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

Project leader: H. H. Lensing (ID-Lelystad)[1]
H. H. Lensing[1], M.-E. Esposito-Farese[2], A. Daas[2] and J-M. Spieser[2]

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)

(1) ID-Lelystad, Institute for Animal Health, PO Box 65, 8200AB Lelystad, The Netherlands.

(2) EDQM, Council of Europe, BP 907, 67029 Strasbourg Cédex 1, France.

(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 l 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.

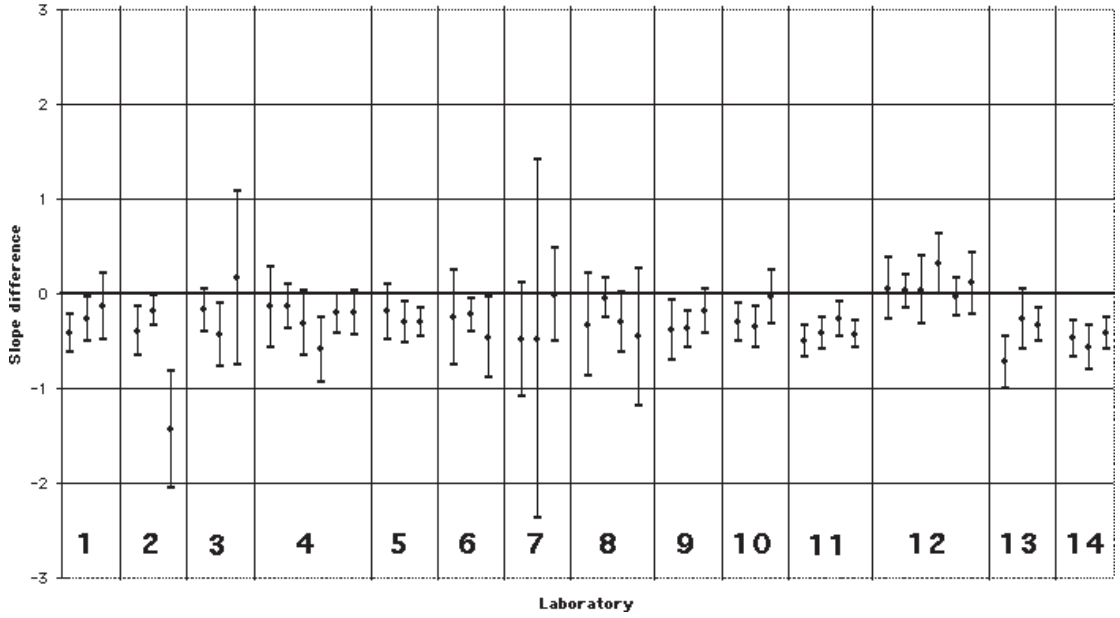

Figure 1a. — *Deviations from parallelism (guinea pig antiserum)*

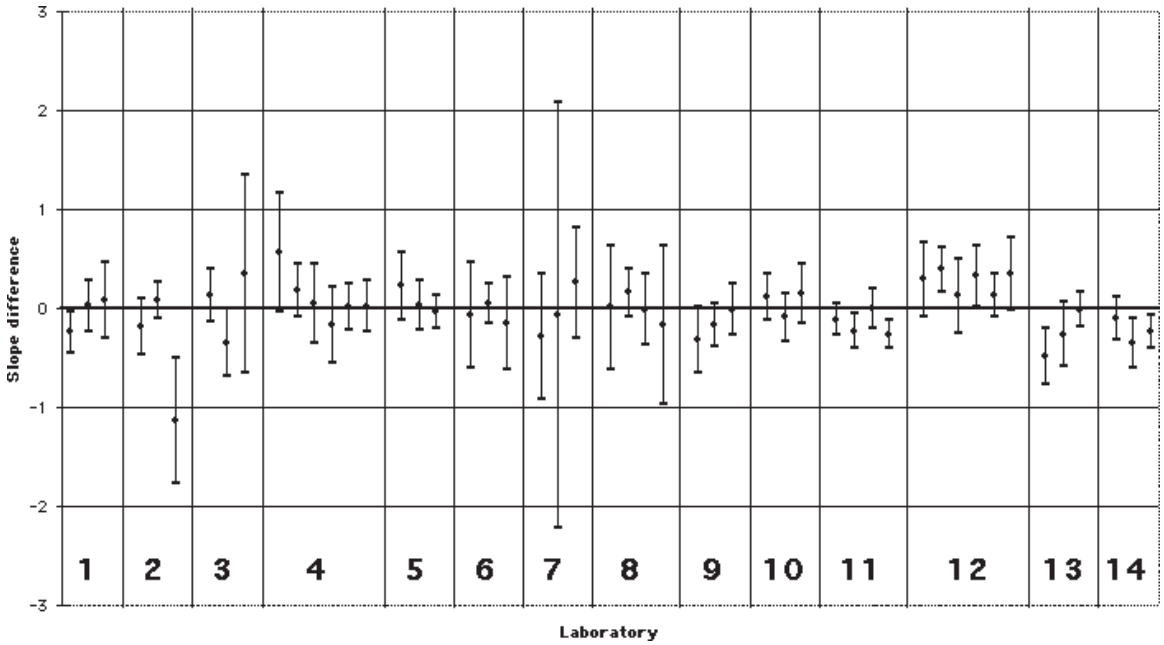

Figure 1b. — *Deviations from parallelism (rabbit antiserum)*

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 = ∞.

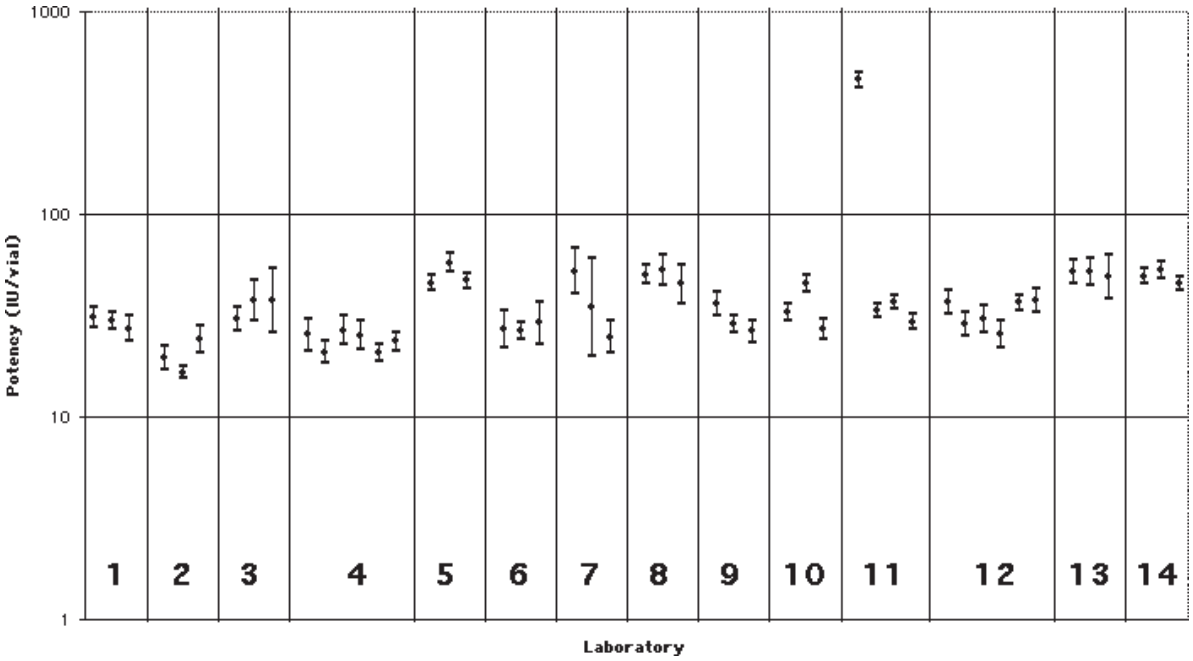

Figure 2a. — *Confidence intervals per assay (guinea pig antiserum)*

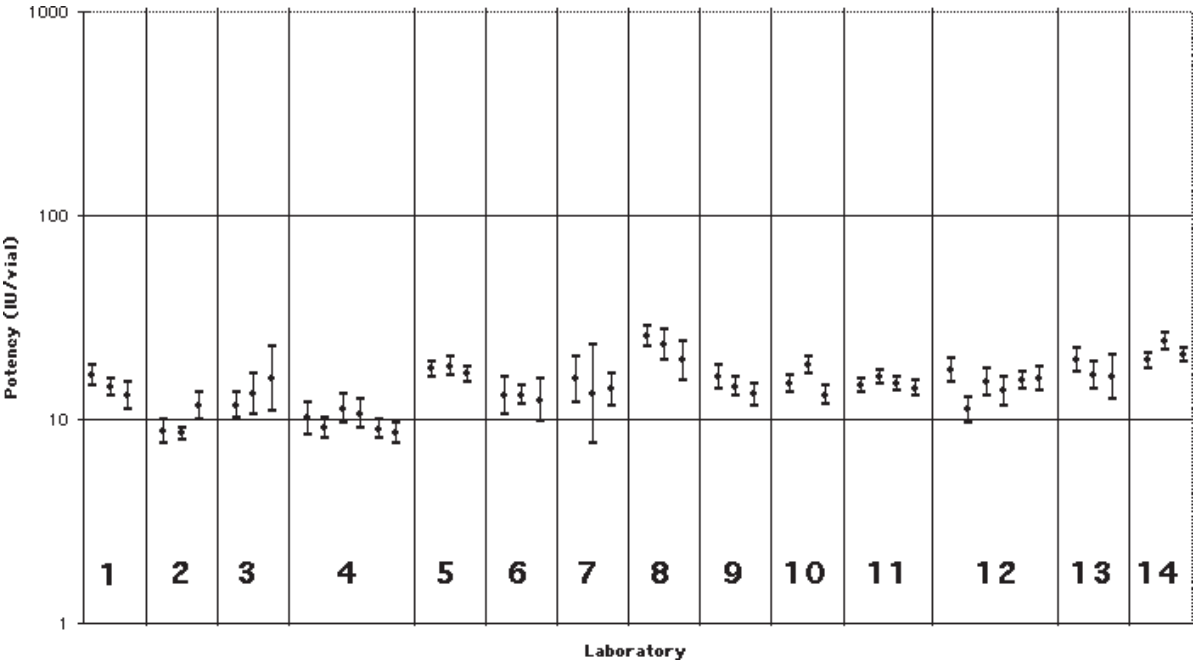

Figure 2b. — *Confidence intervals per assay (rabbit antiserum)*

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.

6. REFERENCES

(1) Tetanus vaccine for veterinary use. Monograph No. 0697, *European Pharmacopoeia*, 3rd Edition (1997).

(2) PA/PH/Exp. 15V/T (95) 12, ANP: Note on the monograph on tetanus vaccine for veterinary use. *Pharmeuropa*, **8.2**, 238-240 (1996).

(3) M. E. Melville-Smith, V. A. Seagroatt and J. T. Watkins: A comparison of enzyme linked immunosorbent assay (ELISA) with the toxin neutralization test in mice as a method for the estimation of tetanus toxin in human sera. *J. Biol. Stand.*, **11**, 137-144 (1983).

(4) A. M. Hagenaars, R. W. van Delft and J. Nagel: Comparison of ELISA and toxin neutralization for the determination of tetanus antibodies. *J. Immunoassay*, **5**, 1-11 (1984).

(5) O. Simonsen, M. W. Bentzon and I. Heron: ELISA for routine determination of antitoxic immunity to tetanus. *J. Biol. Stand.*, **15**, 223-230 (1986).

(6) C. F. M. Hendriksen, J. W. van der Gun, J. Nagel and J. G. Kreeftenberg: The toxin binding inhibition test as a reliable *in vitro* alternative to the toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. *J. Biol. Stand.*, **16**, 287-297 (1988).

(7) C. F. M. Hendriksen, J. W. van der Gun, F. Marsman and J. G. Kreeftenberg: The use of the *in vitro* toxin binding inhibition (ToBI) test for the estimation of the potency of tetanus toxoid. *Biologicals*, **19**, 23-29 (1991).

(8) C. F. M. Hendriksen, J. Woltjes, A. M. Akkermans, J. W. van der Gun, F. R. Marsman, M. H. Verschure and K. Veldman: Inter-laboratory validation of *in vitro* serological assay systems to assess the potency of tetanus toxoid in vaccines for veterinary use. *Biologicals*, **22**, 257-268 (1994).

(9) J. Lyng and I. Heron: Quantitative estimation of diphtheria and tetanus toxoids. VI. Use of different antibody titration methods for evaluation of immunogenicity in animals during potency assay of diphtheria toxoid. *Biologicals*, **20**, 101-108 (1992).

(10) M. Surjan and G. Nyerges: The study of avidity of diphtheria and tetanus antitoxins. *Zschr. Immunitäts Allergie Forschung*, **127**, 75-84 (1964).

(11) Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances. *WHO Techn. Rep. Series*, **800**, 181-213 (1990).

(12) Commission Directive 91/412/EEC laying down the principles and guidelines of good manufacturing practice for veterinary medicinal products. *Off. J. Eur. Com*, **L228**, 70 (1991).

(13) Physical and Physico-chemical Methods. Potentiometric Determination of pH. Chapter No. 2.2.3, *European Pharmacopoeia*, 3rd Edition (1997).

(14) Biological tests. Sterility. Chapter No. 2.6.1, *European Pharmacopoeia*, 3rd Edition, (1997).

(15) Assays. Determination of Water: Semi-Micro Method. Chapter No. 2.5.12, *European Pharmacopoeia*, 3rd Edition (1997).

7. PARTICIPANTS

E. Borrmann, Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Jena (D).

D. Calmels, Mérial, Lyon (F).

I. Claassen, ID-Lelystad, Lelystad (NL).

P. Hauer, Center for Veterinary Biologicals, Ames, Iowa (USA).

C. Hendriksen, Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven (NL).

V. Kardi, Phylaxia-Sanofi Vet Biologicals Ltd., Budapest (H).

D. Mannion, Danish Medicines Agency, Brønshøj (DK).

K. Nöske, Paul-Ehrlich-Institut, Langen (D).

E. Puentes, CZ Veterinaria, Porrino (E).

G. Rautmann, B. Wild, European Directorate for the Qulaity of Medicines, Council of Europe, Strasbourg (F).

N. Reed, Veterinary Laboratories Agency, Surrey (UK).

S. Touffet, C. Schumacher, Vétoquinol, Lure, (F).

A. Van de Loon, Intervet International BV, Boxmeer (NL).

T. Peraferrer Vayreda, Laboratoire Hipra, S. A., Amer (E).

