP) necessary to perform assays by means of five different ELISA methodology groups referred to as A-E (Table 1). For feasibility reasons (availability of reagents), some of them did not strictly follow the provided SOPs.

Table 1 — *Proposed procedures (Phase I)*

Method	Plates	Conjugates	Substrate and others
A	Dynatech Immulon II	Goat anti-mouse IgG-ALP (Zymed or equivalent)	BSA (SIGMA)* Goat serum (Cappel) PNPP (SIGMA)*
B	NUNC Immunoplate F96 Maxisorb	Biotinylated anti-mouse $F(ab')_2$ from rabbit Ig (Dakopatts or equivalent) Biotinylated streptavidin-peroxidase complex (Amersham)	OPD (SIGMA)* BSA (SIGMA)
C	Greiner code 655001	Goat anti-mouse Ig antibody (IgM, IgG, IgA, H+L)- ALP -anti-mouse IgG-ALP (Kirkeegard – Perry Laboratories or equivalent)	PNPP (SIGMA)* BSA (SIGMA)* FCS (Gibco BRL)
D	ELISA Maxisorp NUNC	Peroxidase conjugated goat anti-mouse IgG (H+L) (Jackson or equivalent)	OPD (SIGMA)*
E	Dynatech F-form M129A	Anti-mouse IgG (whole molecule) peroxidase (Sigma or equivalent)	BSA (SIGMA)* OPD (SIGMA)*

Abbreviations:

Ig: Immunoglobulin; OPD: Orthophenylenediamine; ALP: Alkaline phosphatase; PNPP: Paranitrophenyl phosphate; BSA: Bovine serum albumin; FCS: Fetal calf serum

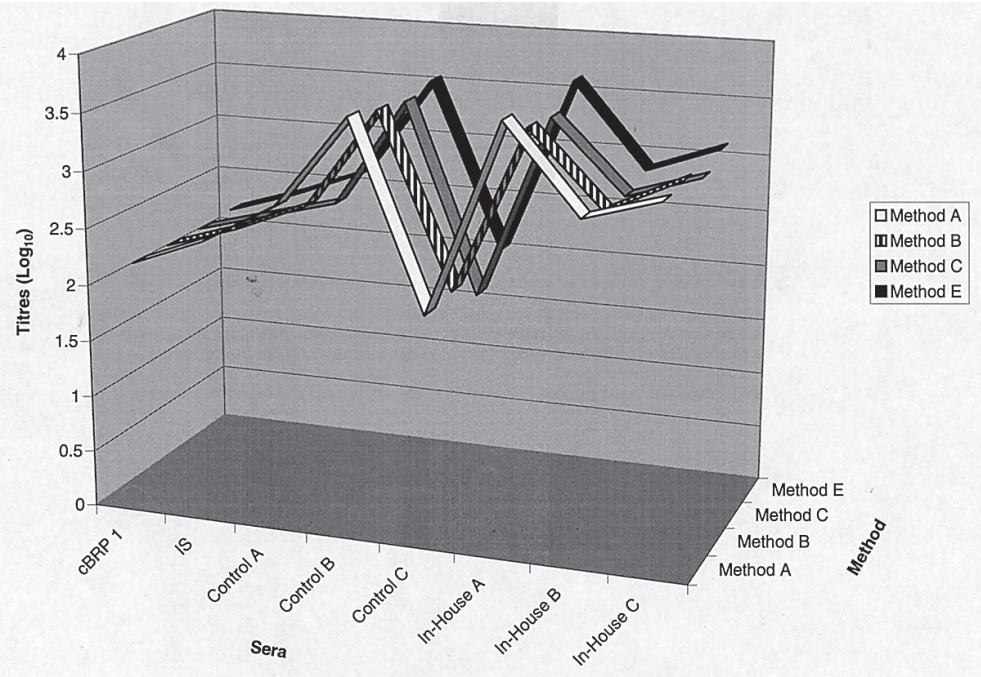
* These reagents replace OPD, PNPP or BSA of different origins mentioned in the manufacturers SOPs.

In phase I, the Fim 2/3 were not analysed due to lack of corresponding ELISA coating antigens and reagents.

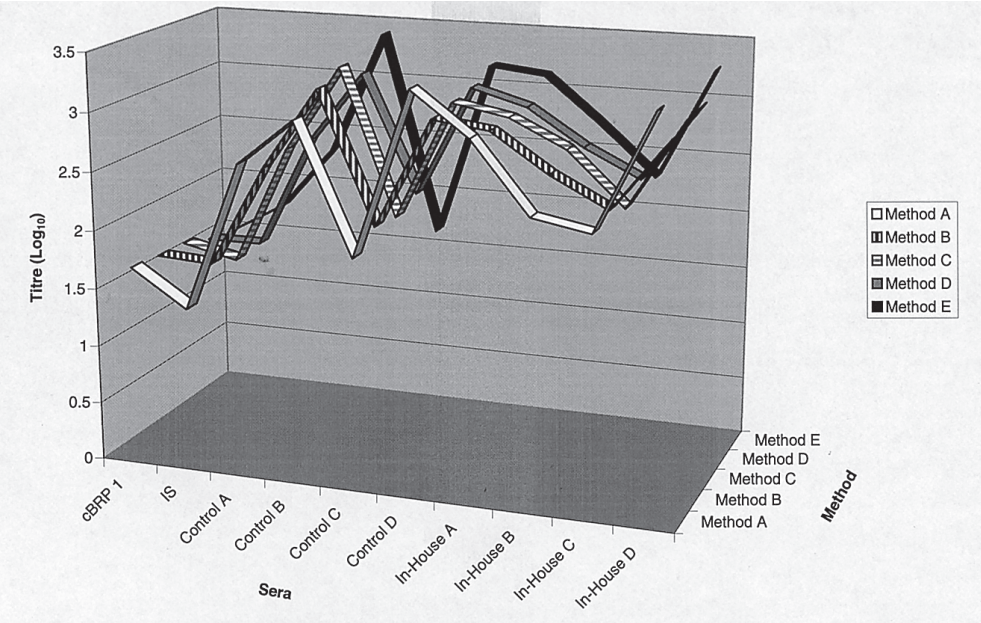
In phase I, the impact of the method factor was analysed by comparing titration results obtained by the different methodologies for all tested sera (internal controls and standards, cBRP, IS) versus reference serum SPAM-1.

It could be observed (Figure 1) that, for all the tested sera, titres and variability according to the method were homogeneous. The maximum titre difference between methods did not exceed $0.5\log_{10}$.

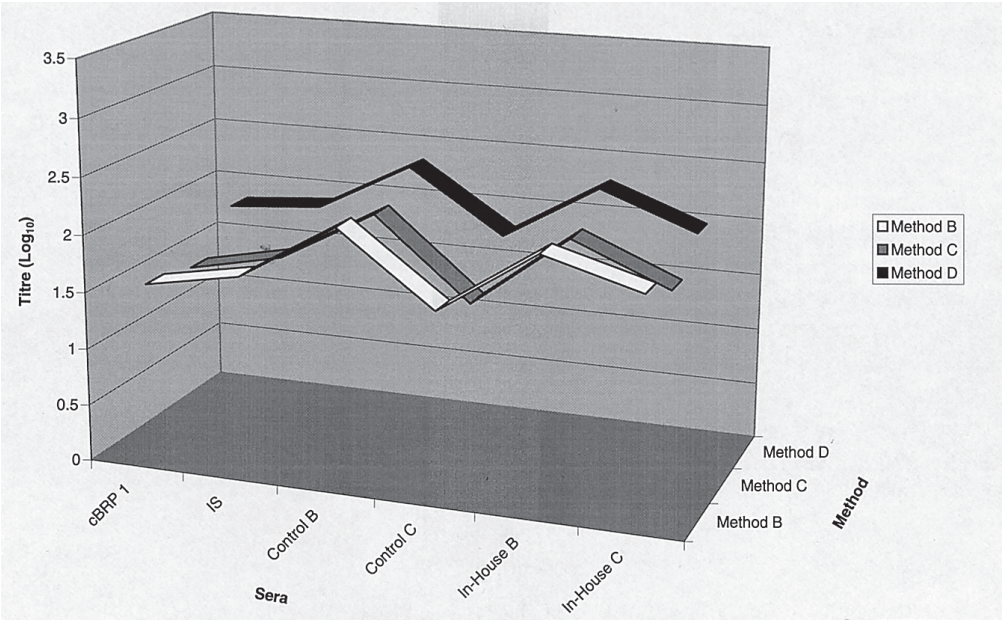
Figure 1. — *Influence of the method in relation to the component tested*



Influence of the method - FHA antigen



Influence of the method - PT antigen



Influence of the method - PRN antigen

Only one method (method E) raised problems of quantification for PT and PRN antigen. This is probably due to the choice of the conjugate, the working dilutions and the incubation time.

The evaluation allowed to conclude that all methodologies tested seemed to be equivalent regarding their ability to calibrate the candidate European, International and in-house references relative to SPAM-1. Repeatability and reproducibility were shown to be satisfactory and no significant bias related to methodology could be identified.

Furthermore, the use of one single methodology did not seem to offer any particular advantage. In general, the variability observed appeared to be representative of the variability encountered in acellular pertussis ELISA assays. Therefore, it was decided to run an enlarged phase, referred to as phase II, allowing participants to use their own in-house method rather than prescribing one single methodology.

2.2. Phase II

Phase II, run between November 1999 and April 2000, was intended to calibrate the *Bordetella pertussis* antiserum Ph. Eur. cBRP in terms of anti-PT, anti-FHA, anti-PRN and anti-Fim 2/3 antibody titres expressed in ELU relative to the USFDA reference serum (SPAM-1) on a sufficiently large database. Furthermore, by including the in-house reference material and control sera currently used by the participating laboratories, Phase II was intended to confirm, under routine assay conditions, the information obtained in Phase I.

3. PARTICIPANTS

Thirteen laboratories participated in phase II of this study. All participants routinely perform ELISA assays measuring anti-FHA, anti-PT, anti-PRN and/or anti-Fim 2/3 antibodies. In this report, the participants are referred to by using an arbitrary assigned number, not related to the order of listing at the end of this report (see 8.).

4. MATERIAL AND METHODS

4.1. Material

The following antisera were provided:

— The USFDA reference antiserum (SPAM-1)

— The WHO International Standard antiserum (IS) (Antipertussis serum, mouse, 97/642)

— The candidate Ph. Eur. Biological Reference Preparation (cBRP).

4.2. Methods

The participants were requested to test the samples using their in-house ELISA methodologies for anti-PT, anti-FHA, anti-PRN and anti-Fim 2/3 antibody content measurement, as available or applicable. Participants followed their control methods using their own reagents and SOPs. For the cBRP, new vials had to be used for each independent assay.

To facilitate independent statistical evaluation, the participants were asked to comply with the ELISA plate layout provided (all the test sera fit onto a single ELISA plate) and to assay eight consecutive dilutions of each reference and test sera.

4.3. Study design

Each participant was requested to perform three independent ELISA assays performed in different weeks.

4.4. Statistical analysis

Participants were asked to provide calculated results using their in-house mathematical models, but an independent statistical evaluation of all the raw data was undertaken by AFSSAPS.

4.4.1. Mathematical models used by the participants

The participants reported both their raw data and the results of their own calculations. The calculation methods used by the participants were as follows:

Laboratories 2, 3, 6 and 13 used a 4-Parameter logistic fit.

Laboratories 9, 11 and 12 used a parallel line model with log dose transformation.

Laboratories 1, 5 and 8 used a parallel line model with log dose / log OD transformation.

Laboratories 4 and 10 used another calculation methodology: “Reference line calculation”.

Laboratory 7 did not report its calculation methodology.

4.4.2. Statistical analysis by AFSSAPS

Raw data from 120 micro-titration plates were independently analysed at AFSSAPS. This comprised data from 13 laboratories which carried out anti-PT assays, 12 laboratories which carried out anti-FHA assays, 11 laboratories which carried out assays for anti-PRN and 4 laboratories which carried out anti-Fim 2/3 assays.

Optical densities corresponding to each individual assay were analysed. Unit calculations were performed with the Biolise 2.0 software (Biolise). ELISA unitage for each sample was assigned relative to the reference sera placed on each microtiter plate (SPAM-1, IS, cBRP or ihr).

A 4-parameter dose response curve[4] was generated for the reference serum by plotting the absorbance as a function of the logarithmically transformed antibody concentration data in ELU/ml. The quantitative range was identified as the absorbance range between 10 and 80 % of the asymptotic absorbances. A serum was considered non-quantifiable when ODs were below or above the quantification limits.

In order to obtain a normal distribution of the antibody unitage data, a logarithmic transformation was applied. The statistical study required Analysis of Variance (ANOVA) and usual statistical tests. The geometric Coefficient of Variation (gCV), defined as $100*(10^s-1)$, where s represents the standard deviation on $\log_{10}$ scale, was calculated. Comparisons between laboratories were evaluated by ANOVA and Newman-Keuls test. The ANOVA decomposes the variability into contributions from various factors. The Newman-Keuls multiple comparison procedure discriminates among the means. With this method, there is a 5.0 % risk of calling one or more pairs significantly different when their actual difference equals 0.

5. RESULTS

5.1. Calibration of the Ph. Eur. cBRP

The cBRP antiserum was calibrated versus SPAM-1 antiserum. Overall analysis of the data generated by all participating laboratories showed the following:

— Anti-FHA antibody titration (Figure 2.1):

Laboratory 10 gave statistically different results compared to the other laboratories. When the results of Laboratory 10 were excluded from the analysis, no significant difference in the results was observed (P = 0.726). The maximal difference observed between any two laboratories' mean titres did not exceed 0.4 $\log_{10}$.

— Anti-PT antibody titration (Figure 2.2):

All laboratories gave statistically different results but none can be shown in evidence as really different (P = 0.0004). The maximal difference observed between any two laboratories' mean titres did not exceed 0.4 $\log_{10}$.

— Anti-PRN antibody titration (Figure 2.3):

There is no statistically significant difference between laboratories (P = 0.362). The maximal difference observed between any two laboratories' mean titres did not exceed 0.3 $\log_{10}$.

— Anti-Fim 2/3 antibody titration (Figure 2.4):

No difference between laboratories achieved statistical significance (P = 0.721). The maximal difference observed between any two laboratories' mean titres did not exceed 0.2 $\log_{10}$.

(4) The equation of the 4-parameter logistic fit is:

$$\frac{a-d}{1+\left(\frac{x}{c}\right)^b}+d$$

— a and d represent the OD values of the asymptotes.

— c represents the logarithmic value of the concentration for which the response value will be centred between the two asymptotes (the inflection point).

— b represents the slope of the linear part of the curve (central part of the curve).

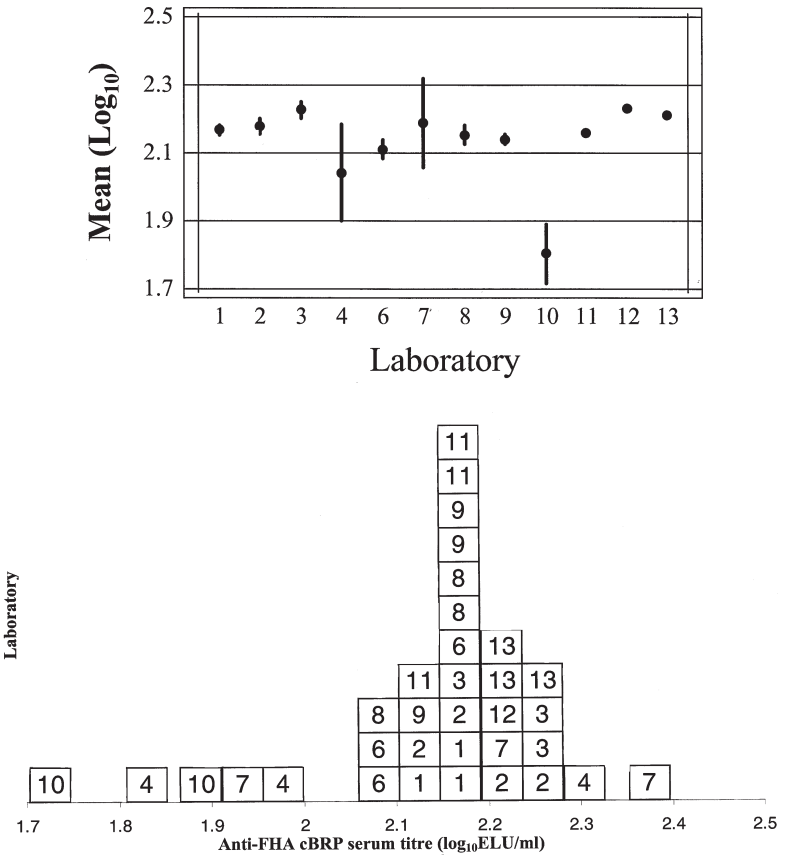


Figure 2.1. — *Results of cBRP antiserum calibration according to the laboratory: anti-FHA titres (mean ± SEM)*

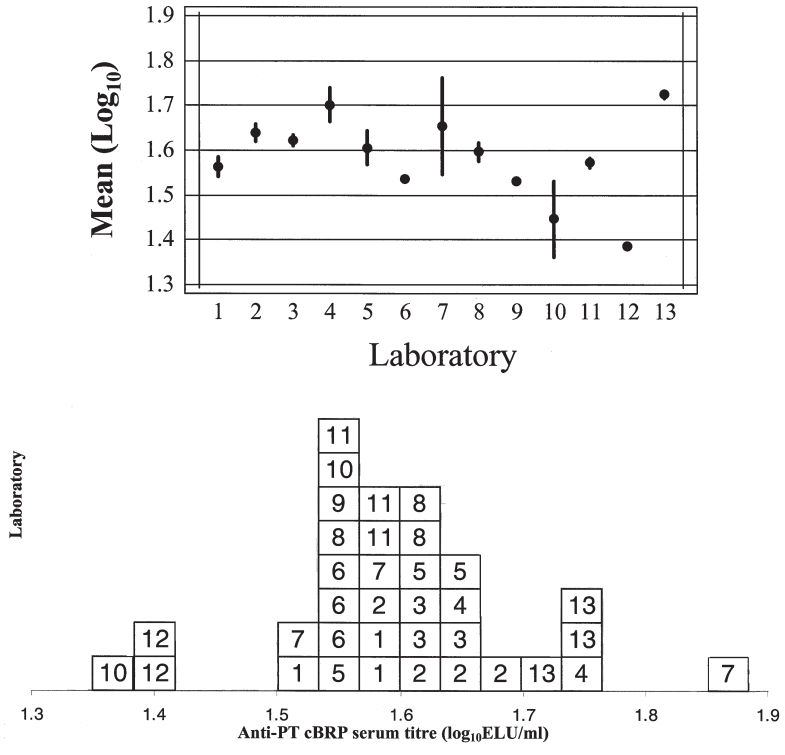


Figure 2.2. — *Results of cBRP antiserum calibration according to the laboratory: anti-PT titres (mean ± SEM)*

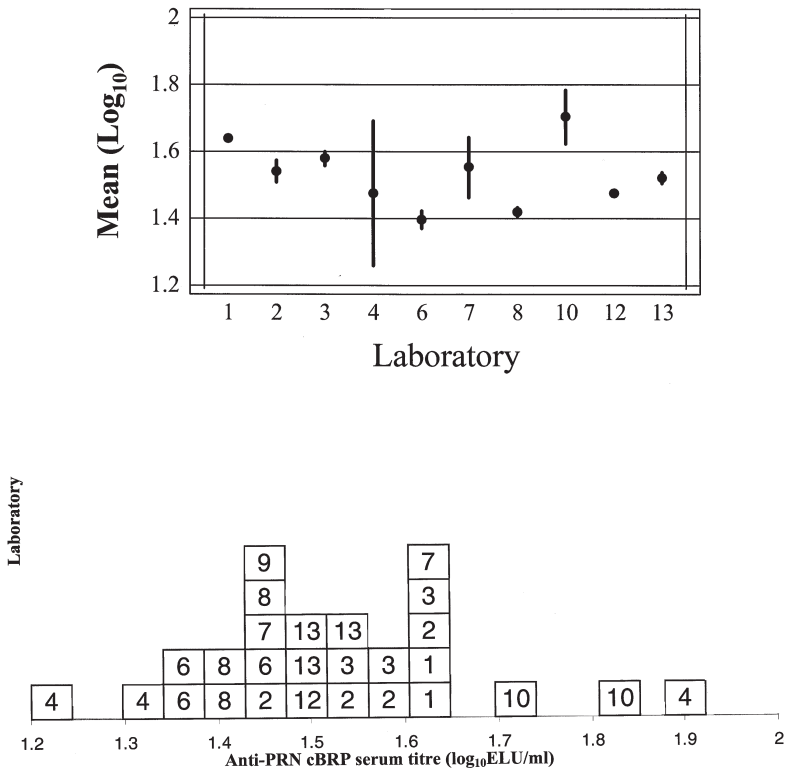


Figure 2.3. — *Results of cBRP antiserum calibration according to the laboratory: anti-PRN titres (mean ± SEM)*

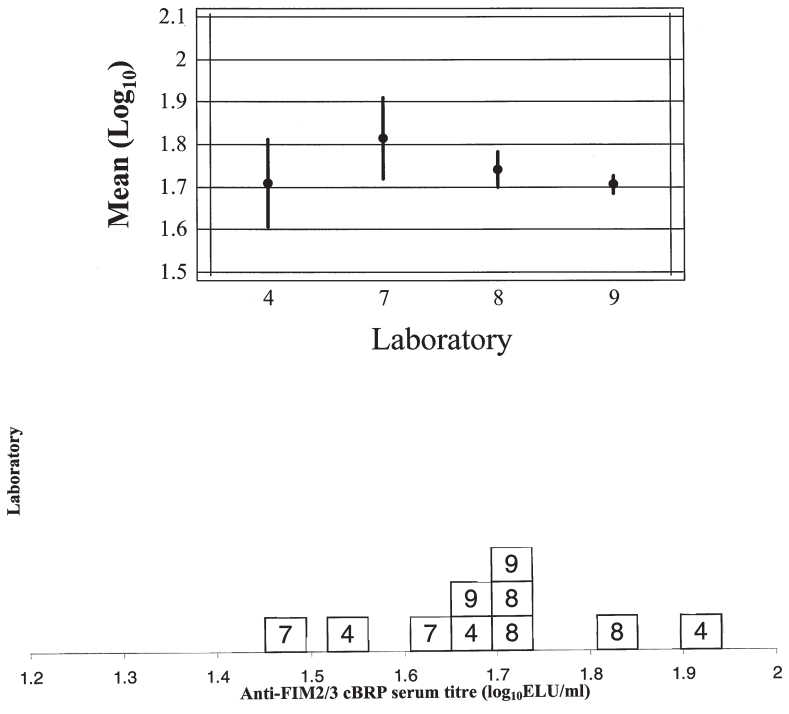


Figure 2.4. — *Results of cBRP antiserum according to the laboratory: anti-Fim 2/3 titres (mean ± SEM)*

Concerning the estimation of the variation of cBRP titrations versus SPAM-1 reference antiserum for all laboratories, the overall gCV is around 36 %, 28 %, 40 % and 31 %, for FHA, PT, PRN and Fim 2/3 components, respectively.

Segregation of variability by laboratory (Figures 2.1-2.4) indicates that the gCVs for each component are roughly equivalent and relatively low. It should be noted, however, that laboratory 4 (for FHA, PRN and Fim 2/3) and laboratory 7 (for FHA and PT) had gCV higher than 50 %. Assays from laboratory 10 showed relatively high variability for all components, but below 50 %.

A combination of all individual results enabled the assignment of antibody titres to the 5 components of acellular pertussis vaccines.

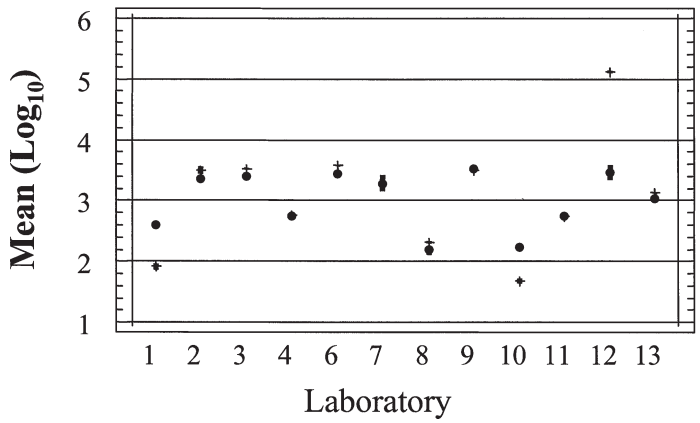
After reconstitution of the lyophilisate with 500 µl of *water R*, the unitage of cBRP antiserum is assigned as follows:

FHA : 138 ELU/ml PT : 39 ELU/ml PRN : 34 ELU/ml Fim 2/3 : 56 ELU/ml

5.2. Behaviour of internal controls and internal references

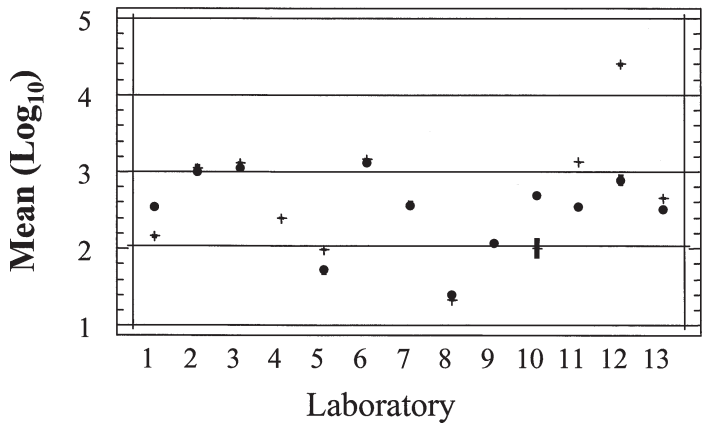
The relative antibody contents of internal references versus SPAM-1 reference obtained in this study showed differences with those previously established by some laboratories (Table 2).

Antibody contents of internal controls were expressed relative to the corresponding in-house reference serum (referred to as homologous situation) or relative to the cBRP antiserum (referred to as heterologous situation). It was shown (Figures 3.1-3.4) that statistically significant differences exist between titres obtained in homologous or heterologous situations. This may be explained by the differences between the unitages of "in-house" antisera estimated in this study versus SPAM-1 and the titres established by some manufacturers; these differences are roughly equivalent (Table 2).



\+ Titre of internal control versus "internal reference" serum (homologous situation)
● Titre of internal control versus cBRP serum (heterologous situation)

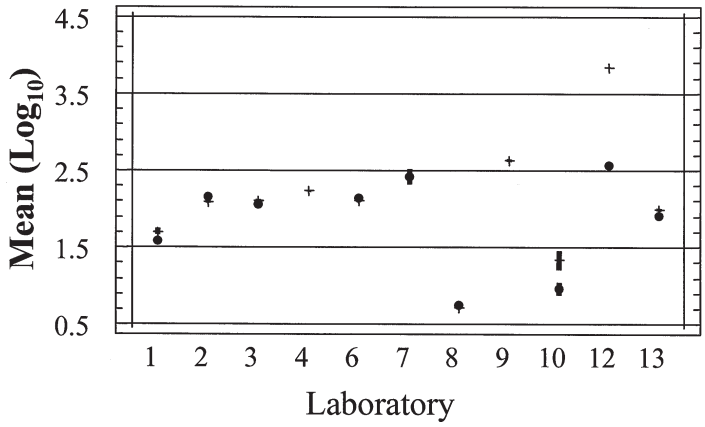
Figure 3.1. — *Behaviour of internal controls in homologous and heterologous situations according to the laboratory: anti-FHA titres (mean ± SEM)*



\+ Titre of internal control versus "internal reference" serum (homologous situation)

● Titre of internal control versus cBRP serum (heterologous situation)

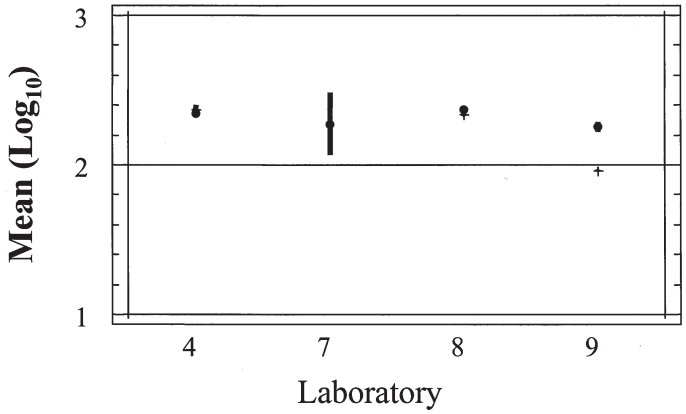
Figure 3.2. — *Behaviour of internal controls in homologous and heterologous situations according to the laboratory: anti-PT titres (mean ± SEM)*



\+ Titre of internal control versus "internal reference" serum (homologous situation)

● Titre of internal control versus cBRP serum (heterologous situation)

Figure 3.3. — *Behaviour of internal controls in homologous and heterologous situations according to the laboratory: anti-PRN titres (mean ± SEM)*



\+ Titre of internal control versus "internal reference" serum (homologous situation)

● Titre of internal control versus cBRP serum (heterologous situation)

Figure 3.4. — *Behaviour of internal controls in homologous and heterologous situations according to the laboratory: anti-Fim2/3 titres (mean ± SEM)*

Table 2. — *Comparison between assigned and calculated titres of "in-house" references*

		Antigen							
		FHA		PT		PRN		FIM 28.3	
		assigned	Calculated	assigned	Calculated	assigned	Calculated	assigned	Calculated
LAB 1	Titres ELU/ml	2600	15466	1400	4028	690	645		
	Titres (Log_{10})	3.41	4.19	3.15	3.61	2.84	2.81		
	Difference (Assigned-Calculated)(1)	– 0 ?77		–0.46		0.03			
	Difference (ihr-cBPR)(2)	– 0.73		– 0.41		0.09			
LAB 2	Titres ELU/ml	713	631	403	447	109	139		
	Titres (Log_{10})	2.85	2.80	2.61	2.65	2.04	2.14		
	Difference (Assigned-Calculated)(1)	0.05		– 0.05		– 0.11			
	Difference (ihr-cBPR)(2)	0.10		0.02		– 0.09			
LAB 3	Titres ELU/ml	713	717	403	427	109	118		
	Titres (Log_{10})	2.85	2.86	2.61	2.63	2.04	2.07		
	Difference (Assigned-Calculated)(1)	0.00		– 0.03		– 0.03			
	Difference (ihr-cBPR)(2)	0.09		0.02		0.01			
LAB 4	Titres ELU/ml	540	500	170	144	150	102	200	179
	Titres (Log_{10})	2.73	2.70	2.23	2.16	2.18	2.01	2.30	2.25
	Difference (Assigned-Calculated)(1)	0.03		0.07		0.17		0.05	
	Difference (ihr-cBPR)(2)	– 0.01		*******		*******		0.01	
LAB 5	Titres ELU/ml			1.832	*******				
	Titres (Log_{10})			0K26	*******				
	Difference (Assigned-Calculated)(1)			*******					
	Difference (ihr-cBPR)(2)			0.24					
LAB 6	Titres ELU/ml	713	528	403	349	109	94		
	Titres (Log_{10})	2.85	2.72	2.61	2.54	2.04	1.97		
	Difference (Assigned-Calculated)(1)	0.13		0.07		0.07			
	Difference (ihr-cBPR)(2)	0.10		.01		– 0.07			
LAB 7	Titres ELU/ml	*******	1349	*******	124	*******	445	*******	268
	Titres (Log_{10})	*******	3.13	*******	2.09	*******	2.65	*******	2.43
	Difference (Assigned-Calculated)(1)	*******		*******		*******		*******	
	Difference (ihr-cBPR)(2)	*******		*******		*******		*******	

(1) Difference between internal reference serum value assigned by the manufacturer and the titre calculated in the collaborative study versus SPAM-1 reference serum expressed in $\log_{10}$

(2) Difference between titres of the internal control in the homologous* and heterologous* situations expressed in $\log_{10}$

* As defined in section 5.2

******** Data not given or not quantifiable.

Not performed

Table 2 (continued). — *Comparison between assigned and calculated titres of "in-house" references*

		Antigen							
		FHA		PT		PRN		FIM 28.3	
		assigned	Calculated	assigned	Calculated	assigned	Calculated	assigned	Calculated
LAB **8**	**Titres ELU/ml**	286	275	34	44	60	52	64	72
	Titres (Log_{10})	2.46	2.44	1.53	1.65	1.78	1.72	1.81	1.86
	Difference (Assigned-Calculated)[1]	0.02		– 0.12		0.06		– 0.05	
	Difference (ihr-cBPR)[2]	0.09		– 0.10		– 0.07		– 0.04	
LAB **9**	**Titres ELU/ml**	1598	1894	90	101	52	44	74	147
	Titres (Log_{10})	3.20	3.28	1.95	2.00	1.72	1.64	1.87	2.17
	Difference (Assigned-Calculated)[1]	– 0.07		– 0.05		0.07		– 0.30	
	Difference (ihr-cBPR)[2]	– 0.06		*******		*******		– 0.31	
LAB **10**	**Titres ELU/ml**	2600	5878	1400	9045	690	409		
	Titres (Log_{10})	3.41	3.77	3.15	3.96	2.84	2.61		
	Difference (Assigned-Calculated)[1]	– 0.36		– 0.81		0.23			
	Difference (ihr-cBPR)[2]	– 0.59		– 0.73		0.34			
LAB **11**	**Titres ELU/ml**	3000	3584	2700	723				
	Titres (Log_{10})	3.48	3.55	3.43	2.86				
	Difference (Assigned-Calculated)[1]	– 0.08		0.57					
	Difference (ihr-cBPR)[2]	– 0.0		0.56					
LAB **12**	**Titres ELU/ml**	111220	5044	20724	384	4901	286		
	Titres (Log_{10})	5.05	3.70	4.32	2.58	3.69	2.46		
	Difference (Assigned-Calculated)[1]	1.34		1.73		1.23			
	Difference (ihr-cBPR)[2]	1.81		1.48		1.24			
LAB **13**	**Titres ELU/ml**	675	718	369	409	144	126		
	Titres (Log_{10})	2.83	2.86	2.57	2.61	2.16	2.10		
	Difference (Assigned-Calculated)[1]	0.03		– 0.04		0.06			
	Difference (ihr-cBPR)[2]	0.05		0.10		0.04			

(1) Difference between internal reference serum value assigned by the manufacturer and the titre calculated in the collaborative study versus SPAM-1 reference serum expressed in $\log_{10}$

(2) Difference between titres of the internal control in the homologous* and heterologous* situations expressed in $\log_{10}$

* As defined in section 5.2

******** Data not given or not quantifiable.

Not performed

5.3. Comparison of results from the WHO and EDQM collaborative studies

5.3.1. IS antiserum titration versus SPAM-1 antiserum (Table 3.1)

Results obtained in both studies are similar.

Table 3.1. — *WHO IS antiserum titration versus SPAM-1*

	EDQM collaborative study		WHO collaborative study	
Antibody	**$\log_{10}$**	**ELU/ml**	**$\log_{10}$**	**ELU/ml**
Anti-FHA	2.35	223	2.46	286
Anti-PT	1.47	29	1.53	34
Anti-PRN	1.66	46	1.78	60
Anti-Fim 2/3	1.66	45	1.81	64

5.3.2. cBRP antiserum titration versus SPAM-1 antiserum (Table 3.2)

The cBRP titres obtained in the phase II study are close to those obtained in the WHO collaborative study.

Table 3.2. — *cBRP antiserum titration versus SPAM-1*

	EDQM collaborative study		WHO collaborative study	
Antibody	**$\log_{10}$**	**ELU/ml**	**$\log_{10}$**	**ELU/ml**
Anti-FHA	2.14	138	2.25	178
Anti-PT	1.59	39	1.68	48
Anti-PRN	1.53	34	1.56	36
Anti-Fim 2/3	1.75	56	1.83	68

5.4. Comparison between laboratories' and AFSSAPS' calculations of unitage relative to SPAM-1

The results obtained by each participant did not differ substantially from those obtained by AFSSAPS with the exception of Laboratory 4 which obtained lower results with its own calculation method (Table 4).

Table 4. — *Individual assay results (calibration against SPAM-1 reference)*

			Serum potency expressed in ELU/ml							
Laboratory	Antigen	Assay	cBRP calculated	cBRP Lab.	IS calculated	IS Lab.	in-house calculated	in-house stated	Internal control calculated	Internal control Lab.
1	FHA	1	138	144	226	219	14167	13885	516	518
1	FHA	2	154	152	238	231	15459	15776	387	385
1	FHA	3	150	157	245	235	16892	15445	434	420
1	PT	1	33	34	23	26	4420	4234	354	326
1	PT	2	39	39	22	25	3975	3878	388	347
1	PT	3	38	38	22	24	3721	3447	312	270
1	PRN	1	44	36	57	56	618	628	47	30
1	PRN	2	*****	34	*****	52	*****	764	*****	33
1	PRN	3	43	38	53	53	673	715	49	41
2	FHA	1	140	138	200	199	481	477	2307	2294
2	FHA	2	135	138	225	224	841	836	2290	2272
2	FHA	3	170	171	278	276	569	568	2985	2970
2	FHA	4	161	160	269	269	687	681	2796	2781
2	PT	1	43	42	35	34	356	344	1047	1010
2	PT	2	39	38	37	36	583	565	1131	1094
2	PT	3	49	48	45	44	434	425	1323	1297
2	PT	4	44	43	42	41	444	433	1206	1173
2	PRN	1	33	33	52	52	128	127	125	125
2	PRN	2	29	29	43	43	115	113	136	133
2	PRN	3	37	37	60	60	177	174	175	175
2	PRN	4	41	41	60	59	143	142	157	156
3	FHA	1	150	150	295	295	768	768	3196	3196
3	FHA	2	181	181	290	290	662	662	2931	2930
3	FHA	3	176	176	296	296	725	725	2994	2994
3	PT	1	44	44	38	38	380	379	1182	1180
3	PT	2	42	42	40	40	437	436	1196	1194
3	PT	3	40	40	47	47	469	468	1426	1424
3	PRN	1	35	35	55	55	100	100	114	114
3	PRN	2	38	38	55	55	126	126	127	127
3	PRN	3	41	41	53	53	131	131	138	138
4	FHA	1	206	192	303	306	1022	1058	1060	1200
4	FHA	2	68	65	145	147	302	302	279	284
4	FHA	3	95	78	175	174	406	362	385	353
4	PT	1	*****	31	*****	22	*****	130	*****	202
4	PT	2	46	30	45	43	153	133	208	165
4	PT	3	55	25	57	41	136	113	169	146
4	PRN	1	80	31	78	54	196	203	209	199
4	PRN	2	21	11	45	28	86	73	85	80
4	PRN	3	16	8	37	34	63	59	68	65
4	FIM 2/3	1	81	46	57	42	359	380	339	346
4	FIM 2/3	2	36	26	31	34	129	145	145	145
4	FIM 2/3	3	46	19	34	16	124	126	168	147
5	PT	1	43	41	34	36	1.28	1.19	64	63
5	PT	2	45	45	46	47	1.24	1.22	58	59
5	PT	3	34	37	26	29	1.24	1.28	60	63
6	FHA	1	124	124	200	200	433	433	2285	2285
6	FHA	2	146	146	234	234	634	634	2902	2902
6	FHA	3	119	119	195	195	531	531	2517	2517
6	PT	1	34	34	23	23	331	331	1166	1166
6	PT	2	35	35	26	26	389	389	1178	1178
6	PT	3	34	34	24	24	331	331	1160	1160
6	PRN	1	23	23	36	36	88	88	92	92
6	PRN	2	24	24	38	38	90	90	94	94
6	PRN	3	28	28	37	37	104	104	115	115
7	FHA	1	88	*****	51	*****	235	*****	840	*****
7	FHA	2	171	*****	167	*****	1953	*****	2160	*****
7	FHA	3	245	*****	268	*****	5350	*****	5844	*****
7	PT	1	32	*****	13	*****	82	*****	357	*****
7	PT	2	39	*****	20	*****	93	*****	252	*****
7	PT	3	73	*****	41	*****	249	*****	934	*****
7	PRN	1	29	*****	16	*****	197	*****	199	*****
7	PRN	2	*****	*****	*****	*****	*****	*****	*****	*****
7	PRN	3	44	*****	58	*****	1006	*****	209	*****
7	FIM 2/3	1	50	*****	25	*****	150	*****	80	*****
7	FIM 2/3	2	55	*****	42	*****	179	*****	572	*****
7	FIM 2/3	3	101	*****	59	*****	719	*****	231	*****

calculated: AFSSAPS calculations

Lab: laboratory calculations

***** Data not given or not quantifiable.

Table 4 (continued). — *Individual assay results (calibration against SPAM-1 reference)*

			Serum potency expressed in ELU/ml							
Laboratory	**Antigen**	**Assay**	**cBRP calculated**	**cBRP Lab.**	**IS calculated**	**IS Lab.**	**in-house calculated**	**in-house stated**	**Internal control calculated**	**Internal control Lab.**
8	FHA	1	156	149	237	237	308	300	208	202
8	FHA	2	126	117	280	277	226	222	138	117
8	FHA	3	147	143	281	285	299	287	190	177
8	PT	1	41	39	29	27	57	56	32	33
8	PT	2	42	40	34	33	39	39	22	22
8	PT	3	36	37	40	39	39	38	25	24
8	PRN	1	26	26	29	27	52	54	4	5
8	PRN	2	28	27	53	51	52	50	4	9
8	PRN	3	25	25	47	48	52	53	5	4
8	FIM 2/3	1	67	68	59	50	83	86	281	289
8	FIM 2/3	2	50	49	60	59	66	61	197	178
8	FIM 2/3	3	50	54	62	64	68	67	217	252
9	FHA	1	129	*****	232	*****	1961	*****	3115	*****
9	FHA	2	144	*****	*****	*****	*****	*****	*****	*****
9	FHA	3	142	*****	208	*****	1830	*****	3456	*****
9	PT	1	*****	*****	*****	*****	*****	*****	*****	*****
9	PT	2	34	*****	35	*****	101	*****	120	*****
9	PT	3	*****	*****	*****	*****	*****	*****	*****	*****
9	PRN	1	*****	*****	*****	*****	*****	*****	*****	*****
9	PRN	2	*****	*****	*****	*****	*****	*****	*****	*****
9	PRN	3	28	*****	38	*****	44	*****	388	*****
9	FIM 2/3	1	50	*****	44	*****	146	*****	183	*****
9	FIM 2/3	2	49	*****	47	*****	152	*****	178	*****
9	FIM 2/3	3	56	*****	43	*****	142	*****	157	*****
10	FHA	1	52	57	131	136	5281	4470	74	96
10	FHA	2	*****	84	*****	88	*****	5378	*****	128
10	FHA	3	78	73	91	104	6542	5382	95	97
10	PT	1	34	33	9	9	6698	7082	591	577
10	PT	2	*****	30	*****	10	*****	6077	*****	421
10	PT	3	23	25	9	9	12215	10684	456	428
10	PRN	1	68	59	45	49	299	305	14	10
10	PRN	2	36	28	33	31	294	307	12	11
10	PRN	3	53	43	32	32	777	743	14	12
11	FHA	1	139	137	255	245	4022	3947	649	645
11	FHA	2	147	143	278	260	3356	3280	530	532
11	FHA	3	148	151	233	238	3412	3450	544	552
11	PT	1	37	36	22	22	793	782	330	366
11	PT	2	36	36	20	20	698	706	322	332
11	PT	3	39	40	20	20	683	689	346	360
12	FHA	1	*****	156	*****	309	*****	5979	*****	6844
12	FHA	2	170	173	283	272	5044	5203	5016	5083
12	FHA	3	*****	163	*****	264	*****	4904	*****	3930
12	PT	1	24	26	64	63	467	709	523	746
12	PT	2	24	24	*****	51	324	555	377	543
12	PT	3	25	25	*****	62	375	559	407	555
12	PRN	1	*****	30	*****	54	*****	312	*****	269
12	PRN	2	30	30	45	45	321	280	332	329
12	PRN	3	*****	36	47	52	255	247	295	291
13	FHA	1	161	161	309	309	729	733	1299	1299
13	FHA	2	163	163	322	322	740	740	1275	1275
13	FHA	3	163	163	295	295	687	687	1256	1256
13	PT	1	54	57	51	53	382	392	460	481
13	PT	2	51	52	49	49	419	414	441	438
13	PT	3	54	57	46	48	429	437	477	517
13	PRN	1	32	32	55	55	121	121	74	74
13	PRN	2	32	32	54	54	124	124	84	84
13	PRN	3	36	36	60	60	132	132	83	83

calculated: AFSSAPS calculations

Lab: laboratory calculations

***** Data not given or not quantifiable.

6. DISCUSSION AND CONCLUSION

Phase I of this study has shown that the Ph. Eur. cBRP for *Bordetella pertussis* mouse antiserum contains significant levels of specific antibodies to the *Bordetella pertussis* antigens PT, FHA, PRN et Fim 2/3 present in acellular pertussis vaccines.

Results obtained during phase I were confirmed by those obtained during phase II. Phase I showed that the use of a unique methodology did not seem to offer particular advantages and this is confirmed by the relative homogeneity of results obtained by different methodologies in phase II.

In phase II, unitages of the sera expressed in reference to SPAM-1 were similar when calculated by AFSSAPS than when calculated by each participant. Results were overall comparable and exhibited an homogeneous variability among laboratories. The most remarkable discrepancies observed (up to 1.7 $\log_{10}$) (Figures 3.1-3.4) concern the titres obtained for the positive controls tested in homologous or heterologous situations. These differences seemed to be linked to the preliminary calibration of internal references. Indeed, it appeared in each case that unitages obtained when testing internal references versus SPAM-1 were different from those established in the procedure (Table 2). These results stress the need to use a common reference.

The analysis of the outcome of this study has shown that the cBRP is suitable to be used as a reference antiserum. Therefore, the candidate preparation has been adopted by the Ph. Eur. Commission during the 107th Session in June 2000 as *Bordetella pertussis* mouse antiserum Ph. Eur. BRP batch 1(5) with the following unitages(6):

— anti-PT: 39 ELU/ml
— anti-FHA: 138 ELU/ml
— anti-PRN 34 ELU/ml
— anti-Fim 2/3: 56 ELU/ml

7. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to the project leaders, Dr. F. Fuchs (AFSSAPS) and Dr. R. Dobbelaer (ISSP-LP), to their collaborators and to all participants for their contributions to this study. The expertise and help of the statistician B. Poirier (AFSSAPS), are gratefully acknowledged. The editorial review was done by Dr. M.-E. Esposito-Farese and Mrs J.A. Trudeau. The study was run by the EDQM with the support of the Council of Europe and the European Commission, under the aegis of the Biological Standardisation Programme (internal project number BSP031) with Dr. M.-E. Esposito-Farese and Dr. G. Rautmann acting as project monitors.

8. PARTICIPANTS

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(5) Cat. No. B1142000

(6) After reconstitution of the lyophilisate with 500 µl of water R.

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF

TWO EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS FOR

CLOSTRIDIUM TETANI ANTISERUM FOR SEROLOGICAL POTENCY TESTING OF TETANUS VACCINES FOR VETERINARY USE

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF TWO EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS FOR *CLOSTRIDIUM TETANI* ANTISERUM FOR SEROLOGICAL POTENCY TESTING OF TETANUS VACCINES FOR VETERINARY USE

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1. INTRODUCTION

A test on potency is required for the batch quality control of finished product of tetanus vaccines for veterinary use. According to the specifications of the current Ph. Eur. monograph *tetanus vaccine for veterinary use* (*0697*) [1], potency is determined either by an indirect or direct challenge test in laboratory animals. In the indirect test (method A), guinea pigs or rabbits are given a primary and booster immunisation with tetanus vaccine and the *Clostridium* (*C.*)[3] *tetani* antitoxin seroresponse is estimated by a toxin neutralisation test (TNT) in mice using neurotoxin of *C. tetani*. In the direct test (method B), guinea pigs or mice are immunised with tetanus vaccine and challenged with *C. tetani* neurotoxin.

These two potency tests require large numbers of animals and cause serious distress to animals [1]. In particular for that reason it is envisaged to replace these tests by tests in which limited numbers of guinea pigs or rabbits are immunised followed by measurement of the seroresponse using a serological/*in-vitro* assay for *C. tetani* antitoxin [2].

In-vitro/serological assays, and in particular enzyme-linked immunosorbent assay (ELISA) and toxin binding inhibition (ToBI) assay for *C. tetani* antitoxin have been described as potential alternatives to the TNT in mice and the challenge test with *C. tetani* neurotoxin [3-7]. Both assays were validated for their suitability of estimating the potency of veterinary tetanus vaccines by an international collaborative study [8].

For the purpose of introducing validated serological assays for measuring the potency of veterinary tetanus vaccines, it was decided to develop appropriate reference preparations. As two laboratory animal species are involved in the proposed potency tests (primary and booster immunisation) and as it is known that different immunisation procedures and animal species may cause differences in antibody affinity [8, 9, 10] two preparations of *C. tetani* antitoxin serum, respectively of guinea pig and of rabbit origin, were to be developed by using immunisation schedules complying with the specifications of the proposed revised monograph *tetanus vaccine for veterinary use* [2].

A collaborative study was initiated in 1998 by the European Directorate for the Quality of Medicines (EDQM) with the objectives of producing (Phase I) and calibrating (Phase II)

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(3) Abbreviations: BRP = European Pharmacopoeia biological reference preparation, c: candidate, C.: Clostridium, EDQM: European Directorate for the Quality of Medicines, EC: European Commission, ELISA: Enzyme-linked immunosorbent assay, GCV: Geometric coefficient of variation, GMP: Good manufacturing practice, ID-Lelystad: Institute for Animal Science and Health, IS: International standard, IU: International Units, OD: Optical density, OMCLs: Official Medicines Control Laboratories, Ph. Eur. European Pharmacopoeia, QC: Quality control, RIVM: Rijksinstituut voor Volksgezondheid en Milieu, SOP: Standard operating procedure, TNT: Toxin neutralisation test, ToBI: Toxin binding inhibition, WHO: World Health Organization.

candidate (c) Ph. Eur. Biological Reference Preparations (BRPs) for *C. tetani* guinea pig and rabbit antisera.

Phase I was carried out along the line of Good Manufacturing Practice (GMP) principles at ID-Lelystad, under the responsibility of Dr. H. H. Lensing, the project leader. The objectives of Phase I were to develop a cBRP *C. tetani* guinea pig antiserum and a cBRP *C. tetani* rabbit antiserum for the performance of the proposed potency tests of the Ph. Eur. monograph *tetanus vaccine for veterinary use* [2]. The main features of phase I are developed in Section 2.

In Phase II an international collaborative study was organised by the EDQM. The objectives of phase II were to establish and to assign the two cBRPS official *C. tetani* antitoxin titres, expressed in International Units (I.U.) using an *in-vitro* technique. Due to the fact that the available WHO IS was equine, while the cBRPs were of rabbit and guinea pig origin the ToBI method, which is the only *in-vitro* method that enables the testing of sera of different species origin in the same test, was chosen as the establishment method. The main features of phase II are developed in Section 3.

2. PHASE I

Phase I aimed at developing the cBRPs and consisted of three steps:

— preparation of bulk materials,

— processing in final containers, and

— characterisation of finished product.

The three development steps were carried out at ID-Lelystad following the WHO guidelines for the preparation, characterisation and establishment of international standards for biological substances [11] and the EC principles and guidelines for GMP of veterinary medicinal products [12].

2.1. Preparation of bulk materials

Guinea pigs and rabbits, purchased from commercial SPF breeding farms, were immunised with a commercial, monocomponent, aluminium hydroxyde adjuvanted purified tetanus toxoid vaccine. The bulk materials (sera pools) of each animal species were sterile filtrated, inactivated for 30 min at + 56 ± 1 °C and stored at – 20 ± 1 °C.

2.2. Processing in final containers

In order to obtain the requested volume of bulk materials for preparing a sufficient amount of vials of the cBRPs, the bulk materials were diluted with *C. tetani* antitoxin negative serum from guinea pigs or rabbits, respectively of the same origin as those used for the preparation of the bulk materials. Thereafter, the diluted homogeneous bulk materials were filled under aseptic conditions into sterile vials (filling volume: 0.8 ml ± 2 %) and freeze-dried. The two resulting batches of BRPs consisted of approximately 2 000 vials each.

2.3. Characterisation of finished products

The characterisation of the finished products of the cBRPs was carried out along the line of Good Quality Control Laboratory Practice [12]. As regards to visual inspection, vacuum testing, identification (animal species of origin, anti-tetanus toxin activity), pH determination [13] and *C. tetani* antibody content, the cBRPs were considered of satisfactory quality. As regards to bacterial and fungal sterility checking (14) inoculation of the content of some

vials of the cBRPs induced growth of germs (contamination level lower than 10 germs per vial) identified as *Bacillus circulus* and *Staphylococcus epidermis*. Repetitions of the sterility test did not confirm the previous findings, therefore it is reasonable to postulate that the results of the first sterility test could be linked to a technical failure and that there is no contamination of the cBRPs. Nevertheless, a storage at - 20 °C of the freeze-dried preparations was recommended and storage of the solution resulting from reconstitution of the cBRPs should be avoided. Moisture content determination [15] and stability checking have also been carried out and it could be concluded that the moisture content and the stability of both cBRPs were in compliance with the specifications set for international reference material [11].

3. PHASE II

3.1. Participants

Fourteen laboratories (6 manufacturers and 8 OMCLs) participated in the collaborative study to establish the *C. tetani* antitoxin potencies of the two cBRPs between January and April 2000. Throughout this report the laboratories are referred to by their code-numbers (1 to 14). The code does not necessarily correspond to the order of appearance in the table of participants (see 7.).

3.2. Materials and method

3.2.1. Materials

All participants were supplied with the following reagents:
— tetanus toxin,
— WHO Second IS for tetanus antitoxin, equine,
— equine anti-tetanus IgG,
— equine anti-tetanus IgG peroxidase conjugate,
— c*Ph. Eur. BRP C. tetani* guinea pig antiserum(1),
— c*Ph. Eur. BRP C. tetani* rabbit antiserum(1).

3.2.2. Method

The method chosen to titrate the cBRPs was the ToBI test. Briefly, the principle of this method can be described as following: on a polystyrene microtitration plate, 2-fold dilution series of the test sera and of the reference serum are made. These dilutions are mixed with a fixed quantity of *C. tetani* toxin and incubated overnight. The day after, the non-bound toxin is determined on a tetanus antitoxin-coated ELISA plate. The *C. tetani* antitoxin titres are estimated by comparing the dose-response curves, based on optical densities (ODs), of the test sera and of the reference serum. The detailed description of the SOP that was used for the collaborative study is available from Division IV of the EDQM.

3.3. Study design

Laboratories were requested to carry out three independent assays on different days.

Deviations from the protocol

All laboratories carried out the assays as requested with the following modifications. Laboratories 4 and 12 used 2 plates per assay. Each plate was treated in this report as an

(1) For the purpose of performing experiments, reconstitution of the final lyophilised product has been achieved by addition of 800 µl of *water for injections* per vial.

independent assay. Laboratory 11 carried out 4 assays because the result of the first assay was so far out of range that methodical inconsistencies were suspected. Laboratory 8 used a pre-dilution of 1/5600 for the IS instead of the requested 1/1400, and a pre-dilution of 1/80 for the rabbit antiserum instead of the requested 1/20. No further modifications were reported.

3.4. Statistical analysis

Complete statistical analysis of out-come of the collaborative study was performed at the EDQM but all the participants were requested to provide both raw data and results of their own calculations. At the EDQM, the raw data were submitted to a 6-parameter logistic curve fit (PROC NLIN, The SAS-System). The first 4 parameters indicate the upper and lower asymptotes of the curves, the slope and the point of inflexion of the IS. Two additional parameters measure the horizontal distance between each of the cBRPs and the IS.

3.5. Results

With exception of laboratories 7 and 12, the participants obtained OD values that were in the expected range for negative and positive controls. However, the maximum level of extinction varied widely between laboratories (ranging from about 0.400 in laboratory 12 to about 1.100 in laboratory 6) but this did not seem to have an important impact on the precision and the accuracy of the assays.

Deviations from parallelism

The data were tested for non-parallelism by fitting three curves, each with their own slope, but with identical asymptotes. The difference between the slopes of the cBRPs and the IS should be zero if the curves are parallel. To allow for random variations, an asymptotically correct 95 % confidence interval was constructed around the estimated difference. A graphical impression is given in Figures 1a and 1b. The figures show for each assay the difference between the slope of the cBRP and the IS. If a confidence interval does not contain the value zero, this indicates a significant departure from parallelism. A striking observation was that the cBRP guinea pig antiserum tended to have a flatter slope than the IS. This was not the case for the cBRP rabbit antiserum.

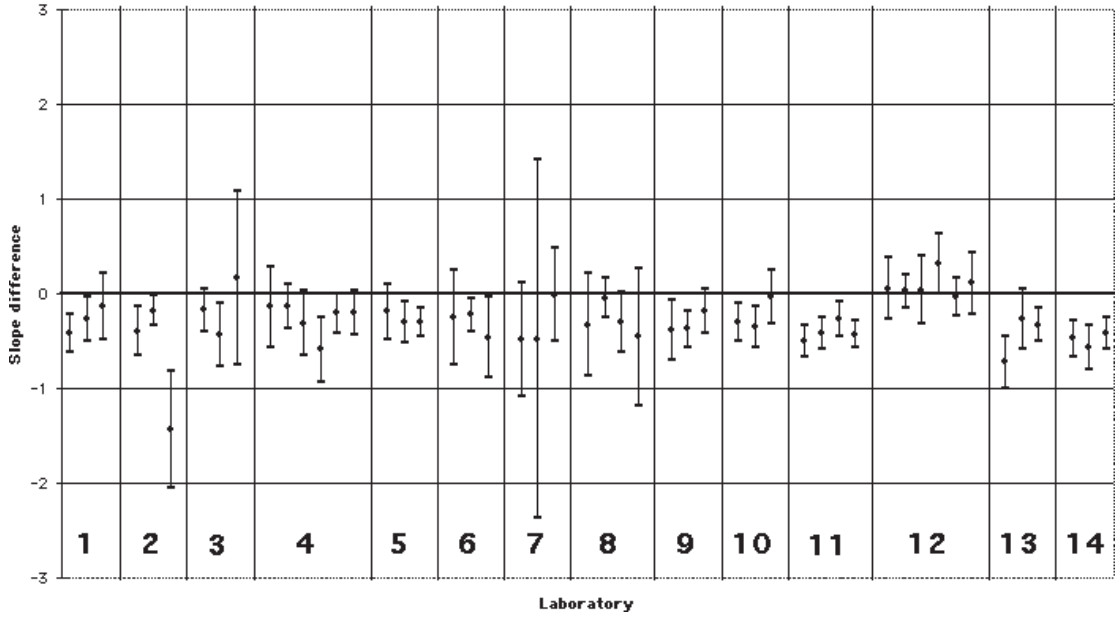

Figure 1a. — *Deviations from parallelism (guinea pig antiserum)*

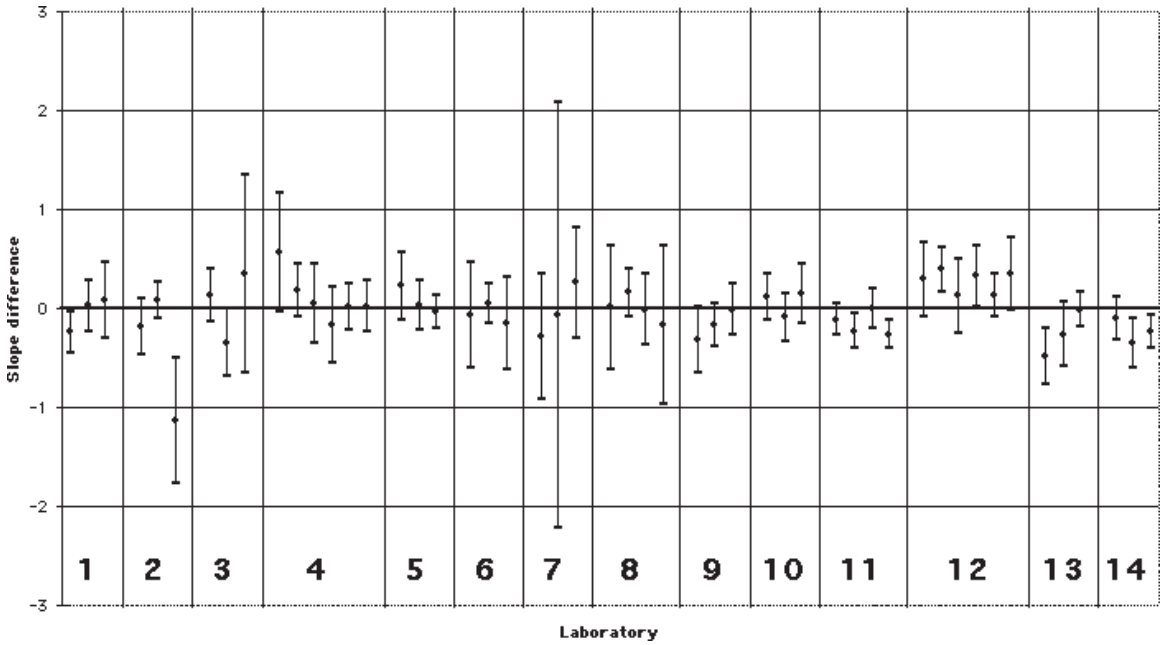

Figure 1b. — *Deviations from parallelism (rabbit antiserum)*

Estimated potencies

Table 1 lists for each assay the estimated potency and the 95 % confidence intervals. Figures 2a and 2b show the same data in a graphical representation. The overall precision (i.e. intra-assay variation) was satisfactory. Assay 1 of laboratory 11 was indeed far out of range and has been excluded. Despite the systematic deviations from parallelism for the cBRP guinea pig antiserum and the fact that the levels of extinction did not always fall within the specified limits, it could be seen that the results were fairly reproducible (i.e. inter-laboratory variation). Therefore, exclusion of these assays would not have been justified.

Table 2 lists the individual and combined potency estimates for each assay. For each laboratory, the assays were combined by taking the unweighted geometric mean of the individual potency estimates. The first assay from laboratory 11 has been excluded. All other estimates were included. The geometric coefficients of variation (GCV) indicated a satisfactory repeatability (i.e. intra-laboratory variation). The mean values per laboratory were combined in one single value for each serum by taking the unweighted geometric mean. This resulted in an estimated potency of 34.43 IU/vial for the guinea pig antiserum and 14.82 IU/vial for the rabbit antiserum.

4. CONCLUSION

It was concluded that the two proposed reference antisera are suitable for the intended purpose, i.e. serological potency testing for batch consistency control of tetanus vaccines for veterinary use. In consequence, they have been adopted at the 107th session of the Ph. Eur. Commission in June 2000 as *Clostridium tetani* guinea pig antiserum BRP Batch 1(1) (34 IU/vial) and *Clostridium tetani* rabbit antiserum BRP Batch 1(2) (15 IU/vial).

(1) Cat. Nr. C2424500.

(2) Cat. Nr. C2425600.

Table 1. — *Confidence intervals per laboratory*

Lab	Guinea pig antiserum			Rabbit antiserum		
	Low	Estimate	High	Low	Estimate	High
1	27,62	31,01	34,81	14,72	16,53	18,56
	26,88	29,65	32,72	12,98	14,32	15,80
	23,51	27,35	31,82	11,21	13,04	15,16
2	17,16	19,53	22,23	7,68	8,75	9,95
	15,43	16,53	17,70	7,96	8,53	9,14
	20,55	24,03	28,10	10,02	11,71	13,69
3	26,48	30,49	35,12	10,20	11,74	13,52
	29,75	37,37	46,96	10,67	13,40	16,84
	25,93	37,31	53,69	11,06	15,92	22,90
4	21,33	25,48	30,45	8,46	10,11	12,07
	18,52	20,89	23,56	8,05	9,07	10,23
	22,79	26,74	31,38	9,63	11,30	13,26
	21,72	25,35	29,60	9,15	10,68	12,47
	18,72	20,74	22,98	8,11	8,98	9,95
	20,97	23,50	26,33	7,70	8,63	9,67
5	42,09	45,96	50,19	16,15	17,63	19,25
	52,18	57,79	64,00	16,50	18,27	20,23
	43,02	46,90	51,13	15,27	16,65	18,15
6	22,07	27,22	33,57	10,56	13,03	16,06
	24,16	26,66	29,42	11,96	13,20	14,57
	22,86	29,14	37,14	9,76	12,44	15,85
7	40,35	52,45	68,18	12,13	15,77	20,50
	20,04	35,01	61,15	7,58	13,25	23,15
	20,76	24,81	29,66	11,77	14,07	16,82
8	45,18	50,39	56,21	22,72	25,51	28,64
	45,01	53,39	63,34	19,69	23,36	27,71
	36,50	45,21	55,99	15,69	19,44	24,07
9	31,39	35,90	41,05	14,08	16,10	18,41
	26,04	28,77	31,78	13,13	14,50	16,02
	23,34	26,52	30,13	11,66	13,24	15,05
10	29,93	32,88	36,12	13,58	14,92	16,39
	41,13	45,53	50,41	16,71	18,49	20,48
	24,40	27,24	30,41	11,83	13,20	14,74
11	424,64	461,00	500,46	13,49	14,59	15,78
	31,29	33,78	36,46	14,87	16,05	17,33
	33,94	36,79	39,89	13,85	15,01	16,28
	27,29	29,56	32,03	13,12	14,22	15,40
12	32,14	36,87	42,29	15,30	17,56	20,14
	25,09	28,80	33,06	9,70	11,13	12,78
	25,92	30,28	35,38	13,11	15,32	17,90
	22,04	25,76	30,12	11,75	13,74	16,06
	33,44	36,59	40,04	14,25	15,59	17,06
	32,80	37,64	43,18	13,88	15,93	18,27
13	45,39	51,97	59,49	17,14	19,62	22,47
	44,60	51,93	60,48	14,22	16,56	19,28
	38,40	49,17	62,96	12,56	16,08	20,60
14	45,39	49,48	53,93	17,87	19,48	21,24
	48,25	53,15	58,56	21,93	24,15	26,61
	42,45	45,75	49,30	19,24	20,73	22,34

Potencies are expressed in IU/vial.

Values are calculated at EDQM on the basis of raw data provided.

95 % confidence intervals are asymptotically correct with degrees of freedom = ∞.

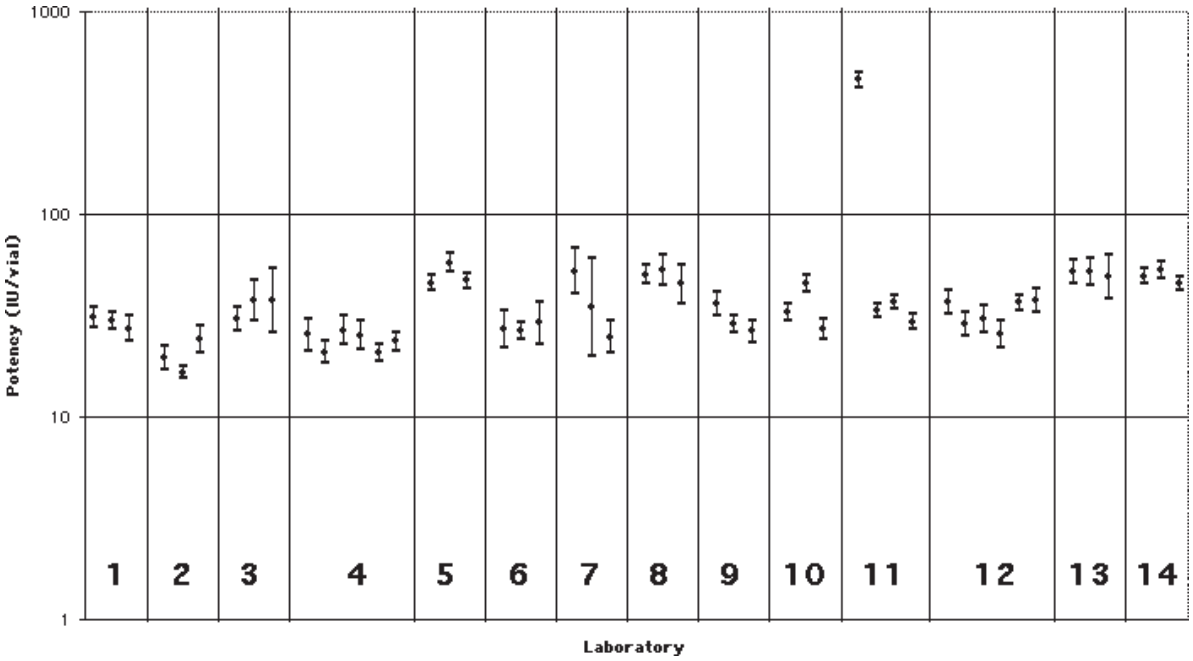

Figure 2a. — *Confidence intervals per assay (guinea pig antiserum)*

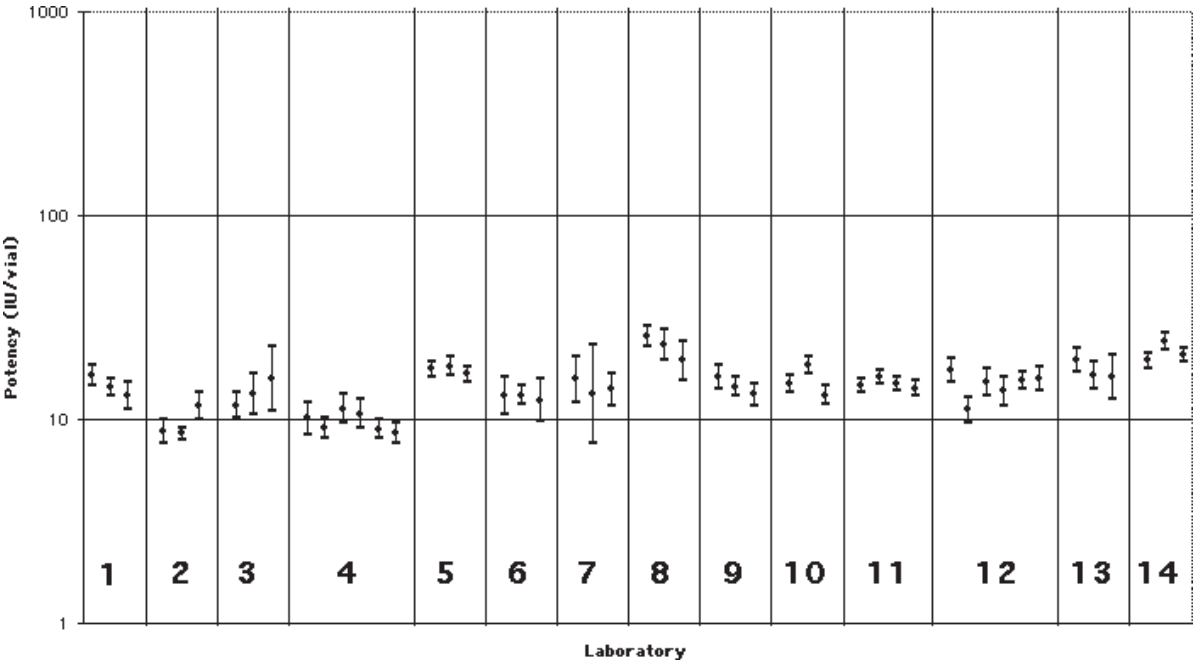

Figure 2b. — *Confidence intervals per assay (rabbit antiserum)*

Table 2. — *Combination of results*

	Guinea pig antiserum				
Lab	Assay 1	Assay 2	Assay 3	Combined	GCV
1	31,01	29,65	27,35	29,30	6,4 %
2	19,53	16,53	24,03	19,80	18,7 %
3	30,49	37,37	37,31	34,90	11,7 %
4	25,48	20,89	26,74	23,67	10,8 %
	25,35	20,74	23,50		
5	45,96	57,79	46,90	49,94	12,7 %
6	27,22	26,66	29,14	27,65	4,7 %
7	52,45	35,01	24,81	35,72	37,5 %
8	50,39	53,39	45,21	49,55	8,4 %
9	35,90	28,77	26,52	30,14	15,7 %
10	32,88	45,53	27,24	34,42	26,0 %
11	*461.00	33,78	36,79	33,24	11,0 %
	29,56				
12	36,87	28,80	30,28	32,32	15,8 %
	25,76	36,59	37,64		
13	51,97	51,93	49,17	51,01	3,2 %
14	49,48	53,15	45,74	49,37	7,5 %
Combined				**34,43**	

	Rabbit antiserum				
Lab	Assay 1	Assay 2	Assay 3	Combined	GCV
1	16,53	14,32	13,04	14,56	12,0 %
2	8,75	8,53	11,71	9,56	17,6 %
3	11,74	13,40	15,92	13,58	15,3 %
4	10,11	9,07	11,30	9,75	10,8 %
	10,68	8,98	8,63		
5	17,63	18,27	16,65	17,50	4,7 %
6	13,03	13,20	12,44	12,88	3,1 %
7	15,77	13,25	14,07	14,33	8,9 %
8	25,51	23,36	19,44	22,63	13,9 %
9	16,10	14,50	13,24	14,57	9,8 %
10	14,92	18,49	13,20	15,39	17,1 %
11	14,59	16,05	15,01	14,95	6,1 %
	14,22				
12	17,56	11,13	15,32	14,73	15,8 %
	13,74	15,59	15,93		
13	19,62	16,56	16,08	17,35	10,7 %
14	19,48	24,15	20,73	21,37	11,1 %
Combined				**14,82**	

Potencies are expressed in IU/vial.

Combined potencies are the unweighted geometric mean of individual results.

GCV = Geometric coefficient of variation.

* = Value has been excluded.

5. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to the project leader, Dr. H. H. Lensing, (ID-Lelystad) and to all participants for their contributions to this study. Dr. C. Hendriksen (RIVM) and Dr. J. van der Gun (RIVM) who developed the ToBI protocol and reagents used for this study, are gratefully acknowledged. The statistical analysis was performed by A. Daas (EDQM). The editorial review was done by Dr. M.-E. Esposito-Farese and Dr. K-H. Buchheit.

The study was run by the EDQM with the support of the Council of Europe, the European Commission and the "Alternatives to Animal Experiments Platform, The Netherlands", under the aegis of the Biological Standardisation Programme (internal project number BSP024) with Dr. M. Berg-Candolfi and Dr. M.-E. Esposito-Farese acting as project monitors.

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COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF

RABIES VACCINE (INACTIVATED) FOR VETERINARY USE

EUROPEAN PARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION BATCH No. 3

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF RABIES VACCINE (INACTIVATED) FOR VETERINARY USE EUROPEAN PARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION BATCH No. 3

C. Milne[1], A. Daas[1], J-M. Spieser[1], L. Bruckner[2]

1. INTRODUCTION

The current test for potency of rabies vaccine (inactivated) for veterinary use specified in the European Pharmacopoeia involves an immunogenicity assay based on the NIH[3] test, in which mice are vaccinated and the protection obtained is measured by subsequent intracerebral challenge by live virus. The potency of the vaccine, calculated in international units, is obtained by comparison in parallel to the protection provided by a defined standard. The first Ph. Eur. BRP for this purpose was established in 1982 in a collaborative study under the aegis of the EDQM, by comparing the potency of the BRP candidate to the International Standard. A second Ph. Eur. BRP for rabies vaccine (inactivated) for veterinary use was established in a collaborative study in 1990. A third lot of the BRP is now required to replenish the stock of this important reference material.

2. AIM OF THE STUDY

This collaborative study was designed to calibrate the candidate rabies vaccine (inactivated) for veterinary use BRP batch No. 3 against the International Standard and to assign a potency value in International Units in order to replace BRP batch No. 2, the stocks of which had been depleted.

3. PARTICIPANTS

Eight laboratories from six countries, including representatives from both OMCLs and manufacturers participated. Throughout this report the laboratories are referred to by their code-numbers allocated at random and not necessarily corresponding to the order of listing at the end of the report.

4. MATERIALS AND METHODS

4.1. Materials

Each participant received 3 vials of candidate batch No. 3 and 3 vials of the 5th International Standard for rabies vaccine.

Sample No. 1:

The 5th International Standard (established in 1991) for rabies vaccine was obtained from NIBSC. It is a freeze-dried vaccine derived from the Pitman Moore strain of rabies virus

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(3) Abbreviations: NIH: National Institute of Health; Ph. Eur.: European Pharmacopoeia; EDQM: European Directorate for the Quality of Medicines; BRP: Biological Reference Preparation; OMCLs: Official Medicines Control Laboratories; NIBSC: National Institute for Biological Standards and Control; IU: International Units; PM: Pitman Moore; QC: Quality Control; CVS: Challenge Virus Standard; ID_{50}: dose infecting 50% of subject animals; IS: International Standard.

produced in Vero cells and inactivated by beta-propiolactone (J. Lyng et. al. 1992). The stated potency is 16 IU per vial to be reconstituted in 1 ml. A basic dilution of 1 in 16 was performed to give 1 IU per ml (0.5 IU per 0.5 ml).

Sample No. 2:

The candidate BRP batch No. 3 of rabies vaccine (inactivated) for veterinary use is a current production batch of a commercial vaccine of live attenuated rabies virus which was kindly donated by Mérial (Ribiffa ® lot 8RBA5B88), the same manufacturer who provided BRP batch No. 2. The current candidate vaccine has the same general characteristics as the previous BRP. The vaccine is a freeze-dried preparation derived from the PM strain of rabies and is produced in NIL-2 cells and inactivated by beta-propiolactone. It was manufactured in August 1998 with an estimated potency of 9 IU per vial as established by the QC department of the donor. The lyophilised material was to be reconstituted in 1 ml. A basic dilution of 1 in 9 was then performed to give 1 IU per ml (0.5 IU per 0.5 ml).

Vaccine dilutions were prepared in phosphate buffered saline pH 7.0.

Challenge virus

The Challenge Virus Standard strain of rabies was used as the challenge virus. Each participating laboratory provided its own preparation of CVS strain prepared as outlined in the Ph. Eur. monograph *Rabies vaccine (inactivated) for veterinary use (1998:0451)*.

4.2. Methods

Participants were asked to perform the challenge test on mice as described in the Ph. Eur. monograph *Rabies vaccine (inactivated) for veterinary use (1998:0451)*. The challenge test outlined in the monograph is an immunogenicity assay based on the NIH test. Groups of mice are vaccinated once with graded doses of vaccine followed by intracerebral challenge with CVS strain rabies virus on day 14 after vaccination. The animals are then monitored for signs of rabies over the next 14 days.

Study design

Each participant was requested to carry out 3 independent assays according to the Ph. Eur. monograph *Rabies vaccine (inactivated) for veterinary use (1998:0451)*. Participants were asked to carry out the 3 assays in succession such that one test did not begin until the previous one had been concluded, thus allowing any adjustments to the dilutions if required.

Test animals

Groups of at least 10 healthy female mice approximately 4 weeks of age were used:
a) 4 groups to test the 4 vaccination doses of the International Standard,
b) 4 groups to test the 4 vaccination doses of the candidate BRP,
c) 4 groups for titration of the challenge virus.

Four dilution steps of the vaccine were used in this study**.**

Suggested vaccination doses:
Dose No 1: Basic dilution,
Dose No 2: Basic dilution diluted 1 in 5,
Dose No 3: Basic dilution diluted 1 in 25,
Dose No 4: Basic dilution diluted 1 in 125.

0.5 ml of each relevant dilution was injected per mouse.

Other dilutions were used according to the experience of the participating laboratory or the results of the first trial.

Participants were allowed to carry out, in addition, any other *in vitro* methods they routinely use for the assay of rabies vaccine.

Statistical analysis

The results were submitted for statistical evaluation at the EDQM. The probit method was used to fit the maximum likelihood curves. If the algorithm failed to converge, the Spearman/Kärber method was used. Results of calculations were, as far as possible, compared with the calculations of the participants to avoid misinterpretation of the raw data. Small differences in outcome can be explained from the fact that most participants used the Spearman/Kärber method only.

Combination of the assays was carried out by fitting the maximum likelihood regression curves to the pooled set of data within laboratories allowing for inter-assay variation. With this technique, the data are used more efficiently than by combining the potency estimates of the individual assays.

The final potency estimate of Ph. Eur. BRP Batch No. 3 was obtained as the weighted combination of the potency estimates per laboratory according to chapter 5.3 of the European Pharmacopoeia.

5. RESULTS

Each participant carried out the assays as requested. No results for *in vitro* assays were submitted by any of the laboratories as additional data.

The complete set of raw data is listed in Tables 1 and 2. The potency estimates of the candidate reference are listed in Table 3, together with the combined potency estimate, 95 per cent confidence intervals and the probability values for non-linearity and non-parallelism. The estimated ID_{50} content of the challenge suspensions are listed in Table 4.

Based on their experience and observations laboratory 3 adjusted the dilutions in the 2nd and 3rd assay: an extra dilution of 1/10 in terms of the reconstituted solution was used for the IS and the basic dilution for the Ph. Eur. BRP was obtained as a 1/8 dilution instead of 1/9.

Laboratory 7 carried out only 2 assays due to lack of time, but the second assay failed due to an error in the concentration of the injectable narcotic which caused most mice to die.

Laboratory 8 carried out only one determination of the ID_{50} content of the challenge suspension, which was reported to apply to each of the three potency assays.

Based on their previous experience with the assay laboratory 5 used only 5 mice per dilution for the titration of the CVS challenge strain in place of the recommended 10 mice per dilution.

6. DISCUSSION

From Table 4 it can be seen that the individual assays 1 and 2 in laboratory 2, and assay 1 in laboratory 5 did not contain the 10 ID_{50} / 0.03 ml in the challenge suspensions as required in the Ph. Eur. monograph. However, the minimum requirement is met in all laboratories if the results of the 3 assays are combined, so it may be argued that this non-compliance is due to statistical fluctuation.

Table 1 – *Raw data for the potency assays*

Lab code	Dilution	5th International Standard			Cand. 3rd Ph. Eur. BRP		
		Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
1	1/1	2/10	3/9	5/10	4/10	5/9	4/10
	1/5	0/10	3/9	5/10	2/10	5/10	3/10
	1/25	0/10	1/10	0/10	1/10	2/10	0/10
	1/125	0/10	2/10	2/10	0/10	0/8	0/10
2	1/1	3/10	10/10	0/10	4/7	5/9	2/10
	1/3	7/14	11/14	2/14	8/14	8/14	0/14
	1/9	7/16	7/16	0/16	4/16	10/16	0/16
	1/27	5/14	10/14	0/16	0/14	4/14	0/14
	1/81	0/10	3/10	0/10	0/10	4/10	0/10
3	*footnote		10/10	7/10			
	1/1	6/10	6/10	5/10	6/10	8/9	8/10
	1/5	3/10	4/10	5/10	4/10	1/10	4/10
	1/25	4/10	0/10	1/10	4/10	3/10	0/10
	1/125	0/10	1/10	2/10	1/10	1/10	0/10
	1/625	0/10			0/10	0/10	0/10
4	1/1	5/9	11/11	11/12	7/10	12/12	9/12
	1/5	3/10	11/11	6/12	3/10	9/12	6/10
	1/25	2/9	6/12	4/12	0/9	3/12	5/12
	1/125	1/10	1/12	0/12	0/10	1/12	4/12
	1/625	0/10			0/10		
5	1/1	9/10	6/9	5/10	7/9	9/10	9/10
	1/5	5/9	2/10	4/10	2/10	8/10	0/9
	1/25	4/10	1/10	3/9	4/10	1/9	1/9
	1/125	1/10		0/8	0/10		0/10
6	1/1	7/10	6/10	9/10	8/9	7/10	8/9
	1/5	5/9	6/10	8/10	6/10	5/10	7/9
	1/25	3/10	2/10	2/10	4/9	4/9	5/10
	1/125	1/10	0/9	2/10	1/10	0/10	2/10
7	1/1	6/12			6/12		
	1/5	0/12			5/12		
	1/25	3/12			1/11		
	1/125	1/12			0/12		
8	1/1	5/12	3/12	2/12	5/12	5/12	3/12
	1/5	3/12	0/12	3/12	3/12	0/11	1/12
	1/25	0/12	0/12	0/11	2/12	0/12	0/12
	1/125	0/11	0/11	0/11	0/12	0/12	0/12

Survival ratios are listed.

Dilutions are expressed in terms of the basic dilutions.

*Laboratory 3 adjusted the dilutions in the 2nd and 3rd assay. An extra dilution of 1/10 in terms of the reconstituted solution was used for the IS and the basic dilution for the Ph. Eur. BRP was obtained as a 1/8 dilution instead of 1/9.

Table 2 – *Raw data for calculation of ID_{50} for the challenge suspension*

Lab code	Dilution	Challenge suspension		
		Assay 1	Assay 2	Assay 3
1	1/1	0/10	0/10	0/10
	7/10	0/10	0/10	0/10
	7/100	2/10	3/10	4/10
	7/1000	8/10	7/10	8/10
2	1/1	2/10	3/10	0/10
	1/10	6/10	7/10	2/10
	1/100	8/10	7/10	5/10
	1/1000	10/10	8/10	7/10
3	1/1	0/10	0/10	0/10
	1/10	1/10	0/10	1/10
	1/100	9/10	6/10	7/10
	1/1000	9/10	10/10	9/10
4	1/1	0/10	0/9	0/10
	1/10	3/10	0/10	5/10
	1/100	10/10	4/10	10/10
	1/1000	10/10	8/9	10/10
	1/10000	10/10	8/8	10/10
5	1/1	0/5	0/5	0/5
	1/10	3/5	1/5	1/5
	1/100	5/5	4/5	4/5
	1/1000	5/5	5/5	4/4
	1/10000	5/5	5/5	5/5
6	1/1	0/10	0/10	0/10
	1/10	2/10	3/10	3/10
	1/100	6/10	9/10	7/10
	1/1000	10/10	10/10	10/10
7	1/1	0/10		
	1/5	0/10		
	1/50	1/10		
	1/500	7/10		
	1/5000	10/10		
8	20	0/12		
	2	0/12		
	1/5	1/12		
	1/50	5/12		
	1/500	11/12		
	1/5000	12/12		

Survival ratios are listed.

Laboratory 7 carried out only one assay.

Laboratory 8 carried out one assay, which would apply to each of the three potency assays

Table 3 – *Potency estimates per laboratory*

Lab code	Individual assay (I.U./vial)			Combined (I.U./vial)			p-values	
	1	2	3	Estimate	Lower limit	Upper limit	Non-linearity	Non-parallelism
1	69.3	21.5	1.9	11.8	3.1	48.7	0.120	0.088
2	4.7	1.9	7.6	3.5	1.0	9.3	0.133	0.409
3	14.3	9.4	5.8	8.7	3.6	19.8	0.440	0.586
4	5.4	4.1	19.0	7.5	3.6	15.2	0.884	0.495
5	3.0	39.6	5.9	9.2	4.1	20.6	0.346	0.082
6	18.5	14.1	14.2	15.4	6.7	36.8	0.361	0.993
7	13.2	.	.	13.2	1.7	141.6	0.133	0.226
8	15.4	n.c.	5.7	11.9	4.6	32.2	0.498	0.883
Combined (Including all data)				9.1	6.5	12.7		
				100.0%	71.4%	139.6%		
Combined (Excluding laboratories 1, 2, 5, 7 and 8)				9.6	6.1	15.3		
				100.0%	63.1%	158.5%		

Explanations: p-values should not be less than 0.05. Combined potency estimates per laboratory were obtained by pooling the data (see text).
n.c. = non-calculable

Table 4 – *ID_{50} content of challenge suspensions*

Lab code	Individual assay (ID_{50}/0.03 ml)			Combined (ID_{50}/0.03 ml)			p-value Non-linearity
	1	2	3	1	2	3	
1	47.8	47.6	31.0	41.2	25.0	73.6	0.510
2	5.6	8.7	179.8	19.8	7.0	50.7	0.574
3	44.9	80.6	67.7	62.2	37.3	104.2	0.170
4	15.2	167.2	10.0	30.4	17.7	52.1	0.691
5	8.1	31.6	31.7	20.3	9.7	42.4	0.949
6	48.5	20.0	31.6	31.6	18.8	53.1	0.498
7	248.3	.	.	248.3	97.5	634.6	0.992
8	59.2	.	.	59.2	23.8	147.2	0.993

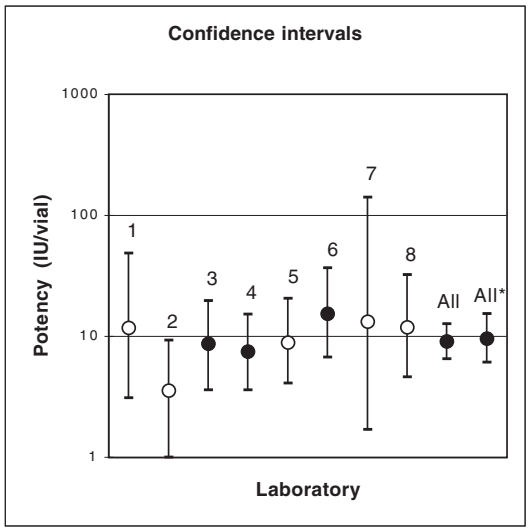
Table 1 shows that the 50 per cent protective dose in laboratories 1 and 8 does not fall between the smallest and the largest dose given to the mice as required in the Ph. Eur. monograph specifications. Responses in laboratories 2 and 7 are not monotonous. Assay 3 in laboratory 5 also shows non-monotonous responses.

Table 3 shows that all assays were statistically valid with respect to linearity and parallelism. The confidence limits obtained for laboratories 1 and 7 exceed the maximum allowed width of 25 per cent and 400 per cent of the estimated potency. Assay 2 from laboratory 5 also exceeds these limits, assays 1 and 3 already being excluded.

The procedure applied by laboratory 8 did not strictly follow the design specified by the protocol and based on the Ph. Eur. monograph. This laboratory carried out only one determination of the ID_{50} content of the challenge suspension and reported that this would apply to each of the three potency assays, but it would appear that the potency assays were not carried out simultaneously.

If the above mentioned validity criteria are applied strictly, it has to be concluded that only results from laboratories 3, 4 and 6 can be used for further calculations.

The following plot (Figure 1) shows the individual 95 per cent confidence intervals per laboratory and their weighted combination both ignoring the validity criteria (All) and excluding invalid results (All*).



Values for individual laboratories (1-8)

The weighted combination ignoring the validity criteria (All) and excluding invalid results ie laboratories 1, 2, 5, 7 and 8 (All*). Excluded laboratories are marked with an empty dot.

Figure 1 – *95 % Confidence intervals for potency estimates*

7. CONCLUSION

The potency estimates from individual assays show a high variability (ranging from 1.9 to 69.3 IU/vial, see Table 3). By applying an adapted statistical model, which uses the information contained in the assays more efficiently than the standard models, it is possible to reduce the variability (potency estimates ranging from 3.5 to 15.4 IU/vial). The confidence intervals are in agreement with each other and with the expected potency of 9 IU/vial. The weighted combination of these intervals leads to a potency estimate of 9.1 IU/vial with 95 per cent confidence limits of 6.5 and 12.7 IU/vial, which represents 71.4 and 139.6 per cent of the estimated potency.

If the validity criteria as mentioned in the European Pharmacopoeia are strictly applied however, only data from 3 laboratories can be used. This would result in an estimate of 9.6 IU/vial with 95 per cent confidence limits of 6.1 and 15.3 IU/vial, which represents 63.1 and 158.5 per cent of the estimated potency. It is highly questionable if the results from only 3 laboratories are sufficient to assign a potency for a European Pharmacopoeia Biological Reference Preparation.

The results from the remaining 5 laboratories, though not adhering strictly to the validity criteria for individual assays are not devoid of useful information in the context of the larger study. It can be argued that the results of the other 5 laboratories, when evaluated and weighted appropriately, contribute valuable information about the true potency. In any case, none of the assays would be inconsistent with the expected potency of 9 IU/vial.

It is therefore recommended to assign a potency of 9 IU/vial to Ph. Eur. BRP Batch No. 3.

The former BRP batch No. 2, established in June 1990 was shown to be stable when stored at – 20 °C at EDQM. Information regarding stability of the candidate BRP is presently being collected.

The variability in results observed and the difficulty in obtaining valid results in individual assays serves to emphasise that the use of this assay, which is based on crude techniques, is not optimal and that serious effort should be made, using new technologies available, to develop a more robust assay that would be both more consistently reliable and reduce the use of animals.

8. REFERENCES

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9. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to Mérial for donating the BRP material, to all participants for their contribution to this study and to Dr. Lukas Bruckner for his guidance as project consultant. The study was run by the EDQM with the internal number BSP036.

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COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF

A EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION FOR

CLOSTRIDIA ANTISERUM FOR SEROLOGICAL POTENCY TESTING OF CLOSTRIDIAL VACCINES FOR VETERINARY USE

COLLABORATIVE STUDY FOR THE ESTABLISHMENTOF A EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATION FOR *CLOSTRIDIA* ANTISERUM FOR SEROLOGICAL POTENCY TESTING OF CLOSTRIDIAL VACCINES FOR VETERINARY USE

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1. INTRODUCTION

Potency tests for veterinary clostridial toxoid vaccines specified in the current European Pharmacopoeia (Ph. Eur[3]) have two stages: vaccination of rabbits with the vaccine to be assayed followed by titration of antibodies raised in the rabbit sera by a toxin neutralisation test in mice (TN). For the second stage of the potency test there is sufficient information to support a general move away from TN to *in-vitro* determination, in the interest of animal welfare. In recognition of this, the relevant monographs (*0361, 0362, 0363, 0364, 0697*) have been amended in the 2001 supplement of Ph. Eur. to permit the use of immunochemical (IC) methods for the quantitation of antibody responses in rabbits.

In making these changes to the monographs it was recognised that development and regulatory approval of *in-vitro* methods would be facilitated if an approved rabbit reference serum with established activities against the relevant toxins was available. In order to establish such a reference serum a collaborative study was conceived with the aim of producing a reference serum with soundly established anti-toxin activities against the five most frequently encountered toxoid antigens incorporated into commercial vaccines, i.e. *Clostridium (C.) perfringens* Type B/C, *C. perfringens* Type D, *C. septicum, C. novyi* and *C. tetani.*

Since a large number of potency tests are conducted by manufacturers and regulatory authorities the reference serum established by this collaborative study was intended for use in calibrating in-house reference sera rather than for routine use in batch testing. Nevertheless, the preparation needed to be appropriate for all manufacturers irrespective of the use of:

a) different ELISA or other *in-vitro* testing methods,

b) different formulations and combinations of multi-component vaccines to which these methods may be applied,

c) different target species for which the vaccines may be recommended.

Following discussion between manufacturers, regulatory authorities, representatives of Ph. Eur. and external experts in the field it was agreed that as no single serum would be representative

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(3) Abbreviations: BRP: European Pharmacopoeia Biological Reference Preparation, c: candidate, C.: Clostridium, C. perf.: Clostridium perfringens, EDQM: European Directorate for the Quality of Medicines, ELISA: Enzyme linked immunosorbent assay, IC: Immunochemical, IS: International standard, IU: International unit, OMCL: Official Medicines Control Laboratory, Ph. Eur.: European Pharmacopoeia, QC: Quality control, SOP: Standard operating procedure, RSD: Relative standard deviation, TN : Toxin neutralisation test in mice, WHO: World Health Organization.

of the full spectrum of components used by all manufacturers, a pool of sera derived from routine potency tests for a range of vaccines from different manufacturers would represent the best chance of producing a reference preparation which fulfilled these objectives. In addition, a reference serum prepared in this way would not require the use of additional animals specifically to raise sufficient quantities of serum and would potentially be suitable as a reference serum for other toxoid components as and when suitable IC methods became available. However, it was also accepted that the eventual replacement of the serum by an identical preparation would be impossible but that a similar preparation, albeit with different specific activities, could, if necessary, be developed.

The European Directorate for the Quality of Medicines (EDQM) has organised an international collaborative study, divided in 2 phases, aimed at producing and establishing a suitable reference serum for serological potency testing of clostridial vaccines for batch consistency demonstration. In phase 1 a series of pools produced from sera provided by each manufacturer and raised against the broadest range of antigens possible were blended to obtain TN titres which were representative of the range of titres normally elicited by the vaccines under test. This pilot scale blend together with samples of the individual pools of sera were then titrated by each of the donor laboratories using their own *in-vitro* assay systems against all the components for which such assays were available. It was intended that the results obtained from phase 1 would determine whether a pool of sera prepared by blending of materials from multiple manufacturers, using a variety of different vaccine formulations and combinations would indeed serve as a suitable reference material allowing different laboratories, using their own *in-vitro* techniques, to obtain results which were comparable with each other and with the activity as determined by the currently applied TN.

Detailed statistical analysis of the data was not possible since only a few laboratories were able to participate and because limited replication of the assays was possible. Nevertheless, sufficient data was collected to conclude that the blend of sera would provide a suitable reference material for use by all manufacturers and regulatory authorities in respect of *in-vitro* assay methods for the five components for which it was primarily designed.

On the basis of these results a production scale blend of the serum pools was prepared. This blend was identical in composition to that prepared on a pilot scale for phase 1 and was filled and freeze-dried by EDQM as a single batch, referred to as candidate (c) Ph. Eur. Biological Reference Preparation (BRP).

Phase 2 of the study required that the antitoxin activity of this blend be defined in respect of the relevant antibodies. In order to achieve this a larger group of laboratories, including both manufacturers and official medicines control laboratories (OMCLs), were invited to participate in a study comparing the activity of the proposed rabbit multicomponent reference serum with the existing equine monovalent ISs. These studies necessarily had to be conducted according to the methods described in the currently applicable monographs i.e. by TN in order to provide a definitive value for the antitoxin activity of the reference preparation in respect of the 5 components studied.

2. PARTICIPANTS

Nine laboratories (4 OMCLs and 5 manufacturers) among which 4 participated both in phases 1 and 2 took part in the collaborative study. They are referred to by a code letter which is not related to the order of the listing presented in Section 9 of this report. It is to be noted that some laboratories were unable to perform testing in respect of all of the five components of interest.

3. MATERIALS, METHODS AND STUDY DESIGN

3.1. Materials

3.1.1. Phase 1

In phase 1 each participant was provided with six coded samples. These materials were five rabbit serum pools together with a blend of these pools constructed in such a way that the theoretical antitoxin activity of each component was in the same order as that expected for the sera resulting from routine potency tests for each component and ensuring that, if appropriate, the reference serum could be constructed in an exactly identical manner. Each sample was provided as a freeze-dried preparation lyophilised from 1ml of serum.

The serum pools provided by each manufacturer derived from a number of potency tests of clostridial vaccines (different products containing a variety of combinations of antigens and incorporating a number of different adjuvants). Each manufacturer provided results of antitoxin titrations for their own serum pool using the methods routinely employed by them in batch release. One manufacturer generated these data using IC assays but all the others used TN. Immunisation schedules used to produce all the sera were in compliance with the relevant Ph. Eur. monographs for clostridial vaccines.

Table 1 shows the recorded activity of each serum pool (sera A-D and F) against the relevant components contained within it (as provided by the donators) together with the calculated activity of the blend prepared from the individual pools (Serum E).

Table 1. — *Antitoxin Activity (IU/ml) of Sera included in Phase 1*

Component	*C. perf* B/C	*C. perf* D	*C. septicum*	*C. tetani*	*C. novyi*
Serum A	not inc.	8.7	10	48	13.3
Serum B	not inc.	6.2	3.4	3.7	6.3
Serum C	19.2	10.1	7.4	5.4	9.1
Serum D	22.4	25.2	9.3	12.1	13.6
Serum E	13.0	14.5	8.0	9.4	12.1
Serum F	27.2	20.2	6.9	22.3	15.9

not inc: not included (indicates that the serum was raised against vaccines which did not contain this antigen).

3.1.2. Phase 2

3.1.2.1. Samples

Each participating laboratory was provided with a total of twenty freeze-dried vials of the cBRP together with one vial of each of the following equine, monovalent WHO ISs:

- 3rd IS for Gas Gangrene antitoxin (*Clostridium novyi*), equine;
- 2nd IS for *Clostridium perfringens* Epsilon antitoxin, equine;
- 3rd IS for Gas Gangrene antitoxin (*Clostridium septicum*), equine;
- 2nd IS for Tetanus antitoxin, equine;
- 2nd IS for *Clostridium perfringens* Beta antitoxin, equine.

3.1.2.2. Reagents

Wherever possible the laboratories were encouraged to use their own clostridial toxins in order to ensure that the cBRP would perform satisfactorily under the conditions normally applied in routine testing. Upon request, clostridial toxins were provided in order to enable testing for *C. perfringens* Beta and Epsilon, *C. septicum*, *C. novyi* and *C. tetani* components.

3.2. Methods and Study Design

3.2.1. Phase 1

Participants were requested to provide results of assays for each component of the six samples provided using their in-house *in-vitro* methods. Results from three separate assays on two containers each were requested. However, not all manufacturers had developed IC assays for all components and hence results are available for different components from different participants. In addition, participants were requested, if possible, to provide the results of one assay for each component performed by TN. In order to minimise the numbers of animals used to perform *in-vivo* testing by TN participants were provided with guidance to the expected ranges of activity for each component.

3.2.2. Phase 2

Although the purpose of the cBRP is to facilitate the use of IC methods for the routine assay of potency test samples the cBRP nevertheless must perform efficiently in the TN test because the activity of the material can be defined only by comparison with the existing ISs using the TN test. Therefore, within phase 2 of the study, the activity of the cBRP was measured by TN using, if available in-house standard operating procedures (SOPs) complying to the Ph. Eur. monographs on clostridial vaccines or, for those participants who did not routinely perform these assays for the purposes of batch release, by means of the SOP provided by EDQM.

3.2.2.1. Determination of test dose of the clostridial toxins

All participants had to conduct the TN assays using the same test dose of toxin as determined by titration against the existing WHO IS antitoxins. Therefore all laboratories were requested to conduct some preliminary titrations to verify the test dose of toxin used within their own routine method by direct titration against these ISs. For the benefit of those laboratories that did not routinely conduct these assays detailed guidance on the methods to be used for determining the toxin dose was provided within the protocol.

Determination of the test dose of each toxin was required by the protocol to be based on the mean of two replicate valid assays.

3.2.2.2. Calibration of cBRP

Having determined the test dose for each toxin each participating laboratory had to compare the activities of the cBRP with the relevant ISs using the TN.

For each antitoxin, a single, valid, preliminary test was required to be followed by four replicate assays (performed on the same day using separately prepared dilutions of toxin and antitoxin or on different occasions). The preliminary assay was required to be conducted using two fold differences in the amount of antitoxin whilst the four replicate definitive assays were to be performed using differences of no more than 20 % in the amounts of antitoxin. Consequently, although only two mice were to be injected with each mixture the result of each assay should have provided an estimate of the activity of the serum which was accurate within ± 10 %.

The principle of the methods applied equally to all components but details of volumes, incubation periods, routes of injection and injection volume varied according to the specific component. In addition, the observation period following injection was defined as 3 days for all components except tetanus where the observation period was set at 4 days. In the case of tetanus the end point for the titration was defined as paralysis whereas for all other components the end point was death. In order to minimise the number of animals required suggested initial dilutions of each of the test preparations were provided.

4. RESULTS

4.1. Phase 1

Four manufacturers, each of which had developed their own serological assay methods for one or more antigens, provided results based on these methods. Three manufacturers also provided results of assays performed using the TN as described within the relevant Ph. Eur. monographs.

Tables 2-5 summarise the results obtained in phase 1 by each of the participants.

Laboratory A provided results in respect of *C. perfringens* Type D, *C. septicum*, *C. tetani* and *C. novyi* for four replicate IC assays each performed on material taken from two separate vials. Detailed results showed that replicate assays yielded a high degree of reproducibility and consistency between vials was obtained. Preliminary results obtained by TN were also reported.

Table 2. — S*ummary of Study Results - Laboratory A*

Serum	Antitoxin Activity (IU/ml) against Clostridial Components									
	C. perf B/C		*C. perf D*		*C. septicum*		*C. tetani*		*C. novyi*	
	IC	TN	IC	TN	IC	TN	IC	TN	IC	TN
A	NT	NT	4.0	NT	7.5	>10	3.3	4	4.6	>7
B	NT	NT	8.1	NT	13.8	7	4.9	6	8.4	>10
C	NT	25	6.1	NT	5.8	<6	5.0	>6	4.4	>7
D	NT	25	17.6	NT	2.7	<8	8.1	>10	5.5	>7
E	NT	15-20	8.0	NT	6.5	8	7.7	>10	5.8	>7
F	NT	>25	9.1	NT	7.0	>5	20.8	26	4.3	>7

NT = not tested.

Laboratory B provided results in respect of *C. perfringens* Type B/C, *C. perfringens* Type D, *C. tetani* and *C. novyi* for three replicate IC assays each performed on material taken from a single vial. Detailed results again showed a high degree of reproducibility. In addition, results obtained by TN were also reported.

Table 3. — *Summary of Study Results - Laboratory B*

Serum	Antitoxin Activity (IU/ml) against Clostridial Components									
	C. perf B/C		*C. perf D*		*C. septicum*		*C. tetani*		*C. novyi*	
	IC	TN	IC	TN	IC	TN	IC	TN	IC	TN
A	<1.1	NT	9.1	6.9	NT	NT	6.7	5.9	13.2	14.5
B	<1.1	NT	9.8	9.1	NT	NT	9.6	7.2	7.1	7.4
C	14.9	15.7	11.9	9.1	NT	NT	10.5	8.0	9.1	11.6
D	19.5	17.2	27.3	18.9	NT	NT	17.7	12.7	12.9	14.5
E	12.0	10.8	14.7	13.1	NT	NT	16.8	12.7	11.1	14.5
F	27.5	24.7	14.5	13.1	NT	NT	45.7	31.2	10.8	10.6

NT = not tested.

Laboratory C provided results in respect of *C. perfringens* Type D, and *C. tetani* for three replicate IC assays each performed on material taken from two separate vials. Detailed results showed a high degree of reproducibility and consistency between vials was obtained. Results obtained by TN were also reported in respect of all five components.

Table 4. — *Summary of Study Results - Laboratory C*

Serum	Antitoxin Activity (IU/ml) against Clostridial Components									
	C. perf B/C		*C. perf D*		*C. septicum*		*C. tetani*		*C. novyi*	
	IC	TN	IC	TN	IC	TN	IC	TN	IC	TN
A	NT	NT	7.5	10-11	NT	15-16	2.0	4-5	NT	16-18
B	NT	NT	11.9	9-10	NT	4-5	4.0	4-6	NT	7-8
C	NT	14-16	9.7	10-11	NT	10-11	3.7	4-5	NT	14-15
D	NT	18-20	24.0	29-31	NT	15-17	6.4	8-9	NT	17-18
E	NT	14-15	13.4	19-20	NT	10-11	5.7	9-10	NT	16-18
F	NT	18-19	15.1	16-17	NT	4-5	15.7	24-26	NT	12-14

NT = not tested.

Laboratory D provided results in respect of all five clostridial antigens for two replicate IC assays each performed on material taken from two separate vials. Detailed results showed a high degree of reproducibility and consistency between the two vials tested was good. This laboratory was unable to perform TN.

Table 5. — *Summary of Study Results - Laboratory D*

Serum	Antitoxin Activity (IU/ml) against Clostridial Components									
	C. perf B/C		*C. perf D*		*C. septicum*		*C. tetani*		*C. novyi*	
	IC	TN	IC	TN	IC	TN	IC	TN	IC	TN
A	3.0	NT	5.5	NT	8.2	NT	4.5	NT	8.2	NT
B	14.4	NT	7.3	NT	7.6	NT	6.6	NT	10.0	NT
C	inv.	NT	10.0	NT	10.4	NT	7.5	NT	11.0	NT
D	21.4	NT	19.9	NT	9.4	NT	12.1	NT	12.9	NT
E	inv.	NT	11.3	NT	8.3	NT	11.7	NT	10.9	NT
F	inv.	NT	12.5	NT	7.0	NT	31.4	NT	12.2	NT

inv. = invalid result due to non-parallelism.

NT = not tested.

4.2. Phase 2

Nine laboratories provided results although one laboratory (B) was able to submit results only in respect of the activity of the *C. novyi* component of the cBRP. One further laboratory (F) was able to provide results only in respect of the *C. perfringens* Type B/C and *C. septicum* components and one laboratory (D) was unable to provide valid results in respect of the *C. perfringens* Type B/C component. Seven of the laboratories which provided results submitted a complete set of results forms in respect of the relevant components. Two laboratories submitted only partially completed results forms. Additional information, i.e. SOP used, experience with the assay method and confidence in the results were also reported. A summary of this survey is presented in table 6.

Table 6. — *Laboratories experience, methods and confidence*

Laboratory	A	B	C	D	E	F	G	H	I
C. perf β									
experience	R		R	R	R	R	R	R	R
protocol	E		C	E	E	E	L	L	L
confidence	+		+		+		+	+	+
C. perf ε									
experience	R		R	R	R	R	R	R	R
protocol	E		C	E	E	E	L	L	L
confidence	+		+	+/-	+	+	+	+	+
C. septicum									
experience	R		R	R	R	NR	R	R	
protocol	E		C	E	E	E	L	L	
confidence	+		+	-	+	+	+	+	
C. novyi									
experience	R	NR	R	R	R	NR	R	R	R
protocol	E	E	C	E	E	E	L	L	L
confidence	+	+/-	+	+	+		+	+	+
C. tetani									
experience	R		NR	R	R		R	R	R
protocol	E		E	E	E		L	L	L
confidence	+		-	+	+		+	+	+

R = performed routinely.
NR = not performed routinely.
E = EDQM protocol followed.
C = own sop adapted to EDQM protocol.
L = own sop followed.
+ = confident.
+/- = unsure.
- = no confidence.

4.2.1. Determination of the toxin test dose

From the data reported, it was possible, for those laboratories and those assays where adequate information regarding the dilutions of toxin and antitoxin used was provided, to calculate the actual toxin dose used in each assay. In those cases where the calculation is possible, the correct nominal toxin dose was used for each component (*C. perfringens* β - 1 IU, *C. septicum* - 0.2 IU, *C. perfringens* ε, *C.novyi* and *C. tetani* - 0.1 IU) with the following exceptions. Laboratory H used a toxin dose of 0.2 IU for the *C. novyi* and *C. tetani* components. Laboratory I used a toxin dose of 0.25 IU for *Clostridium perfringens* ε and *C. novyi*, a dose of 0.2 IU for *C. tetani* and a dose of 1 IU for *C. septicum.*

One laboratory (G) did not verify the test dose of toxins used in the study but relied instead on their own historically assigned value for the toxins. One further laboratory (H) failed to provide the data relating to the determination of the toxin test dose. The remaining laboratories correctly followed the protocol although some experienced difficulty in satisfactorily replicating the results of the first definitive titration and therefore performed more than the required two assays before defining the toxin dose for one or more components. Significantly, for those laboratories which used the toxins provided by EDQM the range of the determined test doses was within 2-3 fold despite the inevitable variation in animal sources and husbandry and in diluent and precise techniques. This is significant because it indicates that this degree of variation is inherent in the existing assay method. However, within any specific laboratory, the results of replicate determinations of the toxin dose was generally far less than the variation between laboratories. Consequently, because the study was designed to directly compare the activity of the cBRP with the existing ISs the use of different toxin volumes by different laboratories should not affect the precision of the estimate of activity nor the ability to combine results from the different laboratories. In contrast, where laboratories failed to follow the protocol and/or to provide evidence that the toxin dose used was as specified, there is substantially reduced assurance that the calculated activity of the cBRP reflects its true value.

4.2.2. Results of potency assays of the equine WHO IS antitoxins

The results obtained in the final assays for antitoxin activity of the IS are summarised in table 7. In principle, because the toxin dose was determined by reference to these materials using the assigned value for each standard, the results obtained in these assays would be expected to fall within 20 % of the assigned value. Some laboratories expressed results as a range when the survival rate changed from 100 % to 0 % in a single dilution. In this table these results have been expressed as the mean value of the range. When results included only 100 % or 0 % survival the result has been expressed as greater than or less than the extreme activity which could have been measured using the dilutions employed in the specific assay.

These extreme values have been included (as end-point values) in the analysis of the intra-laboratory variation because exclusion would bias the results more seriously than inclusion does. Moreover the overall repeatability justifies the assumption that the remaining bias is unlikely to be very important.

Laboratory E did not assay the ISs in direct comparison to the cBRP making it impossible to apply validation criteria to the results obtained in individual assays.

Results of individual assays from some other laboratories were not always fully valid as indicated in the footnotes to the table 7. Results indicated by footnotes 3-9 have been excluded from the calculation of the intra-laboratory mean observed values presented in table 8. Table 8 also includes the calculated intra laboratory residual standard deviation (RSD) for the ISs including the extreme values as indicated above. The grand means and associated RSDs in this table are derived from the mean values obtained by each laboratory but exclude those values shown in parentheses because these were obtained using a toxin dose other than that specified in the protocol.

Table 7. — *Observed Antitoxin Activities (IU/vial) of the Equine IS*

		Component				
		C. perf β	*C. perf* ε	*C. septicum*	*C. novyi*	*C. tetani*
		Assigned Value				
		4770	**1000**	**500**	**1100**	**1400**
Lab.	**Assay**	Calculated Activity				
A	1	4093	1000	555	990	1330
	2	5051	1000	555	1100	1260
	3	5051	1050	555	1100	1260
	4	4789	1000	500	990	>1400[2]
B	1				920	
	2				763[1]	
	3				<763[2]	
	4				920	
C	1	4770	900	550	880	1680
	2	4770[6]	900	500	990	1540
	3	4770[6]	1000	550	990	1540
	4	4770[6]	900	550	990	1540
D	1		1333[1]	>714[8]	768[7]	2083[7]
	2		727[7]	696[8]	909	1923[7]
	3		864	421[8]	909	2272[7]
	4		864	>800[8]	<714[2]	1613
	5			461[8]		
	6			356[8]		
F	1		1000	500		
	2		not sub[3]	not sub[3]		
	3		not sub[3]	not sub[3]		
G	1	4770	1048	435	1153	1687[9]
	2	5489	1000	435	1266	1467
	3	5242	867	549[9]	1153	1213
	4	5489	1048		1050	1335
H[5]	1	5452	1164	350[7]	963[10]	1225[10]
	2	5452	1164	400	963[10]	1225[10]
I	1	4166	740[7,10]	378[7,10]	1000[10]	1111[7,10]
	2	3350[7]	851[10]	421[10]	1053[10]	1290[10]
	3	3774[7]	851[10]	421[10]	862[7,10]	1111[7,10]
	4	3350[7]	851[10]	421[10]	1111[10]	1111[7,10]

Footnotes to table 7:

1 value calculated from result which did not include 100% and 0% survival (i.e. based on 1/2 surviving).

2 value calculated from result including only 100% or 0% surviving.

3 detailed results (survival at each dose level) not provided therefore validity is not confirmed.

4 toxin dose not confirmed as required by protocol.

5 data for toxin dose titrations not provided.

6 result calculated from non-linear survival rates.

7 result deviates by more than 20% from assigned value.

8 result excluded due to poor repeatability.

9 result excluded due to flat slope (i.e. 1/2 surviving at multiple dilutions).

10 result excluded because the specified toxin dose was not used.

Table 8. — *Calculated Mean Antitoxin Activity (IU/vial) and RSD (%) for the IS*

		Component				
		C. perf β	*C. perf ε*	*C. septicum*	*C. novyi*	*C. tetani*
		Assigned Value				
Lab.		**4770**	**1000**	**500**	**1100**	**1400**
A	Mean	4746	1013	541	1045	1283
	RSD	9.5	2.5	5.1	6.1	5.1
B	Mean				920	
	RSD				10.8	
C	Mean	4770	925	538	963	1575
	RSD		5.4	4.7	5.7	4.4
D	Mean		864		909	1613
	RSD		28.0		12.1	14.1
F	Mean		1000	500		
	RSD					
G	Mean	5248	991	435	1156	1338
	RSD	6.5	8.6	0.0	7.6	14.2
H	Mean	5452	1164	400	(963)	(1225)
	RSD	0.0	0.0	9.4	0.0	0.0
I	Mean	4166	(851)	(421)	(1055)	(1290)
	RSD	10.7	6.7	5.2	10.6	7.7
Grand Mean		4876	993	483	998	1452
Inter-lab. RSD		9.2	10.2	13.0	10.3	11.4

4.2.3. Results of potency assays of the cBRP

Table 9 summarises the calculated values of the activity of the cBRP including all the data provided by participants. Within this table those values which derive from assays for which the observed results for the IS are invalid are shown in strikeout. As for the assays of the IS some results are not fully valid as indicated by the footnotes to the table.

The mean result and its associated RSD for each laboratory and each component have been calculated in the same way as for the IS and are summarised in table 10. The mean results for laboratory E are shown in parentheses because, as noted previously, this laboratory did not directly compare the cBRP with the ISs. Similarly the mean results for laboratory H in respect of *C. novyi* and *C. tetani* and for laboratory I in respect of all components except *C. perfringens β* are shown in parentheses because the toxin dose used in these assays was not in accordance with the protocol. Consequently, all the results shown in parentheses have been excluded from the calculation of the overall mean values.

Table 9. — *Observed Antitoxin Activity (I.U./vial) of cBRP*

Lab.	Assay	Component				
		C. perf β	*C. perf ε*	*C. septicum*	*C. novyi*	*C. tetani*
A	1	10.04	10.00	<6.16[1]	11.11	<7.91[1]
	2	10.04	9.52	5.26	11.69	>8.02[1]
	3	10.04	10.55	5.26	11.11	>8.02[1]
	4	10.04	10.01	5.17	11.10	~~8.00~~
B	1				8.9	
	2				~~8.0~~	
	3				~~8.0~~	
	4				8.9	
C	1	11.5	13.0	7.5	13.0[1]	8.0
	2	~~<11~~[1]	12.5	8.0	14.5	9.
	3	~~12.0~~	13.0	7.5	14	9.5
	4	~~11.0~~	13.0[1]	8.0	13.0[1]	9.0
D	1		~~3.33~~[1]	~~>10.6~~[1]	~~4.58~~	~~7.14~~
	2		~~>5.55~~[1]	~~7.62~~	6.52	6.60
	3		~~>6.4~~[1]	~~<6.4~~	7.74	~~<6.06~~[1]
	4		7.0	~~7.0~~	~~7.7~~	6.06
	5			~~<5.3~~[1]		
	6			~~4.85~~		
E	1	~~7.5~~	~~18.75~~	~~11.25~~	~~13.75~~	~~8.75~~
	2	~~8.75~~	~~18.75~~	~~11.25~~	~~13.75~~	~~8.75~~
	3	~~8.75~~	~~16.25~~	~~8.75~~	~~12.5~~	~~8.75~~
	4	~~7.5~~	~~15.0~~	~~11.25~~	~~13.75~~	~~7.80~~
F	1		9.47		5.0[1]	
	2		9.5[2]		5.5[2]	
	3		10.0[2]		5.0[2]	
G[3]	1	10.0	12.1	11.4	12.7	~~9.6~~
	2	11.0	10.0	12.6	11.5	8.7
	3	10.0	10.0	~~10.3~~	11.5	7.1
	4	11.0	10.5		11.5	9.4
H	1	15.5	15.5	~~8.0~~	~~18.0~~	~~8.5~~
	2	13.0	14.0	7.5	~~16.0~~	~~8.5~~
I	1	8.0[5]	~~8.8~~	~~5.5~~	~~7.7~~[5]	~~6.2~~
	2	~~6.9~~	~~8.8~~	~~6.7~~	~~8.8~~	~~7.0~~
	3	~~8.9~~	~~8.8~~	~~6.7~~	~~8.8~~	~~7.0~~
	4	~~6.9~~	~~8.8~~	~~6.7~~	~~10.0~~	~~7.0~~

Footnotes to table 9

[1] value calculated from result which did not include 100 % and 0 % survival (i.e. based on 1/2 surviving).

[2] detailed results (survival at each dose level) not provided therefore validity is not confirmed.

[3] toxin dose not confirmed as required by protocol.

[4] data for toxin dose titrations not provided.

[5] result derived from preliminary titration (2 fold dilutions only).

Table 10. — *Calculated Mean Antitoxin Activity (IU/vial) and RSD (%) for the cBRP.*

Lab.		Component *C. perf β*	*C. perf ε*	*C. septicum*	*C. novyi*	*C. tetani*
A	Mean	10.04	10.02	5.17	11.11	8.0
	RSD	0.0	4.2	9.4	4.3	0.7
B	Mean				8.9	
	RSD				6.1	
C	Mean	11.5	12.88	7.75	13.63	8.88
	RSD		1.9	3.7	5.5	7.1
D	Mean		7.0		7.1	7.2
	RSD		28.9		22.5	10.5
E	Mean	(8.13)	(17.19)	(10.63)	(13.44)	(8.44)
	RSD	8.9	10.9	11.8	4.7	7.4
F	Mean		9.66	5.17		
	RSD		3.1	5.6		
G	Mean	10.5	10.65	12.0	11.8	8.7
	RSD	5.5	9.3	7.1	5.1	13.0
H	Mean	14.25	14.75	7.5	(17.0)	(8.5)
	RSD	12.4	7.2	4.6	8.3	0.0
I	Mean	(8.0)	(8.8)	(6.7)	(8.83)	(7.0)
	RSD	12.6	0.0	9.4	10.6	5.9
Grand Mean		10.9	10.8	7.5	10.5	8.2
Inter-lab.RSD		21.0	24.8	37.1	24.3	9.3

5. ANALYSIS OF THE RESULTS

5.1. Phase 1

Since each laboratory performed at least two separate IC assays within Phase 1 of the study an appraisal of the reproducibility of the assays used was possible. Analysis of the individual results provided by participants revealed that the reproducibility within each laboratory was satisfactory with individual results normally varying only a small amount between occasions.

Invalidity caused by a lack of parallelism was recorded by laboratory D in respect of three of the samples, including the pilot reference blend, (serum E) when tested for activity against *C. perfringens* Type C toxin. Importantly, no invalidity was observed when the serum donated by this laboratory was tested. This observation confirms an earlier preliminary finding which suggested that, for this component, there may be variant strains or toxins which result in non-parallel serological results when assayed in comparison with heterologous antisera. Whilst such a situation would inevitably complicate the development of a single reference serum it does not necessarily mean that two (or more) separate reference sera are required in order that homologous test and reference materials can be used routinely. It should also be noted that the result for serum B from this laboratory indicates a high level of antitoxin activity against *C. perfringens* Type B/C toxin. This may indicate a problem in the specific assay since serum

B was raised against vaccines which do not contain this antigen. The cause of the invalidity recorded is therefore not clear.

Since each participant had developed their own serological assay methods which employ in-house reference reagents, comparison of the absolute values for antitoxin activity against each component was not especially useful. More important was whether the methods used in each laboratory were capable of distinguishing between the samples included in the study and of ranking them in the correct order. In principle, the relative activity of each sample, for each component, should be similar for all laboratories although, since the antigens used in the assays also differ, there may be differences in avidity which result in differences in the observed relative activities. Tables 11a-e provide an overview of the results, in respect of each component, from each participant in which the recorded results obtained both by IC methods and by TN have also been expressed as a proportion of the recorded activity of the pilot blend sample (serum E) and a ranking order assigned.

Table 11a. — *Analysis of Inter-laboratory Variation - C. perfringens Type B/C*

	Serum	**A**	**B**	**C**	**D**	**E**	**F**
Predicted							
	IU/ml	-	-	19.2	22.4	13.0	27.2
	Prpn.	-	-	1.5	1.7	1.0	2.1
	Rank	-	-	3	2	4	1
Lab							
A	TNT						
	IU/ml	-	-	25	25	17.5	>25
	Prpn.			1.4	1.4	1.0	>1.4
	Rank			2=	2=	4	1
B	serology						
	IU/ml	-	-	14.9	19.5	12.0	27.5
	Prpn.			1.2	1.6	1.0	2.3
	Rank				3	2	4 1
B	TNT						
	IU/ml	-	-	15.7	17.2	10.8	24.7
	Prpn.			1.5	1.6	1.0	2.3
	Rank			3	2	4	1
C	TNT						
	IU/ml	-	-	15	19	14.5	18.5
	Prpn.			1.0	1.3	1.0	1.3
	Rank			3	1	4	2
D	serology						
	IU/ml	3.0	14.4	inv.	21.4	inv.	inv.
	Prpn.						
	Rank						

Prpn = proportion of the recorded activity of E.

Inv. = invalid.

Table 11b. — *Analysis of Inter-laboratory Variation - C. perfringens Type D*

	Serum	A	B	C	D	E	F
Predicted							
	IU/ml	8.7	6.2	10.1	25.2	14.5	20.2
	Prpn.	0.6	0.4	0.7	1.7	1.0	1.4
	Rank	5	6	4	1	3	2
Lab.							
A	serology						
	IU/ml	4.0	8.1	6.1	17.6	8.0	9.1
	Prpn.	0.5	1.0	0.8	2.2	1.0	1.1
	Rank	6	3	5	1	4	2
B	serology						
	IU/ml	9.1	9.8	11.9	27.3	14.7	14.5
	Prpn.	0.6	0.7	0.8	1.9	1.0	1.0
	Rank	6	5	4	1	2	3
B	TNT						
	IU/ml	6.9	9.1	9.1	18.9	13.1	13.1
	Prpn.	0.5	0.7	0.7	1.4	1.0	1.0
	Rank	6	4=	4=	1	2=	2=
C	serology						
	IU/ml	7.5	11.9	9.7	24.0	13.4	15.1
	Prpn.	0.6	0.9	0.7	1.8	1.0	1.1
	Rank	6	4	5	1	3	2
C	TNT						
	IU/ml	10.5	9.5	10.5	30	19.5	16.5
	Prpn.	0.5	0.5	0.5	1.3	1.0	0.8
	Rank	4=	5	4=	1	2	3
D	serology						
	IU/ml	5.5	7.3	10.0	19.9	11.3	12.5
	Prpn.	0.5	0.6	0.9	1.8	1.0	1.1
	Rank	6	5	4	1	3	2

Prpn.= proportion of the recorded activity of E.

Table 11c. — *Analysis of Inter-laboratory Variation - C. septicum*

	Serum	A	B	C	D	E	F
Predicted							
	IU/ml	10.0	3.4	7.4	9.3	8.0	6.9
	Prpn.	1.3	0.4	0.9	1.2	1.0	0.9
	Rank	1	6	4	2	3	5
Lab.							
A	serology						
	IU/ml	7.5	13.8	5.8	2.7	6.5	7.0
	Prpn.	1.2	2.1	0.9	0.4	1.0	1.1
	Rank	2	1	5	6	4	3
A	TNT						
	IU/ml	>10	7	<6	<8	8	>5
	Prpn.						
	Rank						
C	TNT						
	IU/ml	15.5	4.5	10.5	16	10.5	4.5
	Prpn.	1.5	0.4	1.0	1.5	1.0	0.4
	Rank	2	5=	3=	1	3=	5=
D	serology						
	IU/ml	8.2	7.6	10.4	9.4	8.3	7.0
	Prpn.	1.0	0.9	1.3	1.1	1.0	0.8
	Rank	4	5	1	2	3	6

Prpn.= proportion of the recorded activity of E.

Table 11d — *Analysis of Inter-laboratory Variation - C. tetani*

	Serum	A	B	C	D	E	F
Predicted							
	IU/ml	4.8	3.7	5.4	12.1	9.4	22.3
	Prpn.	0.5	0.4	0.6	1.3	1.0	2.4
	Rank	5	6	4	2	3	1
Lab.							
A	serology						
	IU/ml	3.3	4.9	5.0	8.1	7.7	20.8
	Prpn.	0.4	0.6	0.6	1.1	1.0	2.7
	Rank	6	5	4	2	3	1
A	TNT						
	IU/ml	4	6	>6	>10	>10	26
	Prpn.	0.4	0.6	>0.6	1.0	1.0	2.6
	Rank	6	5	4	2=	2=	1
B	serology						
	IU/ml	6.7	9.6	10.5	17.7	16.8	45.7
	Prpn.	0.4	0.6	0.6	1.1	1.0	2.7
	Rank	6	5	4	2	3	1
B	TNT						
	IU/ml	5.9	7.2	8.0	12.7	12.7	31.2
	Propn.	0.5	0.6	0.6	1.0	1.0	2.5
	Rank	6	5	4	2=	2=	1
C	serology						
	IU/ml	2.0	4.0	3.7	6.4	5.7	15.7
	Prpn.	0.4	0.7	0.6	1.1	1.0	2.8
	Rank	6	4	5	2	3	1
C	TNT						
	IU/ml	4.5	5	4.5	8.5	9.5	25
	Prpn.	0.5	0.5	0.5	0.9	1.0	2.6
D	serology						
	IU/ml	4.5	6.6	7.5	12.1	11.7	31.4
	Prpn.	0.4	0.6	0.6	1.0	1.0	2.7
	Rank	6	5	4	2	3	1

Table 11e. — *Analysis of Inter-laboratory Variation - C. novyi*

	Serum	A	B	C	D	E	F
Predicted							
	IU/ml	13.3	6.3	9.1	13.6	12.1	15.9
	Prpn.	1.1	0.5	0.8	1.1	1.0	1.3
	Rank	3	6	5	2	4	1
Lab.							
A	serology						
	IU/ml	4.6	8.4	4.4	5.5	5.8	4.3
	Prpn.	0.8	1.4	0.8	0.9	1.0	0.7
	Rank	4	1	5	3	2	6
A	TNT						
	IU/ml	>7	>10	>7	>7	>7	>7
	Prpn.						
	Rank						
B	serology						
	IU/ml	13.2	7.1	9.1	12.9	11.1	10.8
	Prpn.	1.2	0.6	0.8	1.2	1.0	1.0
	Rank	1	6	5	2	3	4
B	TNT						
	IU/ml	14.5	7.4	11.6	14.5	14.5	10.6
	Prpn.	1.0	0.5	0.8	1.0	1.0	0.7
	Rank	1=	6	4	1=	1=	5
C	serology						
	IU/ml	-	-	-	-	-	-
	Prpn.						
	Rank						
C	TNT						
	IU/ml	17.0	7.5	14.5	17.5	17.0	13.0
	Prpn.	1.0	0.4	0.9	1.0	1.0	0.8
	Rank	2=	6	4	1	2=	5
D	serology						
	IU/ml	8.2	10.0	11.0	12.9	10.9	12.2
	Prpn.	0.8	0.9	1.0	1.2	1.0	1.1
	Rank	6	5	3	1	4	2

Prpn.= proportion of the recorded activity of E

5.2. Phase 2

5.2.1. Evaluation of assay validity

In some laboratories the apparent variation in toxin dose between assays was greater than the 20 % incremental doses used in the test. Whether this variation was due to inaccuracies in technique or to greater variability in the susceptibility of the animals in those laboratories is unclear.

However, participants were asked to indicate whether they performed the assays routinely and whether they felt confident in their own results (table 6). Significantly, those laboratories which did not perform the assays routinely or lacked confidence in the results obtained with the reagents provided showed a greater variation between assays. In contrast, laboratories which were very confident in their results tended to observe less variation between assays but did not necessarily succeed in accurately using the toxin dose specified by the protocol.

These data were useful in determining which of the definitive assays may be considered valid. In principle, the existing monographs require the titration to give the expected result with the IS antitoxin within plus or minus 20 %. Consequently, those assays where the observed toxin dose used was not within 20 % of the intended dose (and therefore the observed activity of the IS was not within 20 % of the assigned value), should be considered invalid. However, there were some instances where the results obtained in the assay did not include the 50 % end point. In these cases participants recorded results as greater than or less than a specified value. These assays should also be considered invalid. Furthermore, the results for three of four assays for *C. perfringens* β antitoxin performed by Laboratory C yielded non linear responses and should also be considered invalid.

Similarly, in a number of the assays for the activity of the cBRP the 50 % end point was not detected and results of these assays are indicated in table 9 by a result recorded as greater than or less than a specified value.

In strict statistical terms, only those assays in which the results obtained with the IS were valid and for which linear results encompassing the 50 % end point for the cBRP were obtained can be considered valid.

In order to properly compare the intra-laboratory variation achieved with the existing ISs and with the cBRP all the reported results have been included with the exception of those where non-linearity or failure to encompass the 50 % end point precluded calculation of a result. Using these data tables 8 and 10 respectively show, for each laboratory, the mean value and RSD for the activity of the IS antitoxins and for the cBRP. The material with the lower RSD was identified for each component. These data indicate that in 14 of the 26 possible comparisons the RSD for the cBRP was lower than that for the IS whilst in the remaining 12 cases the IS showed a greater variability. Very importantly, there was no systematic tendency for any of the laboratories or for any component to behave more consistently for either of the preparations. It may therefore be concluded that the cBRP will yield results with an equivalent repeatability compared to the ISs when tested by TN within any single laboratory.

In contrast, comparison of the inter-laboratory variation for the two preparations, as expressed by the overall RSD, indicated that for each of the components other than *C. tetani* the cBRP yielded greater variability as shown in table 12. This observation does not, however, indicate that the cBRP will yield intrinsically more variable results.

In theory, both the intra-laboratory and inter-laboratory RSDs for the ISs should be zero because the toxin dose for each component was calculated by its activity against the relevant IS.

Consequently, the RSD values for the ISs recorded in this study reflect the inherent variability of the assay method. Similarly, the intra-laboratory RSD values for the cBRP reflect the inherent variability of the assay as performed by each individual laboratory. Consequently, the observation that intra-laboratory RSD is comparable for the two preparations was precisely the result that would have been expected. In contrast, the inter-laboratory RSD for the cBRP would have been expected to be larger than for the IS because the toxin dose is determined on the basis of antigen-antibody reaction between the highly avid equine hyperimmune IS and not by reference to the less avid rabbit sera comprising the cBRP. The consequence of this is that an L+ dose of toxin determined against the IS is not necessarily the same as an L+ dose determined against rabbit serum. Furthermore, since each laboratory uses a different source of animals the relationship between the L+ doses determined in these different ways may not be constant. In fact, this same apparent increase in inter-laboratory RSD would be expected if different laboratories were to assay routine potency test samples.

Table 12. — *Inter-laboratory RSD (%) for the ISs and cBRP*

Component	*C. perf* β	*C. perf* ε	*C. septicum*	*C. novyi*	*C. tetani*
IS	9.2	10.2	13.0	10.3	11.4
cBRP	21.0	24.8	37.1	24.3	9.3

In order to further evaluate whether the cBRP would perform as reliably as the ISs the incidence of assay invalidity due to each preparation was considered. Table 13 shows that the incidence of such invalidity is comparable in respect of assays for antitoxin against *C. perfringens* ε and rather lower with cBRP for all other components.

Table 13. — *Proportion and Incidence (%) of Invalid Assays with the ISs and the cBRP*

Component	*C. perf* β	*C. perf* ε	*C. septicum*	*C. novyi*	*C. tetani*
IS	6/18 (33)	3/23 (13)	7/24 (29)	5/24 (21)	8/20 (40)
cBRP	3/22 (14)	4/29 (14)	5/30 (17)	3/30 (10)	4/26 (15)

5.2.2. Determination of the assigned activities of the cBRP in respect of each component

As indicated above, in strict statistical terms, only those assays in which the results obtained with the Equine ISs were valid and for which linear results encompassing the 50 % end point for the cBRP were obtained can be considered valid. Using only these data the mean values for the activity of each of the components of the cBRP were those presented in table 10.

These mean values excluded all of the results from laboratory E because this laboratory did not directly compare the activity of the ISs with the cBRP. In addition, results for the cBRP obtained in assays in which the IS failed to yield valid results have been excluded. It was useful therefore to compare the mean values in table 10 with the mean values obtained when these additional data were included. This comparison is shown in table 14 from which it is clear that the inclusion of these data for which clear validation cannot be provided (basis B) makes very little difference to the overall estimate of activity for any of the components. Furthermore, whether or not these data were excluded but data derived from assays performed using toxin doses other than those specified in the protocol were included (basis C and D), the mean activities calculated are little affected.

Table 14. — *Comparison of Mean Estimate of Activity (IU/vial) for the cBRP excluding and including unvalidatable results.*

Basis of Calculation	Component *C. perf β*	*C. perf ε*	*C. septicum*	*C. novyi*	*C. tetani*
A	10.9	10.8	7.5	10.5	8.2
B	10.3	11.1	7.6	10.6	8.0
C	10.9	10.5	7.4	11.2	8.0
D	10.3	11.4	7.8	11.5	8.1

A excludes all invalid data.

B includes data not confirmed as valid and data from assays where the result for the IS was invalid.

C includes valid data derived from assays using toxin doses other than those specified in the protocol.

D includes data not confirmed as valid and data from assays where the result for the IS was invalid and valid data derived from assays using toxin doses other than those specified in the protocol.

6. DISCUSSION

6.1. Phase 1

6.1.1. C. perfringens Type B/C

Only two laboratories (B and D) provided IC results in respect of antitoxin to *C. perfringens* Type B/C. The results from laboratory D included several assays which were invalid due to lack of parallelism with their own internal reference preparation and one valid result which indicated a high level of antitoxin in a sample which contained no antitoxin. The validity of this specific assay must therefore be questioned. IC results from laboratory B however correlated well with the anticipated activities of the samples and results were ranked in the correct order. Furthermore, three laboratories provided results for this component obtained by TN. Although the ranking of the samples varied slightly between laboratories a good correlation between the results of each laboratory and the anticipated values was obtained.

Accepting that the available data are very limited, it may be concluded that none of the serum samples provided by individual manufacturers nor the blend of these samples led to a break in correlation with TN results and that the results of TN assays themselves were consistent with the expected results whether the donated sera were tested independently (samples A-D, F) or as a blend (sample E).

6.1.2. C. perfringens Type D

All four laboratories provided IC results in respect of antitoxin to *C. perfringens* Type D. The absolute values for activity of individual samples recorded by each laboratory varied by approximately two fold both between laboratories and in comparison to the anticipated values. The relative activities however provided a reasonable correlation. All laboratories found serum D to contain a higher level of antitoxin than any other sample whilst, with the exception of laboratory A, samples E and F were consistently found to contain slightly less activity and were ranked either second or third. Sample C was consistently ranked fourth or fifth by all laboratories and samples A and B, which were anticipated to have the lowest activity were ranked fourth, fifth or sixth, again with the exception of laboratory A. Two laboratories only presented TN results for this component and it is particularly important to note that the variation in absolute values determined by these laboratories was somewhat greater than that recorded by IC methods although again the relative activities were broadly comparable. Using TN, both laboratories found sample D to contain the highest antitoxin level, with samples E and F ranking second or third as anticipated with the remaining three

samples exhibiting slightly lower activities. The serological results obtained by laboratory A showed a weaker correlation than those of other laboratories with the anticipated activities and a weaker correlation when compared to the results of the other laboratories. As noted above, the result obtained in the study for the serum which was provided by this laboratory was significantly different to that notified at the time of donation. Consequently, it may be concluded that the limited correlation obtained by this laboratory is a function of the test system rather than of the nature of the samples. Certainly there is no evidence that any one of the individual serum pools nor the blend of these pools led to a breakdown in correlation with activity as determined by TN assay.

6.1.3. C. septicum

Two participants have each presented serological and TN results for this component. However, one laboratory was able to provide only preliminary results obtained by TN and these did not permit interpretation. The anticipated activities of the samples varied by only three fold and although the TN results from laboratory C showed somewhat different absolute values the relative activities of the samples generally correlated well with the anticipated values. The only exception to this was sample F which seemed to show a lower relative activity than anticipated. By chance, this sample is the one donated by laboratory C and this discrepancy is therefore most likely to be due to assay variation rather than attributable directly to the nature of the samples.

IC results obtained by laboratory A show a poor correlation with the anticipated results and it is particularly noticeable that serum B, which was claimed to have the lowest activity was determined by this laboratory to be by far the most active. In contrast, the results obtained by laboratory D showed a slightly better correlation although the assay still failed to rank the samples in the anticipated order or to show a significant correlation with the single set of TN results provided.

It is unclear from these data whether the nature of the samples themselves has resulted in a breakdown in the correlation between the serological methods and TN or whether the activities of the samples were simply so close that neither the TN nor serological methods possessed the precision to reliably distinguish between them. It can, however, be concluded that blending of the samples did not seem to reduce the correlation further than was seen with the individual samples. Therefore, it could be concluded that the use of a reference serum raised against any specific antigen or strain offers no advantage over one comprising a blend of sera raised against a broad spectrum of strains.

6.1.4. C. tetani

All four laboratories presented IC results in respect of this component and three also provided results obtained by TN. The absolute values obtained by the different laboratories varied by approximately three fold but the relative activities were remarkably consistent and exhibited a very high correlation both between laboratories and in comparison with the anticipated values. Samples were ranked similarly by all laboratories. In addition, the relative activities of the samples as determined by TN also correlated well between laboratories and in comparison with the serological results. Samples were consistently ranked similarly by both methods.

It is clear from these data that none of the serological models were compromised by samples derived from vaccines produced by different manufacturers nor by blending of sera derived from different vaccines. Furthermore, the activity of the pilot blend seemed to represent a good balance between the most and least active of the samples and may therefore be expected to be appropriate as a reference serum for all manufacturers.

6.1.5. C. novyi

Three laboratories have presented IC results for this component and although three sets of TN data were also been submitted, those from laboratory A were preliminary and could not be interpreted.

The anticipated activities of the samples varied by only a little more than two fold and it is therefore not surprising that none of the laboratories ranked the samples in the anticipated order either by IC methods or TN. Relative activities as determined by laboratories B and C using TN showed a very good correlation although this conflicted with the anticipated results based on the data provided by the donators. Importantly, the results obtained by laboratory B using an IC method also correlated extremely well with the TN data. However, results obtained by IC assays by laboratories A and D showed a weaker correlation caused principally by discrepancies in the observed relative activities of samples B and F both of which were donated by laboratories other than A and D. These discrepancies may be a function of normal variation in the assays especially since the range of activities amongst the samples was small but most importantly, inclusion of these sera within the pilot blend (sample E) did not seem to have adversely affected the overall performance of the assays.

Although the range of activities amongst the samples was small the available data indicates that the pilot blend would serve as an appropriate reference serum for all manufacturers irrespective of the method by which antitoxin activity is determined.

6.1.6. Conclusions from Phase 1

The data assembled within phase 1 of this study was limited but suggested that the pilot blend of sera prepared would be appropriate as a reference material for use by all manufacturers within both TN and serological assays for antitoxin against the five clostridial components considered. It was therefore concluded that phase 2 of the study should proceed and that the cBRP should be constructed in a manner exactly identical to that of the blend (serum E) prepared in phase 1.

6.2. PHASE 2

In defining the assigned activity of the cBRP consideration was given to the exclusion of all invalid data, to data invalid for various reasons and to weighting the results of the different laboratories according to the frequency of assay invalidity. However, since the mean results obtained using only results from assays which were fully valid was not significantly different to those obtained when the results of all assays for which results could be calculated were included it was concluded that it was reasonable to include the maximum amount of data possible. This conclusion could be further justified because in only three instances did individual values included in calculations on basis B, C, or D in table 14 differ from the mean values obtained on basis A by more than 20 % and in none of these cases were the absolute values outside the range of values included within the calculation on basis A.

It was also reassuring to note that these values are consistent with the values predicted by the formulation of the cBRP even though these predicted activities were not validated in the sense that the toxin doses used were not verified and direct comparison with the ISs was not included. In addition, the values obtained in this phase of the study were also consistent with those recorded in phase 1 of the study although again the validity and particularly the precision of the estimates obtained in phase 1 was not controlled. A summary of these comparisons is shown in table 15.

Table 15. — *Comparison of Mean Estimates of Activity (IU/vial) determined in Phases 1 and 2 and Predicted Activities*

Component	*C. perf β*	*C. perf ε*	*C. septicum*	*C.novyi*	*C.tetani*
Predicted	13.0	14.5	8.0	12.1	9.4
Phase 2	10.5	11.0	7.5	11.0	8.0
Phase 1 TN	12.7	17.2	8.8	14.8	12.4
Phase 1 IC	14.8	11.9	7.4	9.3	10.5

TN = Toxin neutralisation test in mice.
IC = Immunochemical methods.

7. CONCLUSION

On the basis of the data presented it was concluded that the cBRP is suitable for use as a definitive preparation in place of the existing Equine ISs. Therefore this material has been adopted at the 108th session of the Ph. Eur. Commission in November 2000 as *Clostridia* rabbit multicomponent antiserum Ph. Eur. BRP Batch 1(1) with an assigned activity of:

C. perfringens (Type B/C) β antitoxin	10.5 I.U. per vial
C. perfringens (Type D) ε antitoxin	11.0 I.U. per vial
C. septicum	7.5 I.U. per vial
C. novyi Type B	11.0 I.U. per vial
C. tetani	8.0 I.U per vial

8. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to the project leader, R. Lucken (Trawsnant) and to all participants for their contributions to this study. The help of Merial, CSL Ltd, Intervet UK Ltd, Schering Plough Animal Health who donated the sera pools used to prepare the cBRP and/or the clostridial toxins used in this study is highly acknowledged. The statistical analysis was performed by A. Daas (EDQM). The study was run by the EDQM with the support of the Council of Europe and the European Commission under the aegis of the Biological Standardisation Programme (internal project number BSP022) with Dr. M. Berg-Candolfi and Dr. M-E. Esposito-Farese acting as project monitors.

9. PARTICIPANTS

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(1) Cat. No. C2424400.

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF

ERYSIPELOTHRIX RHUSIOPATHIAE CHALLENGE STRAINS AS EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS FOR THE IMMUNOGENICITY / EFFICACY TEST OF SWINE ERYSIPELAS VACCINES

COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF *ERYSIPELOTHRIX RHUSIOPATHIAE* CHALLENGE STRAINS AS EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS FOR THE IMMUNOGENICITY / EFFICACY TEST OF SWINE ERYSIPELAS VACCINES

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1. INTRODUCTION

Swine erysipelas is a world-wide bacterial disease of great economic importance. More than 20 different serotypes of *Erysipelothrix(E.)*[3] *rhusiopathiae* were identified [1], with serovars 1 and 2 accounting for about 80 % of all clinical pig isolates [2, 3].

Vaccination is a well-accepted method of preventing infection. Vaccines are produced with strains of serovar 2, but provide cross-protection against most serovars [3]. The efficacy of erysipelas vaccines has to be demonstrated during the licensing procedure for each product using a vaccination-challenge test [4]. A reference method and reference material are necessary to limit practical problems as reported previously [5]. Many factors have to be taken into account when a reference preparation for such an efficacy test has to be established [6].

Regulations concerning the Quality Control (QC) of inactivated erysipelas vaccines are laid down in the European Pharmacopoeia (Ph. Eur.) monograph *0064*. The revised monograph [7] now includes a production section which requires an immunogenicity/potency test in pigs. However, as no reference strains for the challenge test are available in Europe, the Paul-Ehrlich-Institut (PEI) was asked to provide challenge strains of *E. rhusiopathiae* serovars 1 and 2, as candidate (c) Ph. Eur. Biological Reference Preparations (BRPs).

The PEI tested the virulence of 7 *E. rhusiopathiae* strains in pigs: 3 strains of serovar 1, 3 strains of serovar 2, and 1 strain of serovar N which is used in the mouse potency test and is considered to be non-virulent in pigs. Following this step, 2 strains identified as A360 (serovar 1) and NF4 (serovar 2) were selected as cBRPs. The results of the pre-qualification study have been published (8) and conditions for preparing working seeds by using the cBRPs have been defined.

Further, a collaborative study, involving 4 laboratories, has been organised. The participants of the latter study have tested the suitability of the cBRP strains in a pig challenge test model.

2. PARTICIPANTS

Four laboratories (3 OMCLs and 1 research laboratory) tested the suitability of the cBRPs. Throughout this report, each participant is identified by a code number, unrelated to the order of listing shown in the table at the end of the report (section 8).

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(3) Abbreviations: c: candidate, BRP: European Pharmacopoeia Biological Reference Preparation, CFU: Colony forming unit, EDQM: European Directorate for the Quality of Medicines, E.: Erysipelothrix, gCV: Geometric coefficient of variation, ELISA: Enzyme linked immunosorbent assay, IS: International standard, OMCL: Official Medicines Control Laboratory, Ph. Eur.: European Pharmacopoeia, PEI: Paul-Ehrlich-Institut, QC: Quality control, SOP: Standard operating procedure, SPF: Specific pathogen free.

3. MATERIALS, METHODS, STUDY DESIGN

Materials

All laboratories used the cBRP challenge strains according to the instructions for use provided by EDQM. Shortly, the freeze-dried strains were reconstituted with saline to a finale volume of 1.0 ml. A volume of 0.1 ml was used as challenge dose (approx. 6.4×10^7 CFU for serovar 1 and 2.6×10^6 CFU for serovar 2).

Methods and study design

In the study the participants assessed the suitability of the cBRP erysipelas strains in their in-house pig challenge test models, complying with the specification of the revised Ph. Eur. monograph *swine erysipelas vaccine (inactivated).* Shortly, two challenge methods, refered to as methods A and B (see Annex) were used and clinical signs of erysipelas, *i.e.* body temperature increase and/or appearance of typical skin lesions were monitored. However, the set-up of the study varied considerably between the laboratories as summarised in Table 1. Therefore, the experimental conditions and results are described for each laboratory separately.

Table 1. — *Experimental conditions according to the laboratory*

Laboratory	Status of animals		No. of pigs	Challenge procedure	
	Housing conditions	Immunisation status		Strains	Injection technique
1	SPF	V	4	Serovar 1 and 2	A
		V (1/3 dose)	4		
		V (1/10 dose)	4		
		N	3		
2	Conventional	N	6	Serovar 1 and 2	A
3	SPF	N	3	Serovar 1	B
			3	Serovar 2	
4	Conventional	V	5	Serovar 1 and 2	A
			5		B
		N	3		A
			3		B

V = Vaccinated.
N = Non-vaccinated.
A = Needle inserted vertical to the skin approx. 1 cm deep (see Annex).
B = Needle inserted 2-3 mm deep under the epidermis with the needle almost parallel to the skin (see Annex).

4. RESULTS

4.1. Laboratory 1

Laboratory 1 tested the cBRP strains in a vaccination-challenge trial. Three groups of SPF pigs were immunised with graded doses of an inactivated erysipelas vaccine. They were challenged together with an untreated control group.

The challenge procedures was performed 21 days after vaccination and ran as follows: 0.1 ml of each suspension inoculated into the flanks of pigs (on the right side for serovar 1 and on the left side for serovar 2 according to method A (see foot note to Table 1 and Annex). Body temperature was measured daily. For four days after challenge the temperature was measured twice a day.

All pigs vaccinated with a full or 1/3 dose remained healthy for the entire observation period. Pigs vaccinated with only 1/10 dose revealed no skin lesions but a slight fever reaction (< 41 °C) which disappeared without treatment. All 3 unvaccinated animals (409, 411 and 417) developed high fever (> 41 °C) (see Table 2) which necessitated a treatment with Procain-Penicillin G intramuscularly. On the first day after challenge they showed slight reddish spots at both infection sites. On day 2 the symptoms intensified to typical brick-shaped lesions.

Table 2. — *Results of Laboratory 1: Vaccination-challenge test in SPF pigs*

Day	0		19	20	21* chall.	22 a.m.	22 p.m.	23 a.m.	23 p.m.	24 a.m.	24 p.m.	25 a.m.	25 p.m.	26	27	28
Pig No.	Vacci-nation	Dose	Temperature (°C)													
407	yes	1/1	38.5	39.4	39.0	39.1	38.9	38.5	39.1	39.0	39.7	39.4	39.4	39.2	38.9	39.4
410			38.7	39.1	39.0	38.8	39.0	39.0	39.0	39.5	39.5	38.4	39.3	39.3	38.5	38.5
412			38.8	39.0	39.1	38.7	38.3	38.7	39.0	38.7	39.2	38.8	39.0	38.9	38.7	38.3
416			38.7	39.0	39.0	39.6	39.0	38.9	39.2	39.1	39.0	38.7	39.3	39.2	38.9	38.6
404	yes	1/3	38.6	39.0	38.9	38.3	38.8	38.7	39.3	39.0	38.9	39.2	39.1	38.8	38.8	38.6
405			38.7	38.9	38.8	38.7	39.3	38.7	39.2	39.7	39.6	39.5	39.7	39.6	39.2	39.4
406			38.6	38.9	38.9	39.0	39.1	39.6	39.2	38.9	39.7	39.2	39.3	39.0	38.9	39.4
408			38.5	38.8	38.7	38.6	38.9	39.0	38.6	38.4	39.2	39.1	39.5	39.2	39.2	39.0
413	yes	1/10	39.0	38.9	39.2	39.1	**40.5**	39.5	39.6	39.3	39.3	39.1	39.5	39.6	39.1	38.8
414			38.9	38.7	39.0	39.2	**40.9**	39.4	39.8	38.8	39.3	39.3	39.4	39.0	38.9	39.4
415			39.1	39.0	39.0	39.8	**40.9**	39.8	**40.0**	**40.1**	**40.0**	39.7	39.8	39.5	39.1	38.6
409	no	-	39.3	39.0	39.1	39.5	**40.5**	39.1	**40.0**	**40.7**	**41.1**	**40.3**	**41.1**	39.3	39.1	39.0
411			39.0	39.5	39.4	**40.0**	**40.8**	38.5	**40.3**	**40.6**	**40.7**	**41.3**	**41.9**	39.3	38.5	38.5
417			39.0	39.2	39.1	**40.1**	**40.3**	**40.1**	**41.3**	39.7	38.6	38.6	39.1	**41.5**	39.3	38.8

* Day of challenge.
Figures in bold = slight fever reaction (< 41 °C).
Figures on grey back ground = high fever reaction (> 41 °C), treatment with penicillin.

4.2. Laboratory 2

Laboratory 2 performed a combined challenge in six conventional reared pigs. Method A was used for injection of the bacterial cultures.

All animals developed typical erysipelas disease. High fever for two consecutive days (> 41.0 °C) and skin lesions were observed in all pigs. Serovar 1 induced skin lesions in all animals. Skin lesions were observed at the injection site of serovar 2 in 4 of 6 pigs. In two pigs with reactions to both strains the lesions spread from the inoculation site (Table 3).

Table 3. — *Results of Laboratory 2: Body temperature and local skin reactions*

Animal	Body temperature (°C) Day after infection									Local skin reactions		Remarks
No.	–1	0	1	2	3	4	5	6	7	serovar 1	serovar 2	
3650	39.4	39.8	40.7	40.5	40.1	41.5	41.2	40.3	39.0	++ (day 2)*	++ (day 3)*	Lesions spread
966	39.1	39.5	39.8	40.1	42.0	40.5	41.2	41.1	40.8	+++ (day 5)	-	
975	39.5	39.9	39.8	40.9	41.1	41.7	41.7	41.9	41.0	++ (day 3)	+ (day 5)	
974	39.3	39.5	40.9	41.1	40.0	41.9	41.8	40.7	39.2	++ (day 2)	+ (day 2)	Lesions spread
973	39.4	39.6	40.0	39.9	40.1	40.9	41.2	40.8	39.5	++ (day 3)	+++ (day 6)	
972	39.4	40.1	40.3	40.5	40.1	41.4	41.8	41.0	39.8	++ (day 2)	-	

* Day of maximum extent.
+ : Weak positive (1-5 cm).
++: Positive (5-10 cm).
+++: Strong positive (> 10 cm).

4.3 Laboratory 3

Eight non-vaccinated SPF pigs, aged 12 weeks, weighing between 40-50 kg, were divided in two groups of 4 (1 group for serovar 1 and 1 group for serovar 2) housed in two different non-communicating animal facilities.

At the day of challenge, each animal in the challenge groups was injected with 0.1 ml of three different dilutions of the bacterial inoculum at three sites. One pig in each group was used as a negative control which received 0.1 ml of sterile saline at one site. The challenge was performed according to method B (see foot note to Table 1 and Annex).

For both strains, cutaneous reactions localised at the sites of injection and rise in body temperature became obvious the day after challenge. The local reactions and body temperatures observed were recorded over a four day period (see Tables 4 and 5). Three days after inoculation the animals presented typical signs of illness, including loss of weight related to anorexia, and cutaneous lesions outside of the inoculation zones. All the animals were then sacrificed. The pathogen could be re-isolated from inoculation zones and blood samples of all the infected pigs.

Table 4. — *Results of Laboratory 3: Serovar 1*

a) Rectal temperatures (expressed in °C)

	Day after challenge						
Pig No.	**Day 0 a.m.**	**Day 0 p.m.**	**Day 1 a.m.**	**Day 1 p.m.**	**Day 2 a.m.**	**Day 2 p.m.**	**Day 3 a.m.**
6443	39.7	39.3	39.4	39.2	39.5	39.4	39.6
6435	39.2	39.4	**41.3**	**41.1**	39.2	39.5	**41.6**
6461	39.2	39.5	**41**	**41**	40.3	**41**	**41.8**
6466	39.6	39.5	**41.5**	**41**	40	**41.4**	**41.5**

Figures in bold = elevated temperatures (> 41 °C).

b) Local reactions at injection sites

Day after challenge	**Day 0**	**Day 1**			**Day 2**			**Day 3**		
Pig No.	**ABC#**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
6443	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
6435	0K	25 **S C**	15 **S C**	25 **S C**	70.40* **CS**$^{-}$	20 **S**$^{-}$	20/30* **S**$^{-}$	60/25* **I**$^{-}$ **C**	15 **I**$^{-}$	20/10* **I**$^{-}$ **C**
6461	0K	30 **S**	40 **S**	20 **S**	35 **S**$^{-}$ **N**	35 **S**$^{-}$ **N**	45 **S D b**	5 **N**	5 **N**	5 **N**
6466	OK	40 **S**$^{+}$ **N**	40 **S**$^{+}$ **C**$^{-}$	20/30* **S**$^{-}$	40 **S N**	30 **S**$^{-}$ **N**	30 **S**$^{-}$ **N**	25 **N**	20/10* **N**	15 **N**

Injection sites.

S: Swelling; **I**: Induration; **C**: Congestion; **R**: Redness; **b**: Bump; **D**: Diffuse; **N**: Necrosis at inoculation site; **ND**: Not Determined.

+ = Strong reaction, **-** = Weak reaction.

Figures indicate the surface (*) or diameter in square mm or mm respectively.

Table 5. — *Results of Laboratory 3: Serovar 2*

a) Rectal temperatures (expressed in °C)

	Day after challenge						
Pig No.	**Day 0 a.m.**	**Day 0 p.m.**	**Day 1 a.m.**	**Day 1 p.m.**	**Day 2 a.m.**	**Day 2 p.m.**	**Day 3 a.m.**
6434	39.3	39.3	39.8	39.4	39.2	39.5	39.4
6415	39.4	39.8	**41.3**	**41.8**	40.1	40.0	**41.4**
6426	39.7	39.5	39.5	**41.4**	**41.7**	**42**	40.9
6453	39.5	39.6	**41.0**	**41.8**	40.1	40.5	**42.2**

Figures in bold = elevated temperatures (> 41 °C).

b) Local reactions at injection sites

Day after challenge	**Day 0**	**Day 1**			**Day 2**			**Day 3**		
Pig No.	**ABC#**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
6434	OK	C (Shaving)	OK	ND	OK	OK	OK	OK	OK	OK
6415	0K	25 **S⁻ C**	25 **S C**	10 **S C**	70/600* **S⁻ C**	45 **S⁻**	45/30* **S I**	50/60***R N**	60/50* **R N**	35 **C S⁺**
6426	0K	20 **S**	15 **S C**	15 **S**	45 **S⁻I⁻N**	15/10* **C**	55 **S⁻ I**	45/50* **C N b**	5 **N**	5 **N**
6453	OK	25 **S**	40 **S⁺**	15 **S**	45 **S I N**	400 **S C N**	20/25 **I C D**	50/60 **R N**	35 **C N**	40/50* **R**

Injection sites.

S: Swelling; **I**: Induration; **C**: Congestion; **R**: Redness; **b**: Bump; **D**: Diffuse; **N**: Necrosis at inoculation site; **ND**: Not Determined.

+ = strong reaction, **-** = weak reaction.

Figures indicate the surface (*) or diameter in square mm or mm respectively.

4.4 Laboratory 4

The challenge was performed three weeks after vaccination using injection techniques A or B for different animals. Body temperature and clinical signs were recorded twice a day until day 8 after challenge.

All vaccinated pigs were protected against the experimental erysipelas infection. Only 3/10 pigs developed a small local skin reaction (diameter <1 cm) one day after challenge, which disappeared on the following days. These three pigs were all inoculated using method B (Table 6).

Table 6. — *Results of Laboratory 4: Vaccination-challenge test in conventional pigs, vaccinated pigs*

Animal No.	Route of Infection*	Local Reaction Serovar 1	Local Reaction Serovar 2	Systemic Reaction
1		-	-	None
3		-	+/- (day 1)**	None
4	Method B	-	+/- (day 1)	None
6		-	+/- (day 1)	None
7		-	-	None
2		-	-	None
5		-	-	None
8	Method A	-	-	None
9		-	-	None
10		-	-	None

-: Negative.
+/-: Probable (0-1 cm).
+: Weak positive (1-5 cm).
++: Positive (5-10 cm).
+++: Strong positive (> 10 cm).
* See footnote of table 1 and annex.
** Day of maximum extent.

All unvaccinated pigs became clinically ill between day 2 to day 5 post challenge. They had an increase in body temperature of at least 1.5 °C compared to the baseline temperature before challenge. Irrespective of the serovar used, the challenge method B provoked skin reactions in all unvaccinated animals. Challenge method A showed only skin reaction of serovar 1. Overall four of the six unvaccinated animals showed generalised skin reaction (Table 7).

Table 7. — *Results of Laboratory 4: Vaccination-challenge test on conventional pigs, unvaccinated control animals*

Animal No.	Route of Infection*	Local Reaction Serovar 1	Local Reaction Serovar 2	Systemic Reaction ΔT [°C]	Remarks
11		++ (day 3)*	+ (day 3)*	2.47 (day 4)* **G** (day 5)	Therapy
13	Method B	++ (day 3)	+ (day 3)	1.47 (day 4)	Therapy
15		+++ (day 3)	+++ (day 3)	2.68 (day 3) **G** (day 4)	Therapy
12		++ (day 4)	-	2.50 (day 2) **G** (day 5)	Therapy
14	Method A	++ (day 2)	-	1.74 (day 3) **G** (day 3)	Therapy
16		+++ (day 5)	-	1.51 (day 5) **L** (day 4)	Therapy

ΔT: Difference of rectal body temperature between day 0 and time of maximum fever reaction.
G: Generalised diamond skin disease, distant from injection site.
L: Local spread of diamond skin disease at injection site.
-: Negative.
+/-: Probable (0-1 cm).
+: Weak positive (1-5 cm).
++: Positive (5-10 cm).
+++: Strong positive (> 10 cm).
* See footnote of table 1 and annex.
** Day of maximum extent.

5. DISCUSSION

The aim of this study was to establish suitable *E. rhusiopathiae* strains for the pig challenge test required in the Ph. Eur. revised monograph on erysipelas vaccines [7].

In a preliminary study it was proven that each cBRP strain is suitable to induce acute erysipelas disease with fever and/or skin reaction as clinical symptoms in conventional reared pigs free of erysipelas antibodies [8]. This result was now confirmed by Laboratory 3 for SPF pigs. Therefore a combined challenge procedure using both challenge strains on the same animals as proposed elsewhere [4] offers the possibility to require only one challenge to demonstrate protection against both serovars and at a time the number of animals necessary for this kind of tests may be reduced by 50 %.

The results of each laboratory separately confirmed the suitability of the cBRP strains. All unvaccinated pigs challenged with these strains developed typical symptoms of acute erysipelas (high fever > 41.0 °C and local skin reactions).

Laboratory 2 and 3 challenged only unvaccinated pigs. Laboratory 2 reported that serovar 1 induced skin lesions in all pigs. With serovar 2 only 2 low positive and 2 strong positive skin lesions out of a total of 6 could be seen. All pigs had body temperatures over 41.0 °C for at least 2 consecutive days. Laboratory 3 reported typical positive skin lesions the day after challenge as well as a high temperature within 24-48 hours after challenge in 3/3 pigs tested for either serovar 1 or serovar 2 strain.

Laboratory 1 and 4 performed vaccination-challenge tests as proposed in the section *Potency* of the revised monograph [7]. All non-vaccinated pigs showed severe fever as well as lesions at the site of infection, confirming the suitability of the strains for this test.

Variability in the appearance of typical skin lesions of serovar 2 could also be seen in the experiment of Laboratory 4. In this case the injection technique obviously had some influence. Whereas the injection method B resulted in skin lesion in all three unvaccinated animals no pig injected according to method A showed positive skin lesions.

The more superficial intradermal injection of the inoculum by method B seems to result in a better and more reproducible development of skin lesions at least for this strain. However, it cannot be excluded that other parameters such as the SPF status may have some influence. The animal numbers used so far are too low to clarify this issue. Nevertheless, it is proposed to amend the initial challenge protocol and recommend injection technique B.

The use of an intradermal inoculation has the great advantage that typical clinical signs develop at the inoculation site. Often the lesions are typical brick or diamond shaped (Figure 1). However, other more or less irregular shapes may also be found. This has also been described before by others [5]. The depot of fluid in the superficial area of the skin set by method 2 may result in a small unspecific reaction on the first day after injection as described by laboratory 4. However, in contrast to the erysipelas skin infection such unspecific reactions will not enlarge or spread and can be clearly distinguished on the following days.

The use of reference strains in a defined challenge procedure ensures a good reproducibility of the test and allows a better comparison between results obtained by different laboratories. The same applies for testing different vaccines.

The typical clinical signs of erysipelas can be easily monitored. Therefore humane endpoints can be defined which allow to terminate the experiment at an early stage to avoid unnecessary

suffering of the animals. Application of penicillin at a therapeutic level for at least three days as used by Laboratories 1 and 4 results in a quick recovery of the pigs.

If pigs of a non-SPF status are used it is necessary to confirm the status of immunity against erysipelas by checking of the antibody status using a sensitive method such as ELISA [7]. Due to the long-lasting persistence of maternal antibodies and the ubiquitous existence of the bacterium a pre-existing antibody titre in a control animal might occur even in non-vaccinated animals if the animal breeding facility has not an SPF status [8].

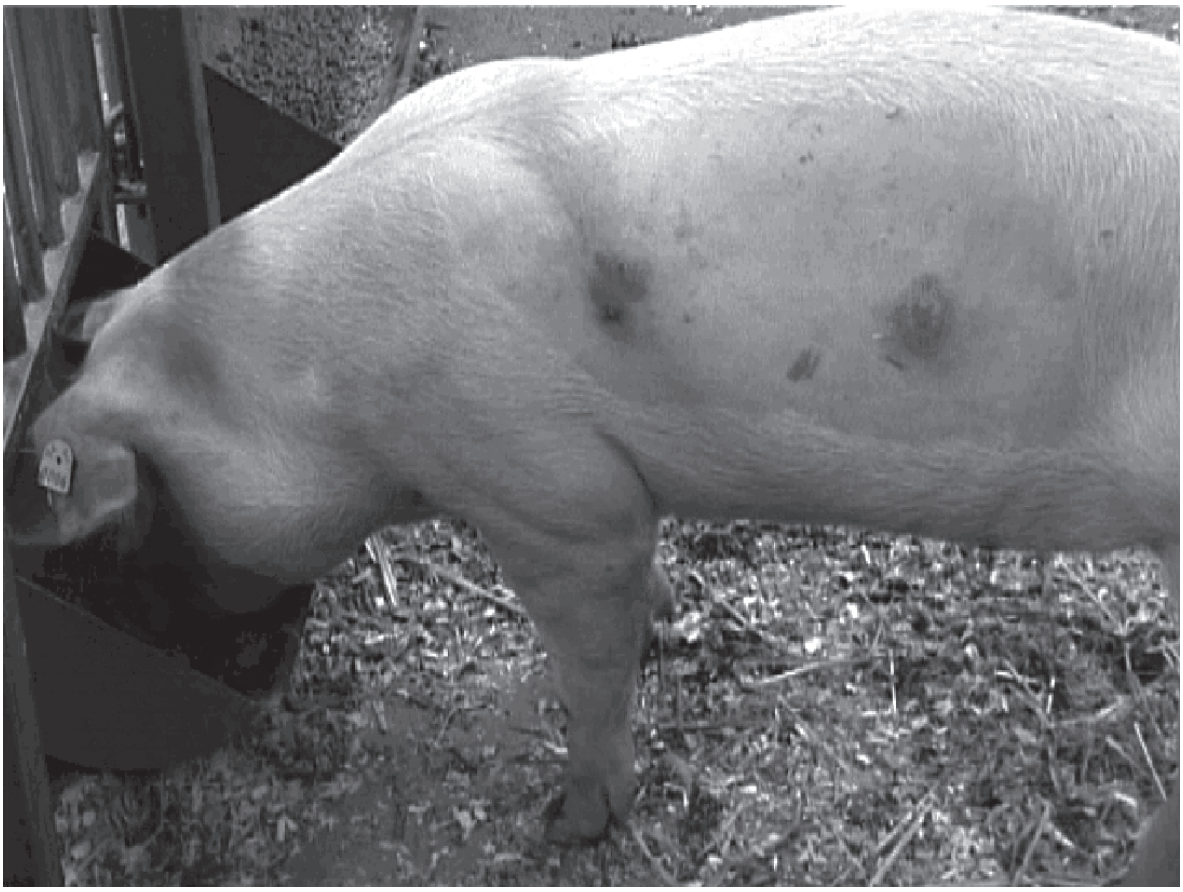

Figure 1. — *Typical rhomboid skin lesion at the injection site three days after infection.*

6. CONCLUSION

The revised monograph *swine erysipelas vaccine (inactivated)* includes now the requirement for a pig challenge test to demonstrate immunogenicity and potency of the vaccine. Two cBRP reference strains were tested in four laboratories in pig challenge tests using the proposed model. In all experiments infected unvaccinated pigs showed local skin reactions and high fever as typical clinical signs of erysipelas disease.

The analysis of the outcome of this study has shown that the cBRPs were suitable to be used as reference strains. Therefore, the candidate preparations have been adopted by the Ph. Eur. Commission during the 105th Session in November 1999 as:

— Swine erysipelas bacteria serotype 1 BRP Batch 1(1) and,

— Swine erysipelas bacteria serotype 2 BRP Batch 1(2)

(1) Cat. No. S5501000.

(2) Cat. No. S5502000.

7. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to the project leader, Dr. K. Cussler (PEI) and to all participants for their contributions to this study. The study was run by the EDQM with the support of the Council of Europe, the European Commission and the German Ministry for Education and Research under the aegis of the Biological Standardisation Programme (internal project number BSP028) with Dr. M. Berg-Candolfi and Dr. M-E. Esposito-Farese acting as project monitors.

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ANNEX

IMMUNOGENICITY / POTENCY TEST OF ERYSIPELAS VACCINES IN PIGS (serotype 1/serotype 2)

1. AIM

Demonstration of immunogenicity/potency of erysipelas vaccines in pigs by intradermal challenge with virulent *Erysipelothrix rhusiopathiae* strains.

2. MATERIAL

- Syringes and needles (1.1 × 25 mm) for vaccination
- Syringes and needles (0.33 × 12 mm) for challenge
- Clinical thermometer
- Water resistent black pencil.

3. ANIMALS

15 pigs of the same origin older than 12 weeks and free of erysipelas antibodies(1).

4. REAGENTS

- Skin disinfectant
- Erysipelas vaccine, inactivated
- Swine erysipelas bacteria, Serotype 1, BRP Batch 1
- Swine erysipelas bacteria, Serotype 2, BRP Batch 1

5. TEST PERFORMANCE

5.1. Randomisation

Pigs are randomly distributed in two groups (10 vaccinated and 5 controls) and marked for identification.

5.2. Immunisation

Administration of the vaccine according to the recommended schedule.

(1) The use of a validated in-house or commercial serological methodology is required.

5.3. Preparation of the challenge strain

Resolve the freeze-dried ampoulle with saline (0.9 %) and mix well. It is recommended to solve the pellet with 1.0 ml which results in a bacterial concentration high enough for direct use in the challenge test (approx. 6.4×10^7 CFU/ml for serovar 1 and 2.6×10^6 CFU/ml for serovar 2).

5.4. Challenge

The animals are challenged intradermally on one of the flanks 3 weeks after vaccination. Depending on the size of the animal, the injection sites should have a minimum distance of 20 to 30 cm. Alternatively, the two strains could be injected on the left and right side. For clear observation of the skin reaction it is recommended to shave the inoculation sites. The injection site is disinfected and marked with a water-resistant pencil. The inoculation of the bacteria was performed with a disposable 1 ml syringe bearing a 0.33×12 mm needle. The bacterial inoculum (0.1 ml) should contain 10^6 - 10^7 CFU/ml.

Method A: The needle is inserted with a right angle 1 cm deep into the skin of the animal, at a minimum distance of 5 cm to the marking and 20 cm to the next injection site.

Method B: The needle is inserted 2-3mm deep, under the epidermis, the needle being almost parallel to skin surface, at a minimal distance of 5 cm of the marking. Injection into the dermis is validated by the appearance of a visible papule at the injection site.

6. OBSERVATION

The animals are monitored for clinical signs at least twice a day over a 7 day period.

7. THERAPY

For animal welfare, pigs with typical erysipelas signs (diamond skin lesions) should be treated with penicillin and/or immunoserum.

8. EVALUATION

The pass/fail criteria are laid down in the revised monograph.