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Corrigendum

to Pharmeuropa 2001-1 page 23 line 13.

Please read

Caruso A., McWilliams T., Wyeth Lederle Vaccines & Paediatrics, New York, USA

instead of

Gupta R., Brock B., Wyeth Lederle Vaccines & Paediatrics, New York, USA

Collaborative Study for the Validation of Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use

Part 1

COLLABORATIVE STUDY FOR THE VALIDATION OF SEROLOGICAL METHODS FOR POTENCY TESTING OF TETANUS TOXOID VACCINES FOR HUMAN USE

Part 1[1]

(reprinted from Pharmeuropa Bio 2000-1)

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1. INTRODUCTION

A collaborative study on the evaluation of alternative methods for potency testing of tetanus toxoid vaccines for human use started in March 1996. This study was performed under the aegis of the Biological Standardisation Programme and supported by the Council of Europe, the European Commission and the European Centre for the Validation of Alternative Methods of the European Commission (ECVAM/IHPC/JRC)[4]. The project is divided in four parts; a brief outline of each is given below. This report describes the results of the validation study and of Phases I, IIa and IIb.

According to the Ph.□Eurmonograph *Tetanus vaccine (adsorbed) (0452)* on tetanus toxoid-based vaccines for human use, assessment of potency is based on a quantitative direct challenge test in guinea pigs or mice. The end-point is taken as paralysis or death of the immunised animals within five days after challenge with 50 times the paralytic or lethal dose of tetanus toxin. The test requires large numbers of animals and causes severe distress to most of the animals involved.

Despite the success of tetanus vaccines for human use, world-wide harmonisation is not yet obtained regarding the methods for testing their potency or immunogenicity. An essential step in the quality control of vaccines for human use containing tetanus toxoid according to the Ph.□Eurand the WHO (WHO Expert Committee on Biological Standardisation 1990) is the potency assay. For that purpose, the Ph.□Eurrequires guinea pig or mice direct challenge testing with tetanus toxin. While the WHO requires either the direct challenge test, or the determination of the antitoxin levels of the individual animals titrated by the *in vivo* toxin neutralisation test (indirect challenge) or *in vitro* methods that have been validated on vaccines of the type being tested. The national control authority must approve the alternative method.

(1) Part 1 describes results of Phases I, IIa and IIb (see Introduction for explanation). Part 2, a summary of Phase III, will be published in a future issue of Pharmeuropa Bio.

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(4) **Abbreviations**: **AVG**: Average; **BRP**: Biological Reference Preparation; **c.i.**: confidence intervals; **c.l.**: confidence limits; **ECVAM/IHCP/JRC**: European Centre for the Validation of Alternative Methods of the Institute for Health and Consumer Protection, Joint Research Centre; **EDQM**: European Directorate for the Quality of Medicines of the Council of Europe; **ELISA**: Enzyme-Linked Immunosorbent Assay; **ERTA**: Ph.□Eur. Biological Reference Preparation for Tetanus vaccine (adsorbed);**FDA**: Food and Drug Administration; **FELASA**: Federation of Laboratory Animal Science Associations; **GPTA-6**: Guinea pig tetanus antiserum produced as standard for the collaborative study; **IS**: International Standard; **IU**: International Units; **LD_{50}**: Dose leading to death of 50□% of the animals; **Lf**: Limes flocculation; **LOD**: Limit of Detection; **LOQ**: Limit of Quantitation; **NIBSC**: National Institute for Biological Standards and Control; **OD**: Optical Density; **OMCLs**: Official Medicines Control Laboratories; **PC_{50} and PC_{99}**: Dose protecting 50□% and 99□% of the animals, respectively;**PD_{50}**: Dose leading to paralysis of 50 % of the animals; **Ph. Eur.**: European Pharmacopoeia; **RIVM**:□Rijksinstituut voor Volksgezondheid en Milieu;**RSD**: Relative Standard Deviation; **SD**: Standard Deviation; **SLK**: Statens Legmiddelkontroll; **SPF**: Specific Pathogen Free; **TNT**: Toxin Neutralisation Test in mice; **ToBI**: Toxin Binding Inhibition test; **WHO**:□World Health Organisation.

For various reasons, guinea pigs were chosen instead of mice for potency determination of vaccines in the present study. As reviewed by Scheibel et al. (1968) previous studies have shown that there is a positive relationship between the laboratory potency assay of tetanus toxoid vaccines and human anti-tetanus antibody response. Similarly, Pittman et al. (1970) found a direct relationship between the direct challenge assay and the anti-tetanus antibody response in guinea pigs following immunisation with tetanus toxoid. In addition, guinea pigs would provide adequate amounts of antisera as it was envisaged that the test system could be used for assaying several vaccine components in combined vaccines, in particular diphtheria toxoid. Furthermore, a previous collaborative study, under the auspices of the Biological Standardisation Programme, on the potency estimation of diphtheria vaccines in mice, indicated great strain differences in the serological responses to diphtheria toxoid, in particular when a whole cell pertussis component was present (Gommer 1996). It was presumed that less strain differences would be seen for guinea pigs, and that the serological responses of guinea pigs to diphtheria toxoid were more similar to human responses. Furthermore, studies by others indicated that guinea pigs, in contrast to Balb/c and NIH strains of mice, had a similar response to fragment B of diphtheria toxin as man (Sesardic et al. 1994).

Serological assays, which are proposed as alternatives to the toxin neutralisation test in mice (TNT) and to the direct challenge procedure, include ELISA (Hagenaars et al. 1984, Simonsen et al. 1986, German-Fattal et al. 1987) and ToBI (Hendriksen et al. 1991), which is a modification of ELISA. From the results of a collaborative study (Hendriksen et al. 1994) on the use of ELISA, ToBI and the haemagglutination test for potency determination of tetanus toxoid for veterinary vaccines, it was concluded that both ELISA and ToBI, but not the haemagglutination test, may be used as valid alternatives to TNT. In the latter study the animals were given a booster dose before antibody analysis.

None of the potency assays mentioned above corresponds directly to the vaccination schedules used in humans, for which a complete primary vaccination consists of 2 or 3 doses. To discriminate between a good and an inferior vaccine in animals, a one-dose immunisation regime has been used in the present study, as it is generally believed that a multi-injection immunisation scheme decreases the discriminating power of the potency assay.

The challenge procedure used in the present study deviates from the Ph.□Eurprocedure with respect to the interval between immunisation and challenge. The interval was prolonged by two weeks because our prevalidation study and a report by others (Gupta et al. 1994) indicated that six weeks gave a better correlation between the results of the direct challenge procedure and ToBI, as well as between TNT and ELISA.

From the evaluation of the use of tetanus toxoid instead of tetanus toxin in the ToBI, no statistically significant differences were observed in antibody titres. Nevertheless it was decided to use tetanus toxin since ToBI is designed to mimic TNT as far as possible, and since previous published studies were performed with toxin.

The project was divided into four consecutive phases with the following objectives:

1) Prevalidation: selection of the optimum time interval between immunisation and bleeding, and evaluation of the use of tetanus toxoid as an alternative to tetanus toxin in ToBI.

2) Phase I: Assessment of the correlations between potencies in the challenge test and the serological tests, between antitoxin titre and protection in the individual animal and of the intra- and inter-laboratory variation in ELISA and ToBI.

3) Phase IIa (3□laboratories) was identical to Phase I, except thaſTNT was not performed. Phase IIb (2□laboratories) was performed because (a) part of the data of the Phase IIa study

was invalid and could not be used and (b) to include a tetanus vaccine of borderline quality which became available during Phase IIa. Furthermore, a combined vaccine with an acellular pertussis component was included.

4) Phase III: Assessment of intra- and inter-laboratory variation in estimation of antitoxin levels by ELISA and ToBI, using a panel of serum samples, in 25 laboratories.

Results of the Phase I and II studies were presented at the International Symposium on Alternatives to Animals in the Development and Control of Biological Products for Human and Veterinary Use, London (Winsnes et al. 1999), and at the International Symposium on Tetanus Vaccine for Human Use, Strasbourg (22-23 June 2000).

2. AIM OF THE STUDY

A collaborative study was performed with the goal to evaluate alternative assay methods for batch release testing of vaccines for human use containing tetanus toxoid. These assay methods should be able to refine the Ph.□Eurpotency test and to reduce the number of animals used for this purpose. Ideally, these alternative assay methods for testing of production consistency, should be acceptable by the manufacturers and the Official Medicines Control Laboratories (OMCLs), as well as by the FDA and the WHO.

3. PARTICIPANTS

Six laboratories from five countries including both manufacturers and public sector laboratories, all experienced in tetanus vaccine quality control, participated in the various parts of this study (see Section *5. Results* for details). The participants are listed at the end of the report and are referred to by code numbers as defined in the following Table.

	Phase I and IIa	Phase IIb
Laboratory code	1	7
	2	8
	3	
	4	
	5	
	6	

4. ANIMALS, MATERIALS AND METHODS

Detailed protocols for ELISA, ToBI, TNT and the challenge test are available from the EDQM upon request.

4.1. VACCINES

Tetanus toxoid vaccines from different manufacturers and representing various types of combined products were used, including the Ph.□Eur BRP for Tetanus vaccine (adsorbed) (ERTA). Composition and Lf content/ml were confirmed at one of the co-ordinating laboratories (Table 1). Vaccines were code-labelled. Vaccine concentrations used for the immunisation of guinea pigs are shown in Tables 12a-c.

Table 1 — *Specifications of tetanus toxoid vaccines used in the collaborative study*

Vaccine	Tested in Phase	Composition	Adjuvant	Lf content*
ERTA	I, IIa & IIb	T	$Al(OH)_3$	54 Lf/ampoule
C	I, IIa	DTP Hib	$AlPO_4$	ca.10 Lf/ml
D	I, IIa	DT	$AlPO_4$	ca.10 Lf/ml
E	I, IIa	DT	$AlPO_4$	ca.15 Lf/ml
F	I, IIa & IIb	DTP	$AlPO_4$	ca.15 Lf/ml
H	I, IIa	DTP	$Al(OH)_3$	ca. 5 Lf/ml
I	IIb	T	$Al(OH)_3$	ca. 10 Lf/ml
K	IIb	DTaP	$Al(OH)_3$	ca. 10 Lf/ml

D: Diphtheria, T: Tetanus, P: Pertussis, Hib: *Haemophilus influenzae* type b, aP: acellular pertussis.
*Data established at one of the co-ordinating laboratories.

4.2. ANIMALS

Guinea pigs used for immunisation were purchased from commercial SPF breeding units. Further details are given in Table 2.

Table 2 — *Specifications of guinea pigs (250-300g) used in the collaborative study*

Specifications	Phase I			Phase IIa			Phase IIb	
	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6	Lab. 7	Lab. 8
Breeder	Harlan (UK)	Charles River (D)	David Hall (UK)	David Hall (UK)	Charles River (D)	n.s.	Harlan (UK)	David Hall (UK)*
Strain	HsdPoc. DH	Crl: (HA)BR	DH	DH	Crl: (HA)BR	n.s.	HsdPoc. DH	DH
Sex	M/F (50/50)	M	F	F	M/F (50/50)	n.s.	M/F (50/50)	F

M: Male, F: Female, n.s.: not specified, *Barrier 2 guinea pigs.

The health status of the animals was recorded on arrival and monitored throughout the experiment. An additional group of guinea pigs from the same batch was housed for obtaining negative control serum. This group also served as sentinel animals for microbiological quality control. The screening criteria list for microbiological control was based on the FELASA health monitoring recommendations (Rehbinder et al. 1996). Animals were randomly distributed into the cages or ground pens and identified individually.

Mice used for TNT (only Phase I) were obtained from different SPF breeding colonies. Specifications are given in Table 3. Animals were housed in polycarbonate boxes with sawdust bedding, under SPF conditions (Lab. 1) or under conventional conditions (Lab. 2 &□3). Cages were located in rooms with controlled lighting, constant temperature and constant relative humidity. Environmental conditions were monitored during the experiments. Animals were fed a commercial diet and tap water was available *ad libitum*.

Table 3 — *Specifications of mice used in the collaborative study*

Specifications	Lab. 1	Lab. 2	Lab. 3
Breeder	Local	Bomholtgård (DK)	Harlan (UK)
Strain	NIH	NMRI	NIH
Sex	F	F	F
Weight at start	17-21 g	18-20 g	17-21 g

F: Female.

4.3. STANDARD TETANUS ANTISERUM (GPTA-6)

A guinea pig standard antiserum (GPTA-6) was prepared. A group of 25 guinea pigs (12 males and 13 females) (HsdPoc.DH), weighing 250-350 g were immunised with 0.5 ml of a 1/50 dilution of the ERTA, after reconstitution of one vial in 1 ml of saline. Animals were bled by cardiac puncture 6 weeks after immunisation. Serum samples were pooled to yield a total volume of 130 ml. In two of the participating laboratories, GPTA-6 was calibrated in the *in vivo* TNT against the WHO IS for Tetanus Antitoxin (Equine, lot 16/4, 1400 IU/ampoule). The potency assigned to GPTA-6 was 0.08 IU/ml.

4.4. IMMUNISATION PROTOCOL

The standard protocol for the immunisation of animals was as follows: groups of 12 (Lab. 1 in Phase I: 13) guinea pigs each were immunised subcutaneously (0.5 ml) with serial two-fold dilutions of the test vaccines and of the ERTA preparation, respectively. Animals were randomly distributed into the cages or pens and identified individually. In addition, 8 guinea pigs were included for toxin challenge control (2 animals per toxin dilution). Forty to 42 days after immunisation, approximately 2.5 ml of blood was collected by cardiac puncture or from the *vena saphena* from each individual animal. The 13th animal per vaccine dilution (Phase I, Lab.1) was terminally bled at day 40 by cardiac puncture. Blood was processed and individual serum samples were prepared and stored according to the protocol. Equal aliquots of the 13th serum samples were sent to the participating laboratories of the Phase I study (Lab. 1, 2 and 3).

Two to 4 days after blood sampling, immunised animals were challenged by subcutaneous injection with 50 guinea pig PD_{50} or 50 guinea pig LD_{50} tetanus toxin (T252, RIVM). Control animals were inoculated with 4 dilutions of the challenge toxin, 2 animals per dilution. Guinea pigs were examined several times a day at regular intervals. Definite signs of tetanus (paralysis of 1 forelimb, signs of scoliosis, grade T3) were used as the end-point and animals were immediately euthanised. The number of animals per vaccine dilution group surviving the observation period was recorded. Deviations of the standard protocol or differences between the laboratories are given in Table 4.

Table 4 — *Specifications and deviations from the immunisation protocol in the participating laboratories*

Specifications	**Phase I and Phase IIb*: Lab. No.**			**Phase IIa : Lab. No.**		
	1 (7)	**2 (8)**	**3**	**4**	**5**	**6**
No. of guinea pigs per dilution	13 (12)	12	12	12	12	12
No. of experiments	2 (1)	1	1	1	1	1
Day of bleeding	40 (42)	42	40/41/42	40/41/42	40/41/42	40/41/42
Blood collection	cardiac puncture	*Vena saphena*	cardiac puncture	cardiac puncture	cardiac puncture	cardiac puncture
Challenge dose	50 LD_{50}	50 LD_{50}	50 PD_{50}	50 LD_{50}	50 LD_{50}	50 LD_{50}
Day of challenge	42 (44)	44	44	44	44	44
No. of guinea pigs for toxin challenge control	4 × 2	4 × 2	3 × 4	4 × 2	4 × 2	4 × 2

*Specifications of the Phase IIb study are given in brackets if they diverged from those of the Phase I study.

4.5. TITRATION MATERIAL AND STUDY DESIGN

Tables 5 and 6 list the design of the tests performed and the material provided by the organisers, respectively. The methods used for the validation and the determination of calculated results of ELISA were somewhat different between Phase I and Phase II of the study. Based on experience of the Phase I study, it was decided to use a fixed OD cut-off value of 0.400 in the Phase II study. ELISA and ToBI were performed in triplicate on different days. In a few of the participating laboratories, some of the test series were split into several parts. In the laboratories of the Phase I study, TNT was performed once, divided over several experiments.

Table 5 — *Design of tests performed in the Phase I, IIa and IIb studies and evaluation of results*

Phase/ Test sera	Lab. / No. of samples	ELISA No. assays	ToBI No. assays	TNT No. assays	Intra-lab. variation	Inter-lab. variation
I/ Individual serum samples	1/336 □□2/288 3/286	3	3	n.p.	d.	n.d.
I/ Serum pools	1/28 2/24 3/24	3	3	1	d.	n.d.
I/ 13th guinea pig serum samples*	1/22 2/20 3/20	3	3	1	d.	d.
IIa/ Serum samples	4/288 5/283 6/286	3	3	n.p.	d.	n.d.
IIb/ Serum samples	7/188 8/190	3	3	n.p.	d.	n.d.

n.p. not performed; d. determined; n.d. not determined.

*Due to shortage in volume, some serum samples were only tested in 1 or 2 laboratories.

Table 6 — *Materials provided by the organising laboratories*

Test system	Materials	Supplier
ELISA	• ELISA plate • GPTA-6 • Rabbit-anti-guinea pig HRP conjugate • Tetanus toxoid, lot MWC S208/A/F-6	Maxisorp, Cat. No. 442404 Greiner EDQM Sigma A5545 NIBSC
ToBI	• PolyStyrene roundbottom microtitre plate • ELISA plate, flat bottom • GPTA-6 • Tetanus toxin, lot T417, 300 Lf/ml • Equine-anti-tetanus IgG, lot GTL34 • Equine-anti-tetanus IgG (HATPO), lot 32-33, peroxidase conjugated	Greiner 650101 Greiner 655092 EDQM RIVM RIVM RIVM
TNT	• Tetanus toxin, T252, 100 Lf/vial • GPTA-6	RIVM EDQM

4.6. STATISTICAL ANALYSIS

Raw data of the tests performed (Phase I: challenge test, ELISA, ToBI, TNT; Phase IIa: challenge test, ELISA, ToBI; Phase IIb: challenge test, ELISA, ToBI) were sent to EDQM and RIVM for further elaboration and statistical analysis. The impact of the use of different calculation programmes and/or of different statistical models on the estimated parameters was assessed. The following parameters were evaluated:

- *Test vaccine potencies obtained by direct challenge procedure*. Potencies were based on the number of animals per test vaccine and per dilution group surviving the 5 days observation period after toxin challenge, using T3 (definite signs of paralysis of one forelimb, signs of scoliosis) as the end-point. Potencies, relative to ERTA, were calculated by a probit analysis, all vaccines calculated in one procedure, using in-house validated software at RIVM and at EDQM. Because different calculation programmes were used, giving slightly different outcomes in dose-response fitting, some deviations in the estimated potencies might be expected.

- *Tetanus antitoxin concentrations of serum samples (individual samples, pooled samples and 13th animal samples) analysed in ELISA and ToBI*. Antitoxin concentrations were calculated based on absorbance readings at 10 dilution steps in ELISA and ToBI, using a 4-parameter model to fit the reference curve (Kineti-Calc V.2.03, Bio-Tek Instruments) at RIVM and using a 5-parameter fit programme (The SAS-System, u.6.12, PROC NLIN) at EDQM. Absorbance curves for each sample were obtained by plotting OD values against the decimal logarithm (log) of the dilution.

  For ELISA the procedure used to calculate antitoxin concentrations differed between Phases I, IIa and IIb. In Phase I, cut-off values were determined for each laboratory and for each test, based on absorbance data of negative serum samples. Absorbance values of the test samples were plotted on the absorbance curve of GPTA-6, RIVM using the range from cut-off to 75□% of maximum absorbance, and EDQM using the whole range above the cut-off value.

  In the Phase IIa and Phase IIb studies, extinctions between 0.400 and 2.300 were used to calculate the antitoxin concentrations. This procedure deviates from the one used in Phase□I. OD of the test samples in the specified range were plotted on the absorbance curve of GPTA-6. Serum samples having an OD below the cut-off value (<□0.400) were assigned to have an antitoxin concentration of 0 IU/ml, or in the case of parallel-line analysis (Phase IIb) an antitoxin concentration of 0.5 times the LOD.

  For ToBI, the absorbance range used was the range within 25-75□% of the sum of the mean absorbance value of positive control samples and the mean of negative control samples on each plate. Serum samples with a maximum absorbance value below 25□% of the mean were considered to have an antitoxin concentration of 0 IU/ml (Phases I, IIa and IIb) or in the parallel-line analysis a titre of 0.5 x LOD (Phase IIb). For parallel-line analysis, antitoxin concentrations were transformed to natural logarithm (ln) in order to obtain a normal distribution of antitoxin titres.

- *Protective concentration (PC_{50} and PC_{99}) values*. PC_{50} and PC_{99} values are the antitoxin concentrations obtained in ELISA or in ToBI, at which 50□% and 99□% of the animals, respectively, were protected against the tetanus toxin challenge. PC_{50} and PC_{99} values were calculated by logistic regression, using the following information from each individual animal: mean antitoxin concentration estimated by ELISA and ToBI, respectively, and tetanus paralysis (T3) within 5 days after toxin challenge. For technical reasons, PC_{99} values were not calculated in Phase IIb.

- *Test vaccine potencies based on serology*. Mean tetanus antitoxin concentrations of triplicate ELISA and ToBIs were submitted to probit analysis after dichotomising these concentrations using the following transformation: a mean antitoxin concentration above the mean PC_{50} value of the participating laboratories was set at 1 (predicting survival), a mean antitoxin concentration below the mean PC_{50} was set at 0 (predicting death). Potencies were calculated using the total score for each vaccine and each vaccine dilution in relation to the total number of animals per dilution and per vaccine for which serum samples were obtained.

  An alternative approach used to calculate vaccine potencies was parallel-line analysis. By this approach, serum samples having an OD below 0.400 in ELISA or below 25□% of the standard tetanus antiserum range in the ToBI were given an arbitrarily low antitoxin titre in IU/ml, e.g.□0.5× LOD.

- *Direct challenge - serology correlation*. Data were log transformed.

- *Intra-laboratory variation in ELISA and ToBI*. The evaluation was based on RSDs (being an indication of intra-assay variation) and on the distribution of precision of triplicate assessment of antitoxin concentrations of individual guinea pig sera (being a parameter of inter-assay variation) in ELISA and ToBI in Lab.□1, 2 and 3.

- *Inter-laboratory variation in ELISA and ToBI (Phase I study only)*. Mean antitoxin concentrations of the 13th guinea pigs obtained in Lab.□1, 2 and 3 were used for the evaluation of inter-laboratory variation. Due to the limited set of data available, only descriptive statistical analysis was performed.

- *Line of agreement and correlation between ELISA and ToBI*. Analysis by Sign test was based on mean (ln-transformed) antitoxin concentrations of three ELISA and ToBI repetitions for each individual animal.

- *In vivo (TNT) antitoxin concentrations (Phase I study only)*. TNT concentrations were estimated in pooled serum samples and the 13th animal serum samples. Correlation coefficients (Pearson) between *in vitro* tests and TNT were only calculated for pooled serum samples, but not for the 13th guinea pigs due to the limited number of serum samples available. For these samples only trends were described.

5. RESULTS

In the Phase IIa study not all the data from 2 of the 3 participating laboratories could be used. The data of Lab. 5 showed that almost all animals immunised with the vaccines D, E and F survived the tetanus toxin challenge. However, tetanus antitoxin concentrations (ELISA and ToBI) of the individual serum samples, obtained a few days before the challenge, were in the expected range. For both vaccine C and the reference preparation the challenge dose response□curves were within the expected range, and the potency of vaccine C could be calculated [285 IU/ml (95□% c.i.:172-448 IU/ml)]. But, as no possible explanation could be given for these findings, it was decided not to include the challenge test data of vaccine C. Vaccine potencies in Lab. 5 could only be calculated based on the results of the serological tests.

No data from Lab. 6 could be used for further analysis, except for ELISA and ToBI data, which were used only for comparison of repeatability. Even in the groups of animals injected with the highest vaccine doses, most animals did not survive the tetanus toxin challenge. Furthermore, a relatively high number of animals already died before the challenge proce-

dure, probably due to the cardiac puncture. Also most, but not all, of the serum samples obtained a few days before challenge, had very low tetanus antitoxin titres, both in ELISA and in ToBI. The reasons for this might be diverse: the guinea pig strain used might be non-responder for tetanus toxoid, animals might have been immuno-suppressed (e.g. by infection) or mistakes in storing, preparing or administrating the vaccine dilutions might have occurred. However, non-responding guinea-pig strains have not been described in the literature. From the microbiological status reports of the animals at the beginning of the experiment it can be excluded that animals were infected with the known immuno-compromising micro-organisms.

5.1. ANTITOXIN CONCENTRATIONS OF THE INDIVIDUAL SERUM SAMPLES

In the Phase I study (Lab. 1-3), retrospective cut-off values were calculated for each ELISA performed on one day, using the mean + 2SD of the ODs of the 1/10 diluted negative serum samples. The values obtained were 0.274, 0.309 and 0.309 for Lab.1; 0.316 for Lab. 2 (values were about the same in each of the triplicate assays) and 0.241, 0.423 (1/20 diluted), and 0.478, 0.421 and 0.271 for Lab. 3 (triplicate ELISAs were performed in 5 assays). Based on these results, the cut-off value for ELISA test was set at an OD of 0.400 for all assays in the Phase II study.

In order to calculate potencies using ELISA and ToBI data, antitoxin concentrations of individual animals estimated at EDQM by the 5-parameter fit and at RIVM by the 4-parameter fit, were dichotomised and submitted to probit analysis. For these purposes, both fits are considered equivalent and generally did not lead to different conclusions although there were exceptions.

For dichotomising concentrations, the PC_{50} was set at 0.0075 IU/ml both for ELISA and ToBI, for data from each laboratory, although the actual PC_{50} values were somewhat higher in the Phase II study. This value approximates the individual PC_{50} values, apart for the Phase IIb study. Potencies and 95% c.i., estimated at RIVM by using the 4-parameter fit, are shown in Table 9a (Phases I and IIa), Table 9c (Phase IIb) and Table 9d (Phase IIb, parallel line analysis). Potencies calculated at EDQM are presented in Table 9b.

Table 7 specifies the range of the mean antitoxin concentrations of the individual serum samples obtained by ELISA and ToBI.

Table 7 — *Range of antitoxin concentrations in ELISA and ToBI (IU/ml)*

Laboratory	ToBI	ELISA
1 (n=364)	0 - 0.78	0 - 0.53
2 (n=288)	0 - 0.56	0 - 0.55
3 (n=286)	0 - 2.21*	0 - 1.18*
4 (n=288)	0 - 0.51	0 - 0.36
5 (n=283)	0 - 1.07**	0 - 2.15**
6 (n=288)	n.v.d.	n.v.d.
7 (n=188)	0 - 0.39	0 - 0.14
8 (n=190)	0 - 6.67***	0 - 2.07***

n.v.d. = no valid data.

* The highest antitoxin concentration determined in Lab. 3 is probably due to the hypersensitivity of one of the animals. If this serum is excluded, the range is 0-0.85 IU/ml for the ToBI and 0-0.66 IU/ml for ELISA.

** The range of antitoxin titres of Lab. 5 included three extreme values. If these were excluded, the range would be 0-0.57 IU/ml for ToBI and 0-0.35 IU/ml for ELISA.

*** The overall antitoxin range in this group of animals was higher than in the other animal groups in this study.

The number and percentage of serum samples from which data could be used for analysis of both intra- and inter-laboratory variation are given in Table 8. Differences in the number of serum samples having a concentration above 0 IU/ml in ELISA and ToBI are mainly to be ascribed to differences in cut-off values used and in LOQ. The highest percentage of animals with an antitoxin concentration above 0 IU/ml is seen in the group of generally high responder animals, which would be expected.

Table 8 — *Number and percentage of serum samples having an antitoxin concentration above 0 IU/ml (based on RIVM calculations)*

	ToBI		ELISA	
Laboratory	**Number**	**%**	**Number**	**%**
1	236	65	255	70
2	163	57	247	86
3	190	66	283	99
4	194	67	237	82
5	270	95	247	87
7	271	47	248	55
8	167	87	185	97

5.2. VACCINE POTENCIES OBTAINED IN THE CHALLENGE TEST AND IN *IN VITRO* SEROLOGICAL TESTS

- *Challenge test*. Vaccine dilutions of product D were slightly adapted for the direct challenge test of the Phase IIa study, as sub-optimal vaccine dilutions in the Phase I study were used. Results of the challenge test are presented in Tables 9a and 9c (RIVM calculations) and Table 9b (EDQM calculations). The ranking order of vaccines based on potency was the same for both sets of calculations, except for vaccines C, E and F (Lab. 1). As a consequence of the different calculation methods used by RIVM and EDQM, both estimates and c.i. of all the vaccines are somewhat different in all three assays. The discrepancy of the two calculation programmes is particularly pronounced for vaccine E (Lab. 1), where the RIVM program gives a 49 % higher estimate than EDQM's software, and is beyond the 95 % c.l. calculated by the EDQM.

  The potency of the respective vaccines tested in one laboratory, and calculated by the same statistical program, is often outside the 95 % c.l. given in another laboratory. The potency estimates of the vaccines in Lab. 1 can be taken as an example. The estimates for vaccines C, D, F and H, respectively, are outside the 95 % c.l. calculated in Lab. 2 and 3. The estimate for vaccine E is outside the 95 % c.l. of Lab. 2.

  A striking feature is that the guinea pigs of Lab. 3 seem to react more strongly than those of Lab. 1 and 2 to vaccine F, estimated from all the three assays. A 612 % higher value of the estimate was found by Lab. 3 compared to the results of Lab. 1 (Phase I study). To obtain an indication of possible strain differences in the guinea pig immune response to this vaccine, vaccine F was included in the Phase IIb study, in which Lab. 8 (= Lab. 2 in Phase I study) used the same strain of guinea pigs as Lab. 3, but with the difference that the guinea pigs in Lab. 8 were "barrier 2-animals" (Rehbinder et al. 1996). Although the guinea pigs in Lab. 8 elicited a high immune response, in general, such an extraordinary high potency as that observed in Lab. 3, was not seen. The maximal range of the 95 % c.l., calculated by RIVM and EDQM, was 52-247 % and 64-153 % of the estimate, respectively (Table 9e).

Table 9a — *Potency results and 95% c.i. of Phase I (Lab. 1-3) and Phase IIa (Lab. 4-5) per test and per laboratory (RIVM calculations). Potency values expressed in IU/ml for ELISA and ToBI obtained by probit analysis (after dichotomising).*

Challenge

	1 A	1 B	2	3
Slope (probit(y)/ln(x))	3.51	5.46	5.26	1.99
p-value parallelism	0.51	0.48	0.45	0.58
p-value linearity	1.00	0.99	1.00	0.72

Laboratory Vaccines	Potencies			
1 A	Estimate	Low	High	
C	**417**	227	811	
D	**159**	84	251	
E	**492**	257	1217	
F	**436**	262	623	
H	**181**	109	320	
1 A	Estimate	Low	High	
C	**877**	609	1331	
D	**528**	317	1065	
E	**610**	422	1041	
F	**868**	611	1286	
H	**260**	200	335	
1	Estimate	Low	High	
C	**838**	554	1529	
D	**296**	183	472	
E	**471**	322	724	
F	**2669**	1812	4096	
H	**330**	222	522	

	4	5
Slope (probit(y)/ln(x))	3.38	3.72
p-value parallelism	0.37	0.60
p-value linearity	0.98	0.96

Laboratory Vaccines	Potencies		
4	Estimate	Low	High
C	**381**	249	577
D	**255**	156	418
E	**230**	150	348
F	**335**	203	547
H	**140**	91	214
5	Estimate	Low	High
C	**X**	X	X
D	**X**	X	X
E	**X**	X	X
F	**X**	X	X
H	**X**	X	X

ELISA

	1 A	1 B	2	3
Slope (probit(y)/ln(x))	3.35	5.14	5.11	3.94
p-value parallelism	0.30	0.20	0.48	0.24
p-value linearity	0.74	0.99	0.97	0.87

Potencies			
0.01	Estimate	Low	High
C	**690**	452	1068
D	**147**	89	225
E	**364**	205	687
F	**515**	357	750
H	**217**	155	307
0.02	Estimate	Low	High
C	**1487**	998	2224
D	**693**	480	1003
E	**784**	524	1176
F	**1342**	920	1959
H	**393**	263	587
0.03	Estimate	Low	High
C	**522**	347	792
D	**193**	130	285
E	**299**	192	470
F	**1936**	1234	3083
H	**290**	194	447

	4 A	4 B	5
Slope (probit(y)/ln(x))	3.55	3.32	4.33
p-value parallelism	0.31	0.13	0.86
p-value linearity	0.66	0.99	0.18

Potencies			
4 A	Estimate	Low	High
C	**431**	283	655
D	**256**	156	421
E	**317**	207	484
F	**321**	199	510
H	**152**	97	238
Recalculated PC50 used			
4B	Estimate	Low	High
C	**394**	257	599
D	**291**	172	492
E	**219**	139	343
F	**340**	202	565
H	**171**	109	270
5	Estimate	Low	High
C	**248**	166	365
D	**165**	113	240
E	**222**	148	327
F	**247**	161	372
H	**138**	93	206

ToBI

	1 A	1 B	2	3
Slope (probit(y)/ln(x))	3.36	5.43	4.80	4.27
p-value parallelism	0.19	0.59	0.28	0.54
p-value linearity	0.93	0.69	1.00	0.75

Potencies			
0.01	Estimate	Low	High
C	**645**	351	1295
D	**141**	83	218
E	**368**	238	572
F	**690**	452	1068
H	**147**	89	225
0.02	Estimate	Low	High
C	**920**	612	1387
D	**507**	348	736
E	**660**	439	995
F	**856**	536	1349
H	**327**	221	484
0.03	Estimate	Low	High
C	**852**	563	1296
D	**303**	205	446
E	**404**	277	589
F	**2487**	1621	3848
H	**347**	231	526

	4 A	4 B	5
Slope (probit(y)/ln(x))	3.17	3.24	4.43
p-value parallelism	0.15	0.35	1.00
p-value linearity	0.72	0.84	0.04

Potencies			
4 A	Estimate	Low	High
C	**351**	219	555
D	**250**	148	418
E	**202**	121	333
F	**397**	247	617
H	**131**	82	208
Recalculated PC50 used			
4B	Estimate	Low	High
C	**377**	242	582
D	**270**	164	443
E	**200**	123	324
F	**415**	264	633
H	**149**	97	230
5	Estimate	Low	High
C	**208**	119	350
D	**162**	98	257
E	**204**	123	327
F	**260**	150	432
H	**127**	76	207

Table 9b — *Potency results and 95% c.i. of Phase I (Lab. 1-3) and Phase IIa (Lab. 4-5) per test and per laboratory (EDQM calculations). Potency values expressed in IU/ml for ELISA and ToBI obtained by probit analysis (after dichotomising).*

Challenge

Slope (probit(y)/ln(x))	1 A	1 B	2	3
p-value parallelism	2.26	2.62	2.54	1.99
p-value linearity	0.00	0.43	0.30	0.58
	0.01	0.98	0.99	0.72

Laboratory		Potencies		
Vaccines	1	Estimate	Low	High
	C	**384**	272	538
	D	**174**	122	246
	E	**330**	234	466
	F	**438**	310	606
	H	**182**	132	252
	2	Estimate	Low	High
	C	**856**	622	1178
	D	**472**	344	650
	E	**584**	424	804
	F	**854**	620	1176
	H	**260**	190	358
	3	Estimate	Low	High
	C	**782**	538	1148
	D	**300**	206	438
	E	**468**	322	686
	F	**2780**	1896	4176
	H	**326**	226	480

Slope (probit(y)/ln(x))	4	6
p-value parallelism	3.58	
p-value linearity	0.37	
	0.96	

Laboratory		Potencies		
Vaccines	4	Estimate	Low	High
	C	**381**	251	575
	D	**257**	170	388
	E	**231**	151	349
	F	**328**	209	503
	H	**140**	92	211
	6	Estimate	Low	High
	C	**X**	X	X
	D	**X**	X	X
	E	**X**	X	X
	F	**X**	X	X
	H	**X**	X	X

ELISA

1 A	1 B	2	3
1.75	2.49	2.33	2.12
0.31	0.44	0.33	0.66
0.51	1.00	0.31	0.78

Potencies			
1	Estimate	Low	High
C	**760**	514	1130
D	**166**	106	248
E	**366**	248	544
F	**552**	392	774
H	**242**	174	336
2	Estimate	Low	High
C	**924**	662	1292
D	**514**	368	716
E	**522**	376	730
F	**656**	468	916
H	**256**	182	356
3	Estimate	Low	High
C	**658**	462	938
D	**236**	164	338
E	**374**	262	536
F	**2446**	1706	3562
H	**356**	248	516

4 A	4 B	6
3.69	3.73	
0.40	0.35	
0.18	0.86	

Potencies			
4A	Estimate	Low	High
C	**524**	349	789
D	**309**	206	467
E	**329**	219	494
F	**383**	250	576
H	**179**	119	271

Recalculated PC50 used

4B	Estimate	Low	High
C	**364**	242	545
D	**261**	174	391
E	**233**	154	349
F	**361**	235	546
H	**160**	107	241
6	Estimate	Low	High
C	**X**	X	X
D	**X**	X	X
E	**X**	X	X
F	**X**	X	X
H	**X**	X	X

ToBI

1 A	1 B	2	3
2.13	1.75	1.82	2.02
0.02	0.98	0.05	0.58
0.45	0.80	0.07	0.97

Potencies			
1	Estimate	Low	High
C	**532**	374	756
D	**160**	108	232
E	**406**	286	578
F	**596**	392	892
H	**208**	140	312
2	Estimate	Low	High
C	**940**	642	1390
D	**558**	380	822
E	**712**	486	1052
F	**996**	678	1470
H	**356**	242	530
3	Estimate	Low	High
C	**810**	564	1170
D	**320**	222	462
E	**398**	276	576
F	**2494**	1726	3652
H	**326**	226	474

4 A	4 B	6
2.92	3.28	
0.14	0.07	
0.05	0.01	

Potencies			
4A	Estimate	Low	High
C	**467**	288	754
D	**313**	193	511
E	**266**	164	428
F	**391**	237	628
H	**140**	86	227

Recalculated PC50 used

4B	Estimate	Low	High
C	**429**	277	664
D	**292**	189	453
E	**213**	135	331
F	**464**	294	721
H	**160**	103	249
6	Estimate	Low	High
C	**X**	X	X
D	**X**	X	X
E	**X**	X	X
F	**X**	X	X
H	**X**	X	X

Table 9c — *Potency results and 95% c.i. of Phase IIb per test per laboratory (RIVM calculations). Potency values obtained for ELISA and ToBI by probit analysis (after dichotomising). All values are in IU/ml*.*

		Challenge test			ELISA			ToBI test		
		7	8		7	8		7	8	
Slope		5.53	6.23		4.71	4.40		4.50	5.73	
p-value parallelism		0.70	0.67		0.36	0.44		0.42	0.87	
p-value linearity		0.96	0.89		0.80	0.94		0.81	0.99	
		Potencies			Potencies			Potencies		
Laboratory	Vaccines	Estimate	Low	High	Estimate	Low	High	Estimate	Low	High
7	F	485	339	679	398	266	591	460	313	671
	I	137	94	192	104	*69*	150	101	*67*	149
	K	232	156	342	193	125	296	190	122	297
8	F	483	350	664	608	358	1001	550	379	778
	I	154	112	212	124	*74*	195	144	101	200
	K	287	199	407	208	116	350	270	192	385

* For re-calculation to IU/human dose, all values should be divided by 2.

Italic: lower levels of 95 % c.i. below the Ph. Eur. minimum requirement of 40 IU/human dose.

Table 9d — *Potency results and 95% c.i. of Phase IIb per test per laboratory (RIVM calculations). Potency values obtained for ELISA and ToBI by parallel line assay calculations**. All values are in IU / ml*.*

		ELISA			ToBI test		
		7***	8		7***	8	
Slope		5.10	4.68		5.90	5.97	
p-value parallelism		0.22	0.37		0.05	0.11	
p-value linearity		0.49	0.00		0.34	0.00	
		Potencies			Potencies		
Laboratory	Vaccines	Estimate	Low	High	Estimate	Low	High
7	F	416	328	516	453	348	577
	I	129	102	161	134	102	171
	K	186	150	229	205	160	260
8	F	408	244	644	462	303	683
	I	118	*70*	187	126	81	187
	K	193	120	305	214	143	316

* For re-calculation to IU/human dose, all values should be divided by 2.

** Zero values are assigned to 0.0005 IU/ml.

*** Upper 3 dilutions of the vaccines used.

Italic: lower levels of 95 % c.i. below the Ph. Eur. minimum requirement of 40 IU/human dose.

- *ELISA*. Both calculation methods gave the same ranking of vaccines C, E and F (Tables 9a and 9b), but not for vaccines D and H (Lab. 1). Except for vaccines C, E, F and H (Lab. 2),all estimates (RIVM calculations) were within the 95 % c.l. given by the EDQM program. As was observed from the challenge test data, estimates obtained in one laboratory often fell outside the 95 % c.l. of another laboratory for the same vaccine. The maximal range of the 95 % c.l. calculated by RIVM and EDQM were 56-189 % and 64-151 % of the estimate, respectively (Table 9e).

- *ToBI*. Both calculation methods gave the same ranking of the vaccines except for vaccines C and F (Lab. 2 and 4a). All estimates (RIVM calculations) were within the 95 % c.l. given by the EDQM programAs was observed from the challenge test and the ELISA data, estimates obtained in one laboratory often fell outside the 95 % c.l. of another laboratory for the same vaccine. The maximal range of the 95 % c.l. calculated by RIVM and EDQM were 54-201 % and 66-150 %, respectively (Table 9e).

- *Challenge test, ELISA and ToBI.* Another approach for calculation of vaccine potencies is to use parallel-line analysis. To this end, zero values of individual antitoxin titres have to be replaced by an arbitrarily low antitoxin titre, e.g. 0.5 × the LOD. This allows log-transformation of antitoxin titres of all serum samples. Vaccine potencies, based on antitoxin concentrations, calculated by parallel-line analysis are additionally presented in Table 9c. Non-linearity occurred in Lab. 8 for vaccine I, both in ELISA and in ToBI.

  In general, the range of the 95% c.i. was similar whether the ELISA or the ToBI results were calculated by probit analysis after dichotomising or by parallel line assay (Table 9d). The maximal 95% c.l. of the challenge test data, calculated by EDQM, did not differ from those of ELISA and the ToBI, whereas a somewhat higher upper limit was seen for the challenge test data calculated by RIVM (Table 9e).

Table 9e — *Maximal range of the 95% c.l. obtained for the various analyses as calculated by RIVM and EDQM*

Study Phase	Test	Max. 95% c.l. (probit analysis, RIVM)	Max. 95% c.l. (probit analysis, EDQM)	Max. 95% c.l. (parallel line analysis, RIVM)
Phases I and IIa	Challenge test	52-247%	64-153%	n.d.
	ELISA	56-189%	64-151%	n.d.
	ToBI	54-201%	66-150%	n.d.
Phase IIb	ELISA	56-168%	n.d.	59-159%
	ToBI	64-148%	n.d.	64-148%

n.d. = not determined.

Table 10 — *No overlap in 95% c.i. of potencies estimated by challenge test (RIVM calculations). Vaccines are indicated by their code.*

		Laboratory				
Method	**Laboratory**	**1**	**2**	**3**	**4**	**5**
Challenge	1		D	F		n.d.
	2	D		F	C, E, F	n.d.
	3	F	F		F, H	n.d.
	4		C, E, F	F, H		n.d.
	5	n.d.	n.d.	n.d.	n.d.	
ELISA	1		D, F	F		C
	2	D, F		C, D, E	C, E, F, H	C, D, E, F, H
	3	F	C, D, E		F	F
	4		C, E, F	F		
	5	C	C, D, E, F, H	F		
ToBI	1		D	F, H		C, F
	2	D		F	C, E, H	C, D, E, F, H
	3	F, H	F		C, F, H	C, F, H
	4		C, E	F, H		
	5	C, F	C, D, E, F, H	C, F, H		

n.d. = not determined.

An overview of the vaccines for which no overlap in 95 % c.i. was seen in the different tests is given in Table 10. When potencies were estimated by the 5-parameter fit, slightly different results were obtained (results not shown). As partly different vaccines were tested in Phase IIb, Lab. 7 and 8 are not included in this table.

Vaccine ranking in the order of decreasing potency is illustrated in Table 11. An inverse ranking order was only observed for the vaccines at the same potency level (vaccines C, E, F and D, H). Considering the influence of the statistical calculations, it is assumed that these differences are not relevant. The ranking order of vaccines based on the challenge test was the same regardless of whether the estimate or the lower c.l. was used.

Table 11 — *Ranking of vaccines based on decreasing potency estimates as obtained in different test systems (RIVM calculations)*

Vaccine ranking	Lab. 1			Lab. 2			Lab. 3			Lab. 4			Lab. 5		
	Ch	El	To	Ch	El	To	Ch	El	To	Ch	El	To	Ch	El	To
1	E	C	F	C	C	C	F	F	F	C	C	F		C	F
2	F	F	C	F	F	F	C	C	C	F	F	C		F	C
3	C	E	E	E	E	E	E	E	E	D	E	D		E	E
4	H	H	H	D	D	D	H	H	H	E	D	E		D	D
5	D	D	D	H	H	H	D	D	D	H	H	H		H	H

Ch = Challenge, El = ELISA, To = ToBI.

5.3. Comparison between titres of individual test sera obtained in ELISA and ToBI and absence of tetanus paralysis in the challenge test

The ratio of the number of animals without tetanus paralysis in the challenge test versus the ratio of number of animals having an antitoxin concentrations higher than 0.0075 IU/ml (the cut-off value) per number of serum samples tested, are shown in Table 12a (RIVM calculations) and Table 12b (EDQM calculations) for the Phase I and Phase IIa study, and in Table 12c for the Phase IIb study (RIVM calculations only). Within each laboratory, a very good agreement can be seen between the results of the challenge test and those of the serological tests. However, between laboratories, there could be a significant difference in ratios, e.g. in the Phase IIb study between Lab. 7 and Lab. 8.

The difficulties of the challenge procedure in Lab. 5 are clearly illustrated in Table 12a. It can be seen that challenge, ELISA and ToBI ratios for ERTA and vaccine C are about the same (apart from ToBI ratio for dilution 2.008 of Vaccine C). However, for the vaccines D, E, F and H, ELISA and ToBI ratios are in close agreement, but challenge ratios do not show the expected dose-response effect. Data of Lab. 6 are presented in Table 12b. Ratios are generally very low for the challenge test, while ratios for antitoxin concentrations above 0.0075 IU/ml per number of serum samples are high. For all vaccines tested at Lab. 6, no dose-response effect is seen.

Table 12a — *Listed are the ratios of animals with a positive response. For the challenge test this means animals without tetanus paralysis/animals challenged. For ELISA and ToBI assays this means: titres higher than 0.0075 IU/ml / number of sera tested.*

Note: the ERTA results of test 2 at laboratory 1 are not listed. (RIVM calculations).

	Laboratory 1				Laboratory 2				Laboratory 3				Laboratory 4				Laboratory 5			
Vaccine	Dose (μl)	Challenge	ELISA	ToBI	Dose (μl)	Challenge	ELISA	ToBI	Dose (μl)	Challenge	ELISA	ToBI	Dose (μl)	Challenge	ELISA	ToBI	Dose (μl)	Challenge	ELISA	ToBI
ERTA	15.625	12/12	11/13	11/13	15.625	12/12	11/12	11/12	15.625	9/10	12/12	11/12	15.625	11/12	12/12	12/12	15.625	11/11	11/11	11/11
	7.813	8/12	11/13	9/13	7.813	4/12	2/12	3/12	7.813	3/11	4/11	2/11	7.813	9/12	9/12	9/12	7.813	12/12	12/12	11/12
	3.906	3/12	3/13	3/13	3.906	0/12	0/12	0/12	3.906	1/12	2/12	1/12	3.906	4/12	7/12	5/12	3.906	10/12	9/12	9/12
	1.953	0/12	1/12	1/12	1.953	0/12	0/12	0/12	1.953	0/11	1/12	0/12	1.953	1/12	0/12	1/12	1.953	5/12	4/12	4/12
C	8.032	12/12	13/13	13/13	8.032	12/12	12/12	12/12	8.032	12/12	12/12	12/12	8.032	10/12	10/12	10/12	8.032	12/12	12/12	12/12
	4.016	7/12	11/13	11/13	4.016	10/12	12/12	10/12	4.016	10/12	10/12	11/12	4.016	7/12	10/12	8/12	4.016	10/12	9/12	8/12
	2.008	0/12	6/13	0/13	2.008	4/12	7/12	3/12	2.008	6/12	6/12	7/12	2.008	4/12	5/12	4/12	2.008	7/11	5/11	1/11
	1.004	0/12	2/13	0/13	1.004	0/10	1/11	0/11	1.004	0/10	0/12	0/12	1.004	0/12	1/12	0/12	1.004	2/12	0/12	0/12
D	10.309	6/12	6/13	5/13	10.309	11/12	12/12	10/12	10.309	10/12	9/12	10/12	15.306	12/12	12/12	12/12	15.788	11/11	11/12	11/12
	5.155	2/12	4/13	3/13	5.155	7/12	8/12	7/12	5.155	2/12	4/12	4/12	7.653	10/12	11/12	10/12	7.894	12/12	12/12	12/12
	2.577	1/12	0/13	0/13	2.577	2/12	2/12	2/12	2.577	2/12	1/12	1/12	3.827	3/12	5/12	5/12	3.947	11/12	7/12	6/12
	1.289	0/12	0/13	0/13	1.289	0/12	1/12	0/12	1.289	0/12	0/12	0/12	1.913	0/12	0/12	0/12	1.974	12/12	1/12	0/12
E	11.173	12/12	13/13	12/13	11.173	12/12	12/12	12/12	11.173	11/11	12/12	10/12	11.173	9/12	11/12	11/12	11.172	12/12	12/12	11/12
	5.587	10/12	10/13	10/13	5.587	10/12	11/12	10/12	5.587	8/12	8/12	8/12	5.587	6/12	10/12	6/12	5.586	12/12	12/12	12/12
	2.793	0/11	0/12	3/13	2.793	3/12	4/12	3/12	2.793	2/11	1/11	2/11	2.793	3/12	3/12	1/12	2.793	12/12	3/12	4/12
	1.397	0/12	0/13	0/13	1.397	0/12	0/12	0/12	1.397	0/12	0/12	0/12	1.397	0/12	2/12	0/12	1.397	12/12	2/12	0/12
F	4.950	8/12	11/13	10/13	4.950	12/12	12/12	12/12	4.950	12/12	12/12	12/12	4.950	9/12	9/12	9/12	5.000	12/12	11/12	11/12
	2.475	1/12	1/12	1/12	2.475	4/12	7/12	4/12	2.475	12/12	12/12	12/12	2.475	2/12	4/12	3/12	2.500	9/9	7/12	6/12
	1.238	0/12	0/13	0/13	1.238	1/12	3/12	0/12	1.238	11/12	11/12	10/12	1.238	0/12	0/12	1/12	1.250	12/12	0/12	0/12
	0.619	0/12	0/13	0/13	0.619	0/11	0/12	0/12	0.619	2/11	3/12	2/12	0.619	0/12	0/12	2/12	0.625	12/12	0/12	0/12
H	30.075	12/12	13/13	13/13	30.075	12/12	12/12	12/12	30.075	12/12	12/12	12/12	30.075	12/12	12/12	12/12	30.066	12/12	12/12	12/12
	15.038	9/12	12/13	9/13	15.038	11/11	11/11	10/11	15.038	11/12	11/11	11/11	15.038	8/12	10/12	8/12	15.033	12/12	12/12	12/12
	7.519	4/12	5/13	4/13	7.519	4/12	7/12	6/12	7.519	8/12	9/12	8/12	7.519	5/12	5/12	5/12	7.517	12/12	9/12	8/12
	3.759	0/12	0/13	0/13	3.759	0/12	1/12	1/12	3.759	2/12	4/12	2/12	3.759	1/12	2/12	2/12	3.758	12/12	5/12	3/12

Table 12b — *Listed are the ratios of animals with a positive response. For the challenge test this means animals without tetanus paralysis/animals challenged. For ELISA and ToBI assays this means: titres higher than 0.0075 IU/ml / number of sera tested.*

Note: the ERTA results of test 2 at laboratory 1 are not listed. (EDQM calculations).

	Laboratory 1				Laboratory 2				Laboratory 3				Laboratory 4				Laboratory 6			
Vaccine	Dose (µl)	Challenge	ELISA	ToBI	Dose (µl)	Challenge	ELISA	ToBI	Dose (µl)	Challenge	ELISA	ToBI	Dose (µl)	Challenge	ELISA	ToBI	Dose (µl)	Challenge	ELISA	ToBI
ERTA	15.625	12/12	10/12	10/12	15.625	12/12	12/12	11/12	15.625	9/10	12/12	11/12	15.625	11/12	12/12	12/12	15.625	4/12	10/12	12/12
	7.813	8/12	10/12	9/12	7.813	4/12	5/12	3/12	7.813	3/11	3/11	3/11	7.813	9/12	9/12	9/12	7.813	1/12	5/12	10/12
	3.906	3/12	3/12	3/12	3.906	0/12	0/12	0/12	3.906	1/12	2/12	1/12	3.906	4/12	6/12	6/12	3.906	0/11	2/12	10/12
	1.953	0/12	0/11	0/12	1.953	0/12	0/12	0/12	1.953	0/11	0/12	0/12	1.953	1/12	0/12	2/12	1.953	1/12	3/12	12/12
C	8.032	12/12	12/12	12/12	8.032	12/12	12/12	12/12	8.032	12/12	12/12	12/12	8.032	10/12	10/12	10/12	8.032	5/12	10/12	12/12
	4.016	7/12	10/12	11/12	4.016	10/12	12/12	10/12	4.016	10/12	10/12	11/12	4.016	7/12	11/12	11/12	4.016	0/8	11/12	12/12
	2.008	0/12	6/12	0/12	2.008	4/12	4/12	3/12	2.008	6/12	6/12	6/12	2.008	4/12	7/12	7/12	2.008	1/11	4/11	11/11
	1.004	0/12	2/12	0/12	1.004	0/12	0/11	0/11	1.004	0/10	0/12	0/12	1.004	0/12	1/12	1/12	1.004	0/11	8/12	10/12
D	10.309	6/12	6/12	5/12	10.309	11/12	11/12	10/12	10.309	10/12	9/12	10/12	30.612	12/12	12/12	12/12	30.612	2/12	6/12	9/12
	5.155	2/12	3/12	2/12	5.155	7/12	8/12	6/12	5.155	2/12	3/12	5/12	15.306	10/12	11/12	11/12	15.306	0/12	6/12	10/12
	2.577	1/12	0/12	0/12	2.577	2/12	2/12	3/12	2.577	2/12	1/12	1/12	7.653	3/12	6/12	6/12	7.653	1/11	8/12	7/12
	1.289	0/12	0/12	0/12	1.289	0/12	1/12	1/12	1.289	0/12	0/12	0/12	3.827	0/12	1/12	3/12	3.827	0/12	5/12	11/12
E	11.173	12/12	12/12	12/12	11.173	12/12	12/12	12/12	11.173	11/11	12/12	10/12	11.173	9/12	11/12	11/12	11.173	1/12	9/12	12/12
	5.587	10/12	10/12	10/12	5.587	10/12	10/12	10/12	5.587	8/12	8/12	8/12	5.587	6/12	10/12	9/12	5.587	0/10	8/12	10/12
	2.793	0/11	0/11	2/12	2.793	3/12	2/12	4/12	2.793	2/11	1/11	2/11	2.793	3/12	4/12	5/12	2.793	0/11	5/12	10/12
	1.397	0/12	0/12	0/12	1.397	0/12	0/12	0/12	1.397	0/12	0/12	0/12	1.397	0/12	1/12	0/12	1.397	0/12	4/12	9/12
F	4.950	8/12	10/12	10/12	4.950	12/12	10/12	12/12	4.950	12/12	12/12	12/12	4.950	9/12	10/12	9/12	4.950	1/11	7/12	11/12
	2.475	1/12	1/11	2/11	2.475	4/12	4/12	4/12	2.475	12/12	12/12	12/12	2.475	2/12	5/12	4/12	2.475	0/12	11/12	12/12
	1.238	0/12	0/12	1/12	1.238	1/12	0/12	0/12	1.238	11/12	11/12	10/12	1.238	0/12	0/12	1/12	1.238	0/12	8/12	9/12
	0.619	0/12	0/12	0/12	0.619	0/12	0/12	1/12	0.619	2/11	3/12	2/12	0.619	0/12	0/12	3/12	0.619	1/11	3/12	6/12
H	30.075	12/12	12/12	12/12	30.075	12/12	12/12	12/12	30.075	12/12	12/12	12/12	30.075	12/12	12/12	12/12	30.075	0/12	9/12	6/12
	15.038	9/12	11/12	9/12	15.038	12/12	10/11	10/11	15.038	11/12	12/12	12/12	15.038	8/12	11/12	11/12	15.038	2/11	6/12	7/12
	7.519	4/12	5/12	4/12	7.519	4/12	6/12	6/12	7.519	8/12	9/12	7/12	7.519	5/12	6/12	5/12	7.519	0/11	6/12	8/12
	3.759	0/12	0/12	1/12	3.759	0/12	0/12	2/12	3.759	2/12	4/12	2/12	3.759	1/12	3/12	2/12	3.759	0/12	9/12	6/12

Table 12c — *Listed are the ratios of animals with a positive response. For the challenge test this means animals without tetanus paralysis / animals challenged. For ELISA and ToBI this means: titres higher than 0.0075 IU/ml / number of sera tested. Results of Phase IIb (RIVM calculations).*

Vaccine	Dose	Laboratory 7			Laboratory 8		
	(µl)	Challenge	ToBI	ELISA	Challenge	ToBI	ELISA
ERTA	15.625	10/11	11/12	12/12	12/12	12/12	11/11
	7.813	8/12	10/12	7/12	12/12	12/12	12/12
	3.906	0/12	1/12	1/12	11/12	11/12	12/12
	1.953	0/11	1/12	1/12	3/12	5/12	8/12
F	4.950	8/12	10/12	9/12	11/11	12/12	12/12
	2.475	2/11	4/12	1/12	11/12	12/12	12/12
	1.238	0/11	0/12	0/12	7/12	9/12	11/12
	0.619	0/12	0/12	0/12	0/12	1/12	6/12
I	15.306	7/12	7/12	6/12	12/12	12/12	12/12
	7.653	1/12	1/12	1/12	11/11	12/12	12/12
	3.827	0/12	0/12	0/12	4/12	5/12	6/12
	1.913	0/12	0/12	0/12	1/12	1/12	4/12
K	15.306	12/12	12/12	12/12	12/12	12/12	12/12
	7.653	5/12	6/12	5/12	12/12	12/12	12/12
	3.827	0/12	0/12	0/12	12/12	11/12	12/12
	1.913	0/12	0/12	0/12	4/12	6/12	6/12

5.4. Relation between individual guinea pig serum antitoxin concentrations and challenge results

For each individual serum sample the relation between mean antitoxin concentrations (ELISA and ToBI) and challenge test results obtained in the participating laboratories (apart from Lab.□6), are shown in Figures 1a1-1h2. PC_{50} and PC_{99} values, calculated by logistic regression of individual guinea pig data (concentration versus survival), are presented in Table 13.

Table 13 — *Antitoxin concentrations (IU/ml) protecting 50□% (PC_{50}) and 99□% (PC_{99}) of the animals after challenge*

Laboratory	**ToBI**		**ELISA**	
	PC_{50}	**PC_{99}**	**PC_{50}**	**PC_{99}**
1	0.0071	0.0302	0.0071	0.0451
2	0.0077	0.0485	0.0076	0.0473
3*	0.0081	0.0471	0.0075	0.0348
4	0.0086	0.0313	0.0115	0.0352
5	0.0025*	0.0211*	0.0036**	0.0229**
7	0.0108	n.a.	0.0080	n.a.
8	0.0120	n.a.	0.0214	n.a.

* excluding data of one outlier.

** PC_{50} and PC_{99} values are based on a limited amount of data (ERTA and vaccine C) and unreliable challenge results.

n.a. = not available.

PC_{50} values obtained in Lab. 4 were in line with those obtained in the Phase I study, although the ELISA PC_{50} values were somewhat higher. PC_{50} values in the Phase IIb study were higher than in the Phase I and IIa study, especially in Lab. 8. The latter might be due to the somewhat higher toxicity of the tetanus toxin used. It was decided to use the PC_{50} value of 0.0075 IU/ml as obtained in the Phase I study also in the Phase IIa and IIb study. Survival or death of the individual animal was predicted based on its antitoxin concentration (death $< PC_{50}$, alive $> PC_{50}$). These predicted values were compared with observed death/survival. Results are shown in Tables 14a-14f.

Figure 1a1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 1)*

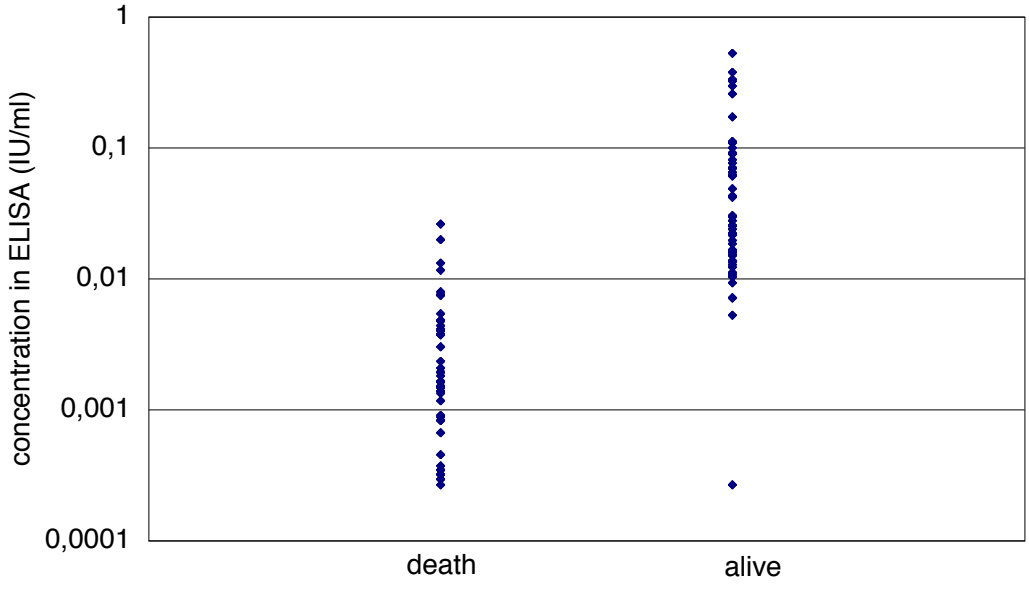


Figure 1a2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 1)*

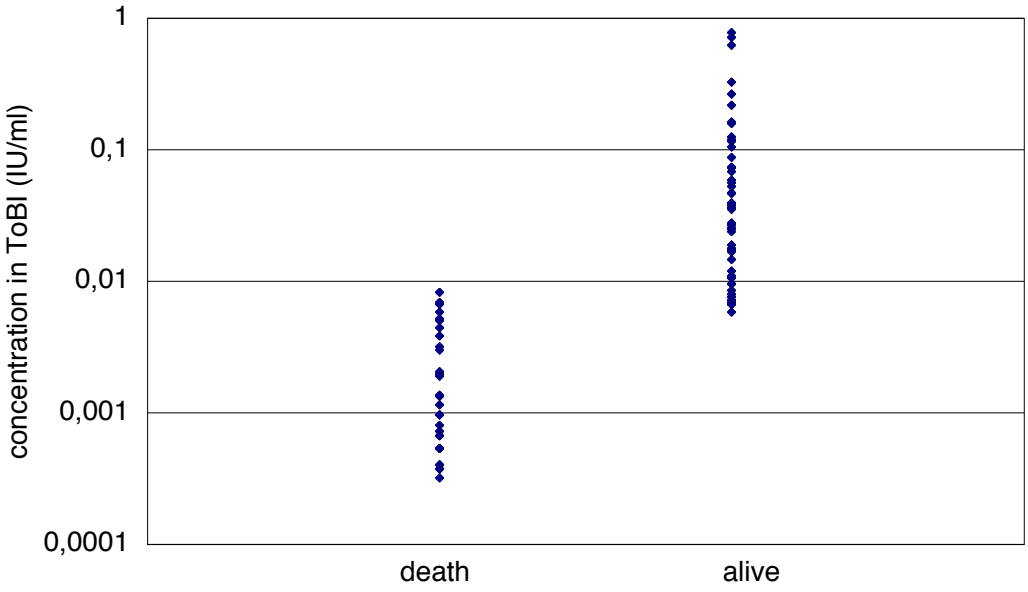


Figure 1b1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 2)*

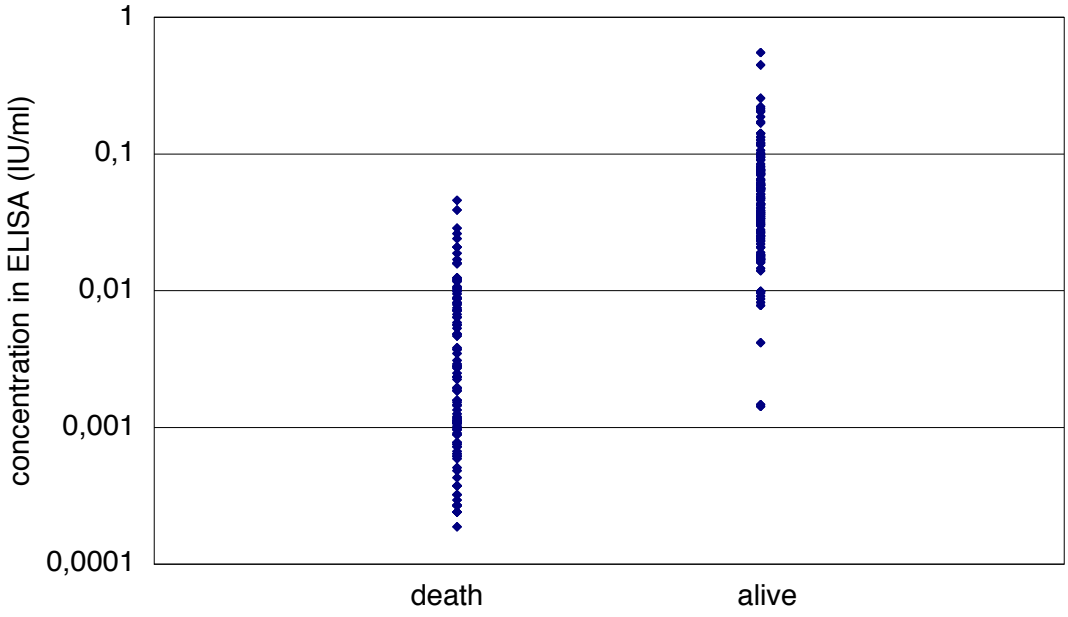


Figure 1b2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 2)*

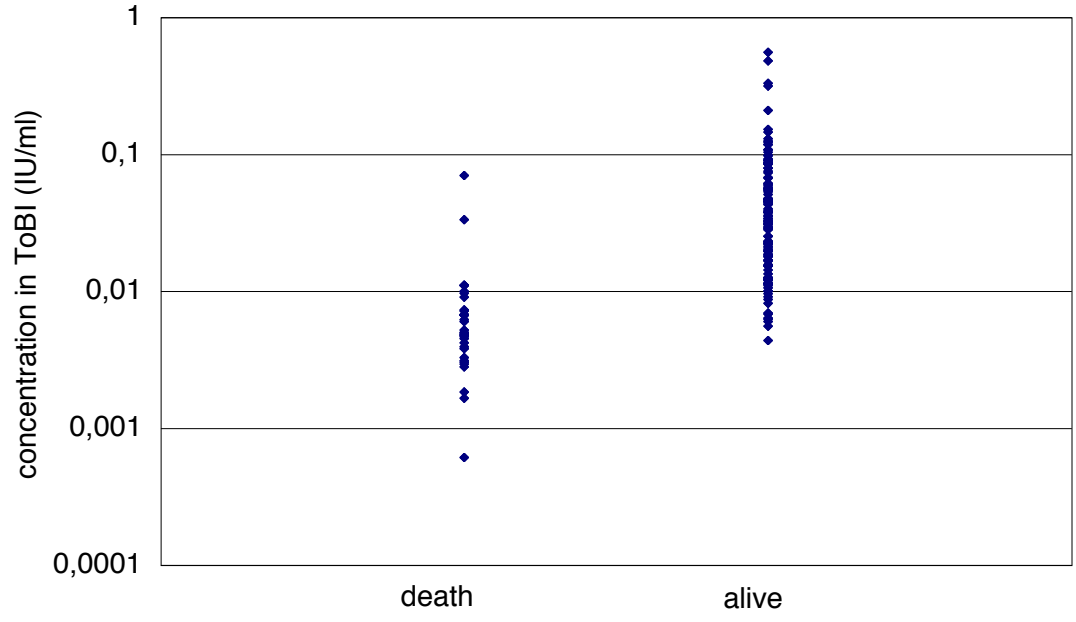


Figure 1c1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 3)*

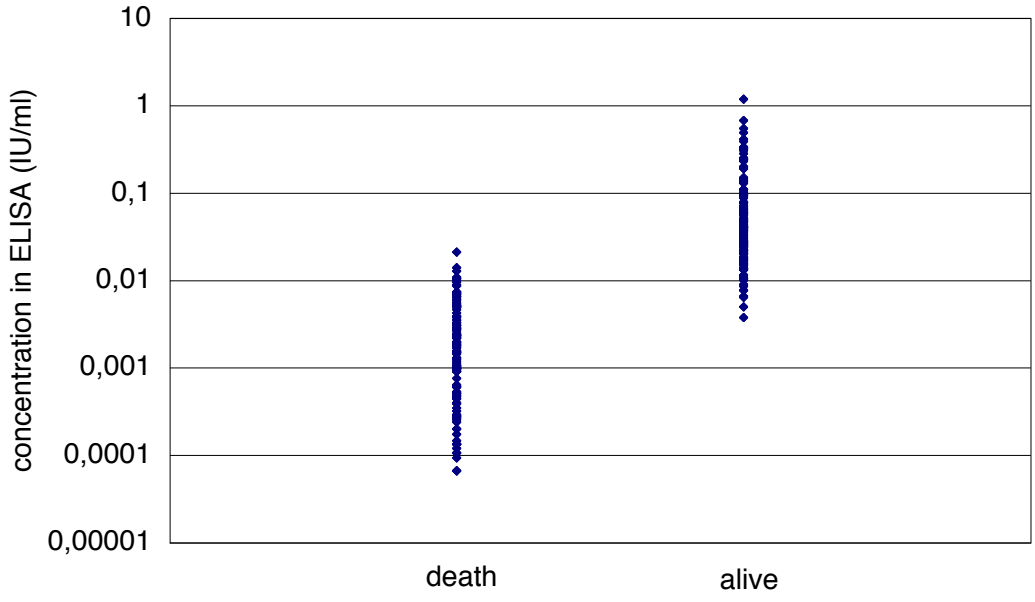


Figure 1c2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 3)*

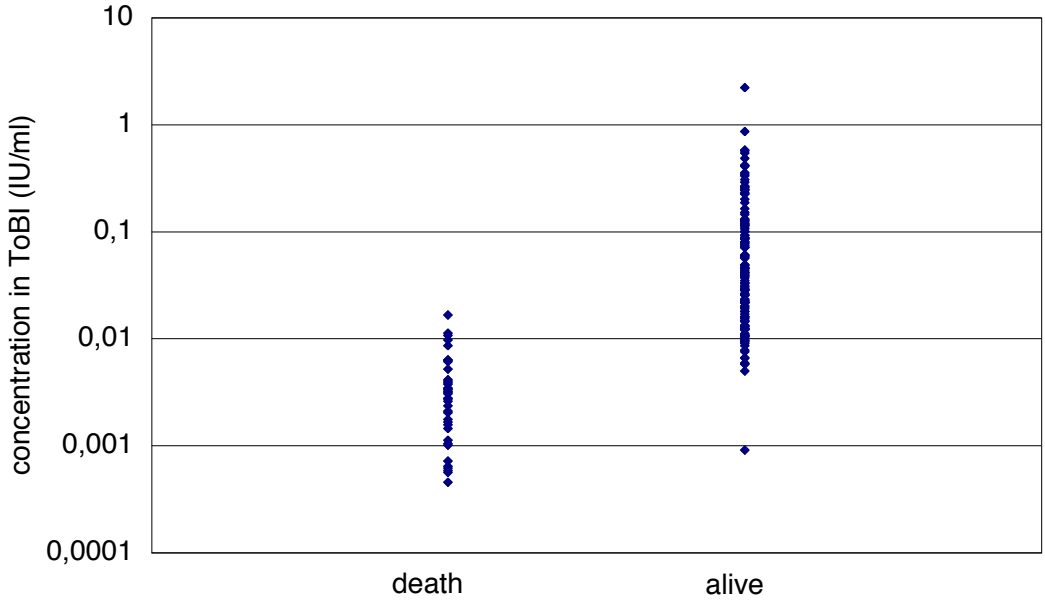


Figure 1d1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 4)*

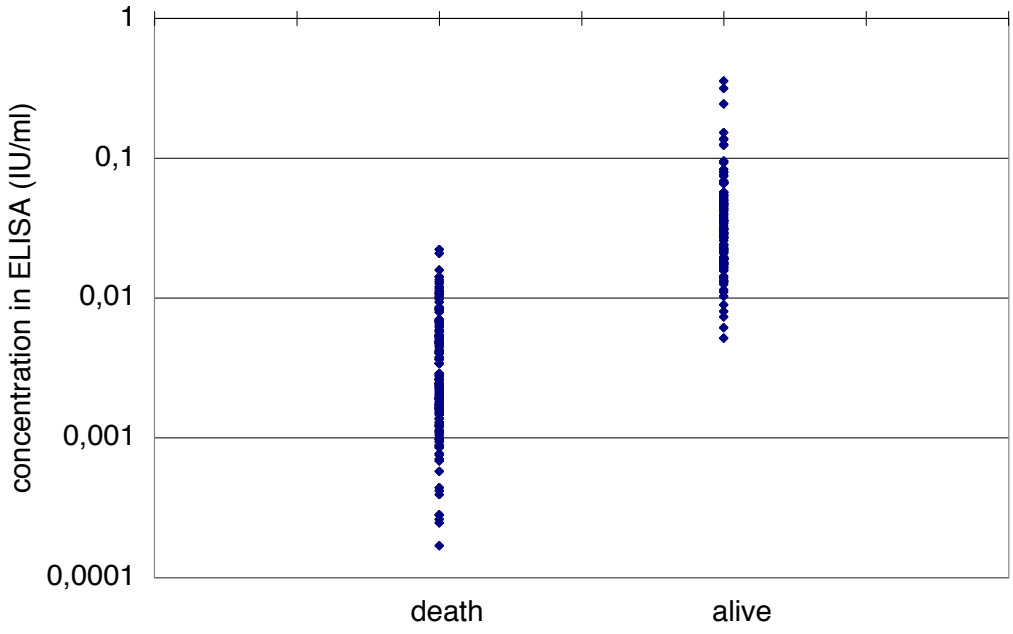


Figure 1d2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 4)*

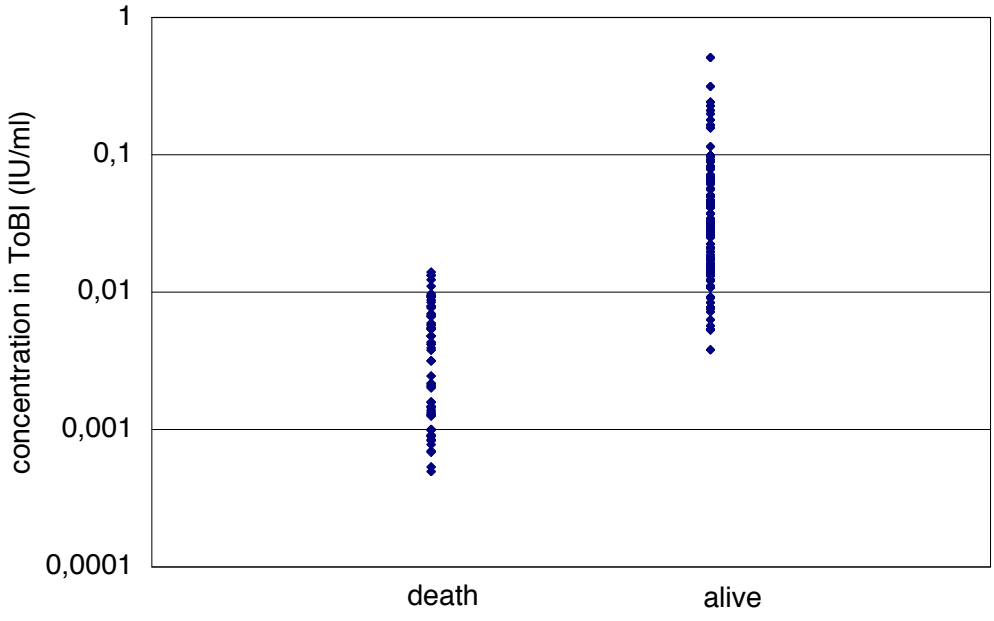


Figure 1e1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 5)*

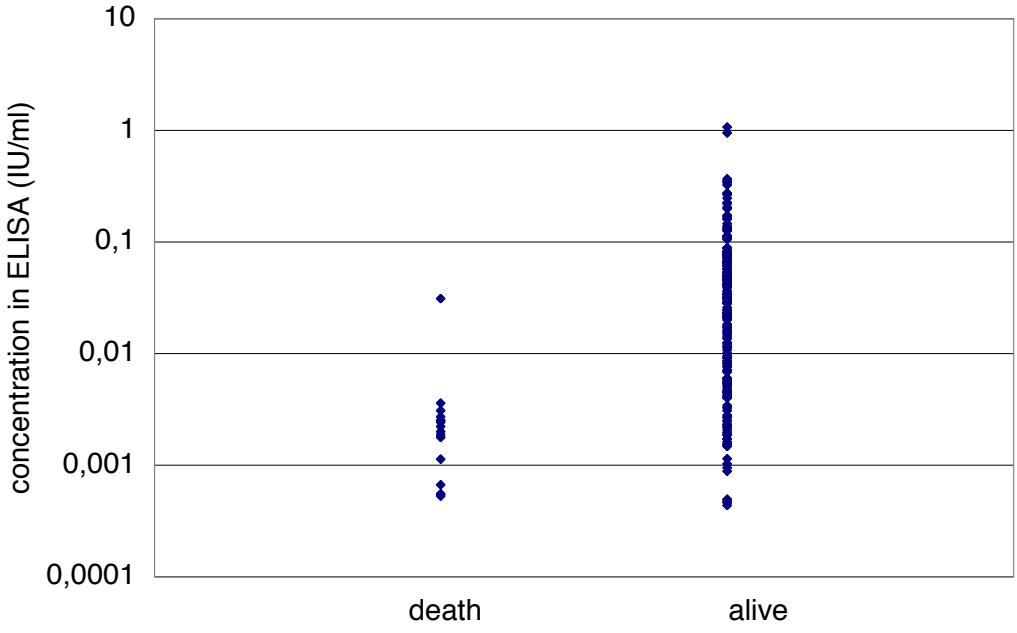


Figure 1e2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 5)*

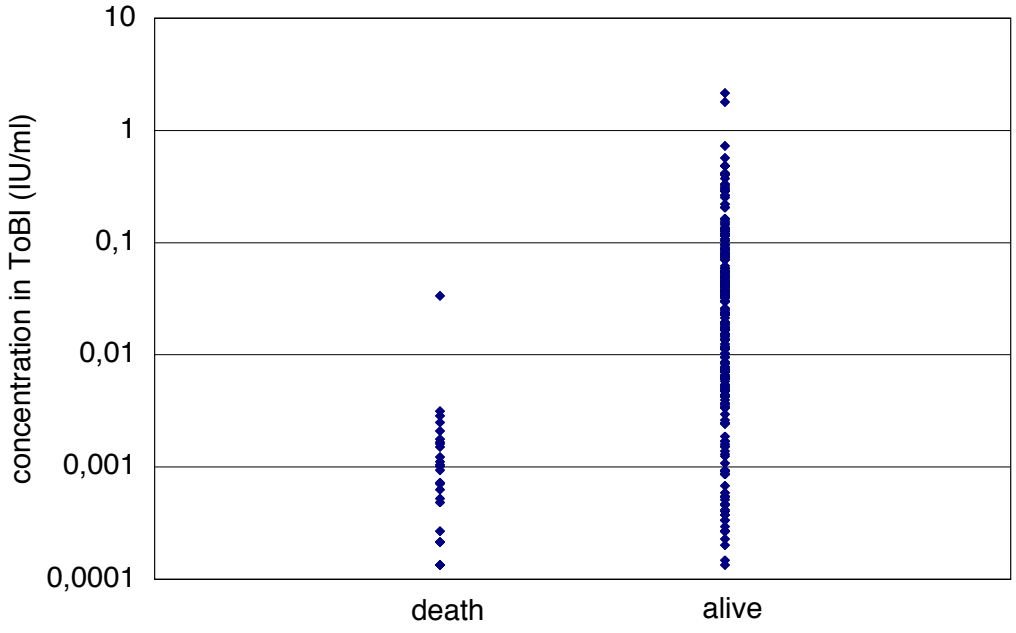


Figure 1g1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 7)*

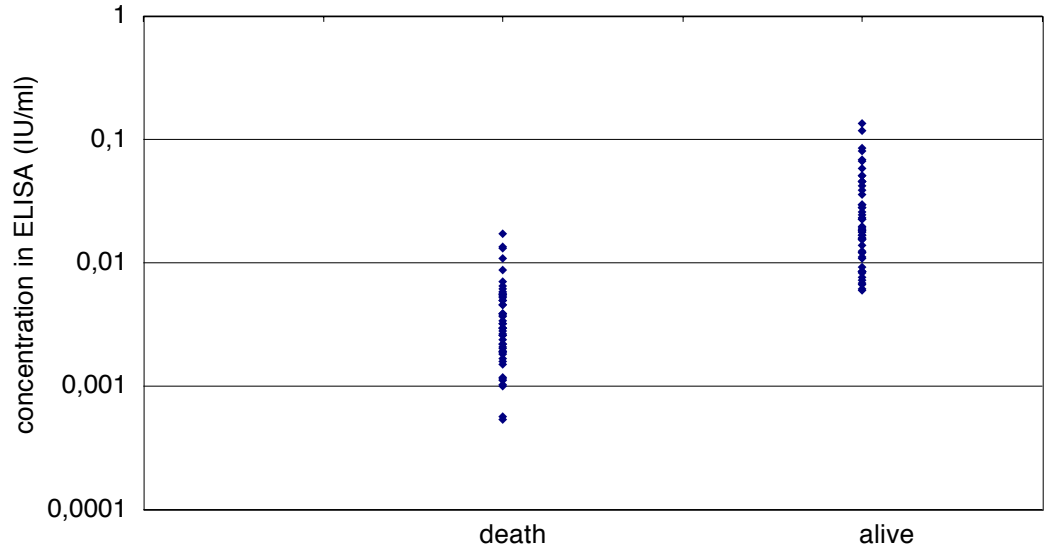


Figure 1g2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 7)*

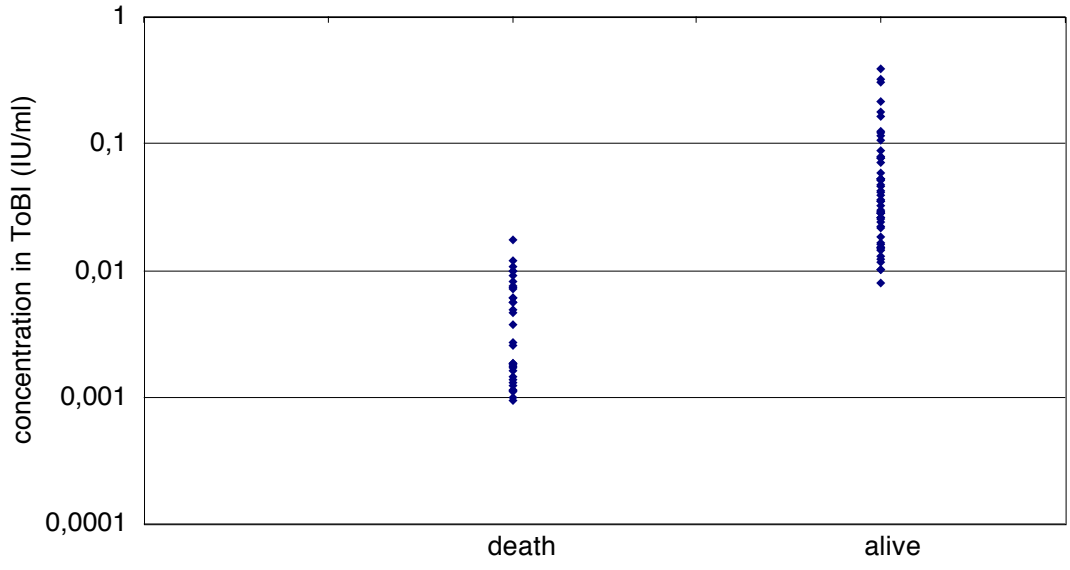


Figure 1h1 — *Relation between mean antitoxin concentration (ELISA) and survival in the challenge test (Laboratory 8)*

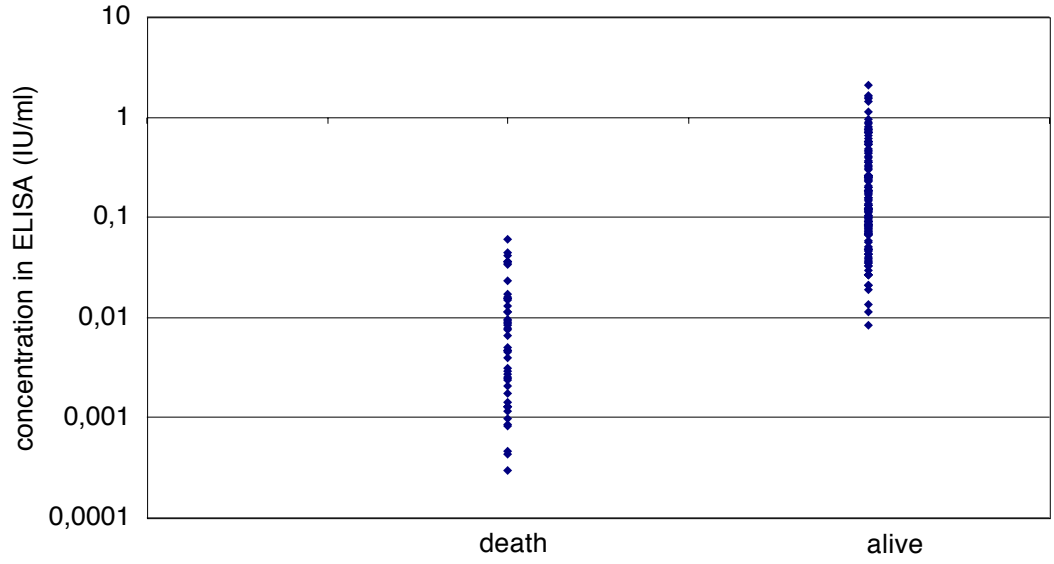


Figure 1h2 — *Relation between mean antitoxin concentration (ToBI) and survival in the challenge test (Laboratory 8)*

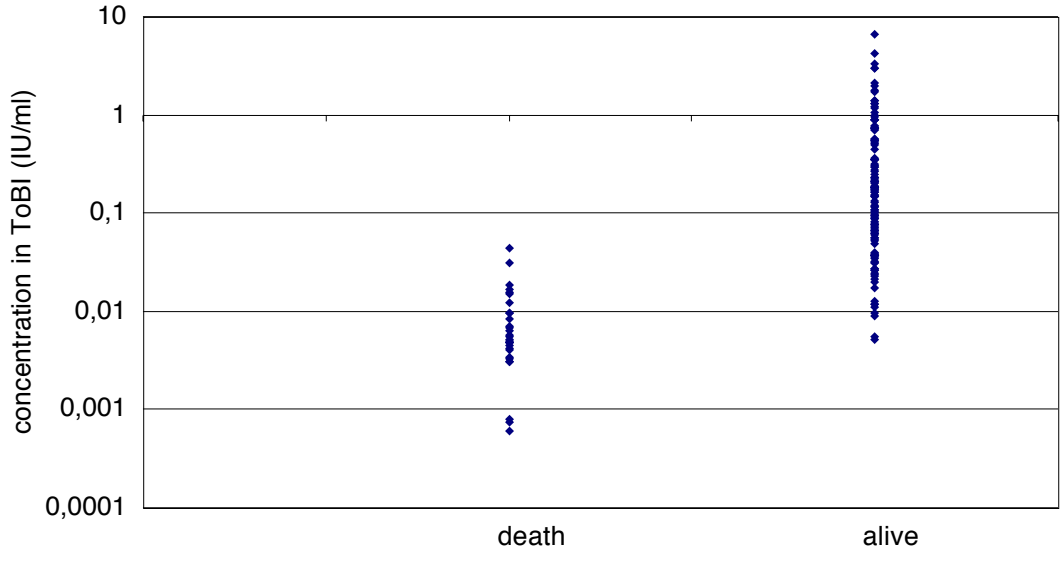


Table 14a — *Laboratory 1 (Phase I): Prediction of survival and death due to tetanus paralysis in individual animals, based on their serum antitoxin concentration in ELISA and ToBI*

Test		No. predicted	Observed death	Observed survival	Percentage correct	Overall percentage
ELISA	Predicted death	86	83	3	96.51	
n = 143	Predicted survival	57	5	52	91.23	93.87
ToBI	Predicted death	87	84	3	96.55	
n = 142	Predicted survival	55	3	52	94.55	95.55

Table 14b — *Laboratory 2 (Phase I): Prediction of survival and death due to tetanus paralysis in individual animals, based on their serum antitoxin concentration in ELISA and ToBI*

Test		No. predicted	Observed death	Observed survival	Percentage correct	Overall percentage
ELISA	Predicted death	159	145	14	91.19	
n = 284	Predicted survival	125	9	116	92.80	91.99
ToBI	Predicted death	164	148	16	90.24	
n = 286	Predicted survival	122	8	114	93.44	91.84

Table 14c — *Laboratory 3 (Phase I): Prediction of survival and death due to tetanus paralysis in individual animals, based on their serum antitoxin concentration in ELISA and ToBI*

Test		No. predicted	Observed death	Observed survival	Percentage correct	Overall percentage
ELISA	Predicted death	135	126	9	93.33	
n = 278	Predicted survival	143	6	137	95.80	94.56
ToBI	Predicted death	134	128	6	95.52	
n = 278	Predicted survival	144	4	140	97.22	96.37

Table 14d — *Laboratory 4 (Phase IIa): Prediction of survival and death due to tetanus paralysis in individual animals, based on their serum antitoxin concentration in ELISA and ToBI*

Test		No. predicted	Observed death	Observed survival	Percentage correct	Overall percentage
ELISA	Predicted death	139	136	3	97.84	90.53
n = 288	Predicted survival	149	25	124	83.22	
ToBI	Predicted death	152	146	6	96.05	92.51
n = 288	Predicted survival	136	15	121	88.97	

Table 14e — *Laboratory 7 (Phase IIb): Prediction of survival and death due to tetanus paralysis in individual animals, based on their serum antitoxin concentration in ELISA and ToBI*

Test		No. predicted	Observed death	Observed survival	Percentage correct	Overall percentage
ELISA	Predicted death	136	130	6	95.59	92.98
n = 188	Predicted survival	52	5	47	90.38	
ToBI	Predicted death	136	133	3	97.79	96.97
n = 188	Predicted survival	52	2	50	96.15	

Table 14f — *Laboratory 8 (Phase IIb): Prediction of survival and death due to tetanus paralysis in individual animals, based on their serum antitoxin concentration in ELISA and ToBI*

Test		No. predicted	Observed death	Observed survival	Percentage correct	Overall percentage
ELISA	Predicted death	52	46	6	88.46	90.94
n = 189	Predicted survival	137	9	128	93.43	
ToBI	Predicted death	53	48	5	90.56	92.72
n = 190	Predicted survival	137	7	130	94.89	

5.5. INTRA-LABORATORY VARIATION FOR ELISA AND TOBI

For each serum, the RSDs of antitoxin concentrations (based on the 5-parameter fit) considered as an indicator for test repeatability, have been calculated from the three ELISA and ToBI repetitions. The distribution of the RSDs obtained by Lab. 1 to 3 (Phase I), Lab. 4 to 6 (Phase□IIa) and Lab. 7 and Lab. 8 (Phase IIb) are plotted in Figures 2a-2h, respectivelyIt should be noted that, although RSDs could be calculated for Lab. 6, no valid ELISA and ToBI data were produced in this laboratory. In all cases, apart from Lab. 8 in Phase IIb, these figures indicate that ELISA gives better repeatability than ToBI in the participating laboratories.

Figure 2a — *Laboratory 1 - Repeatability of the individual assays*

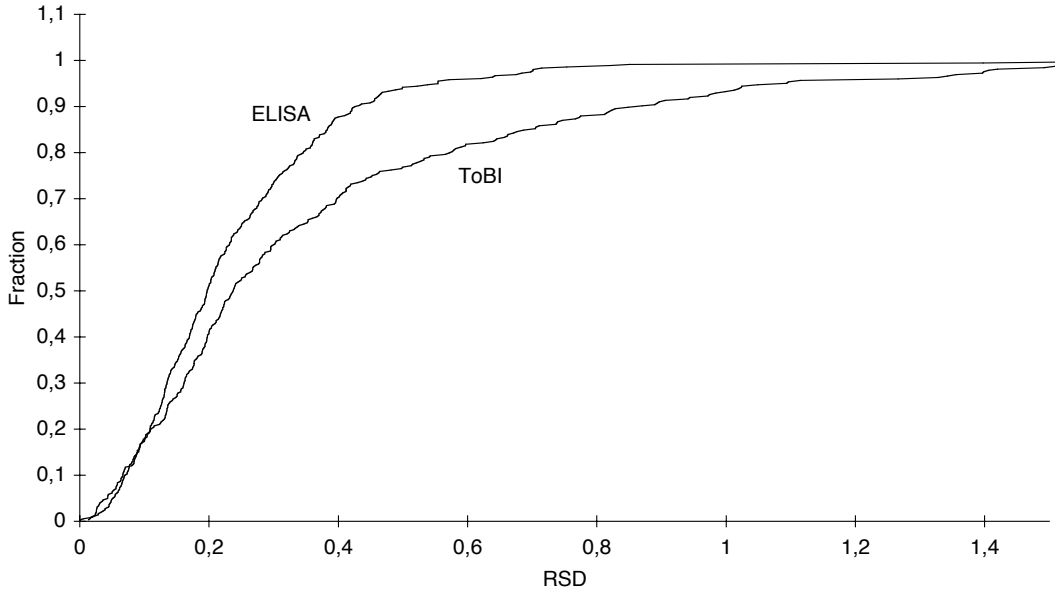


Figure 2b — *Laboratory 2 - Repeatability of the individual assays*

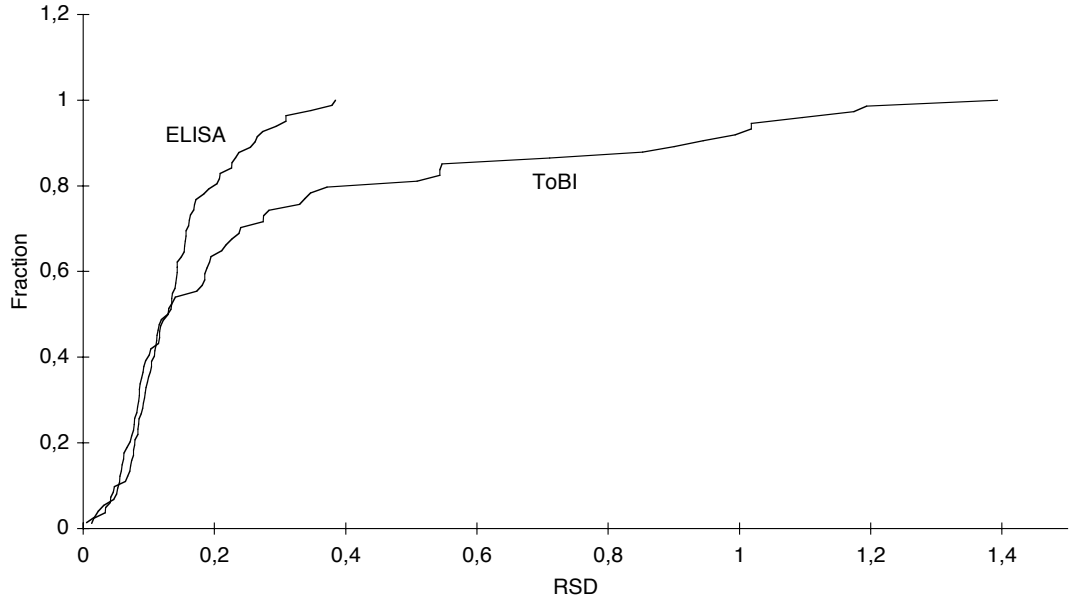


Figure 2c — *Laboratory 3 - Repeatability of the individual asssays*

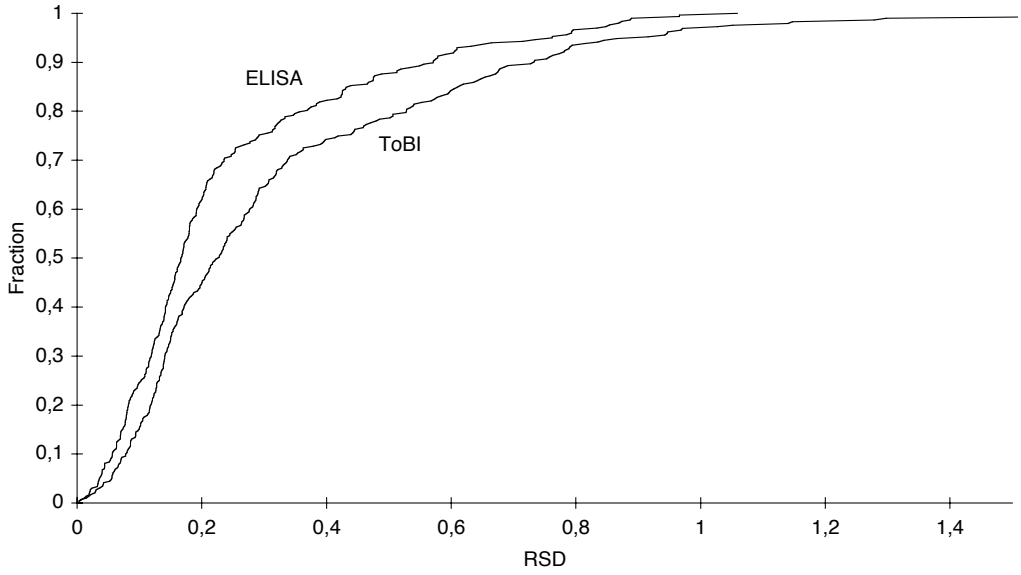


Figure 2d — *Laboratory 4 - Repeatability of the individual assays*

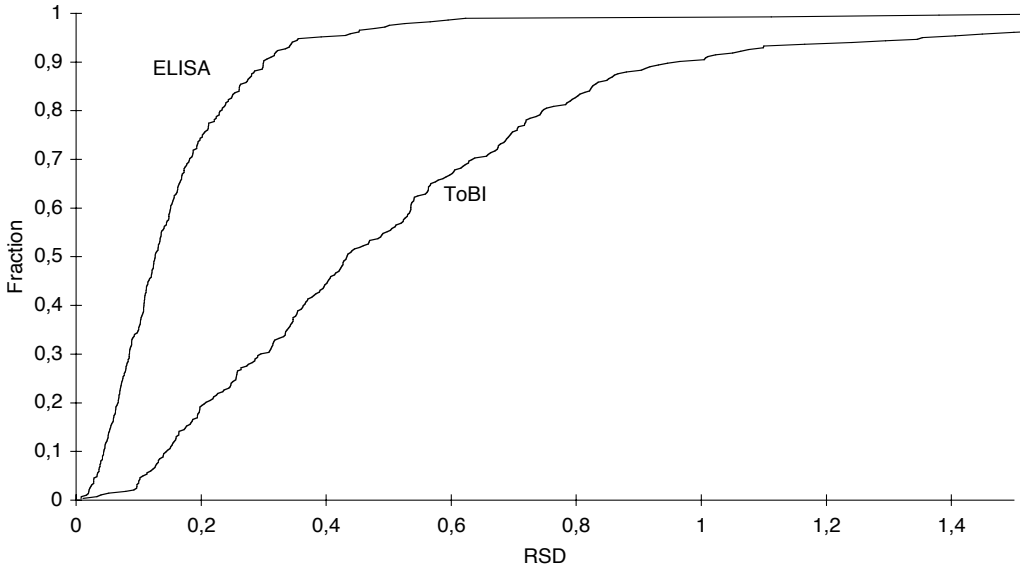


Figure 2e — *Laboratory 5 - Repeatability of the individual assays*

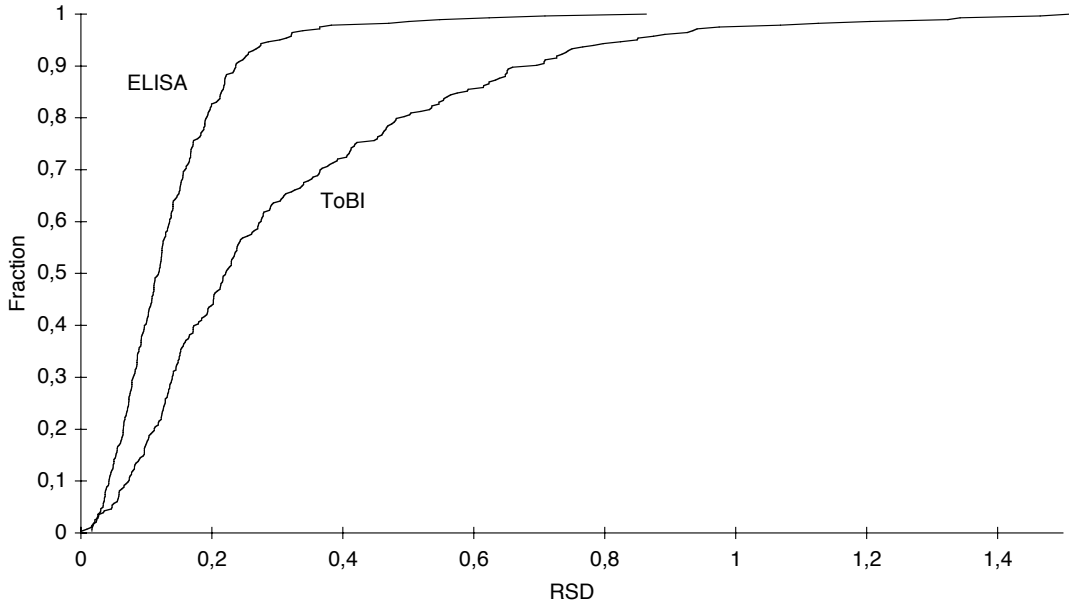


Figure 2f — *Laboratory 6 - Repeatability of the individual assays*

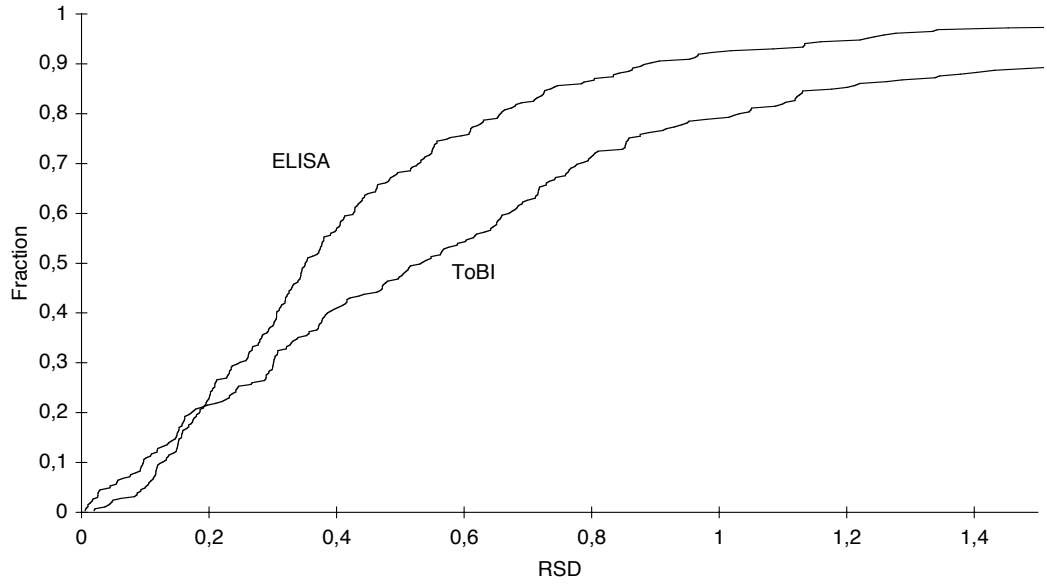


Figure 2g — *Laboratory 7 - Repeatability of the individual assays*

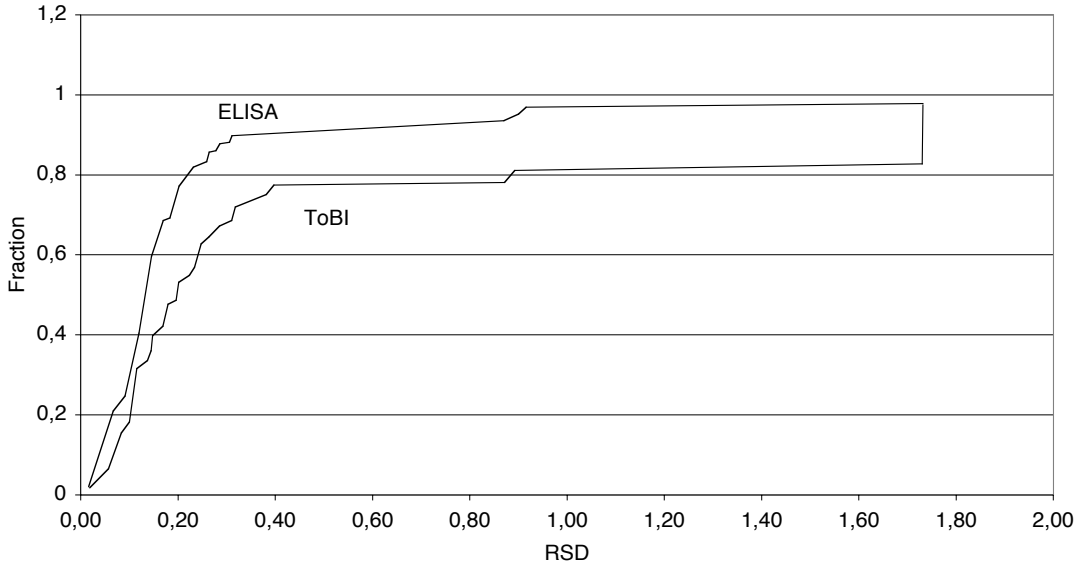
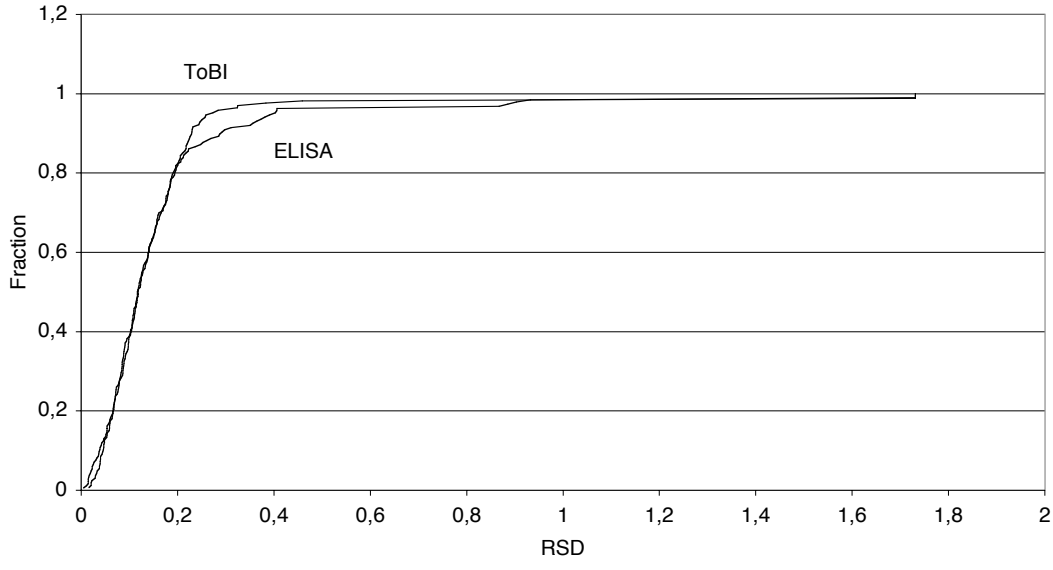


Figure 2h — *Laboratory 8 - Repeatability of the individual assays*



5.6. ELISA-ToBI CORRELATION

For the individual serum samples, log-transformed antitoxin concentrations determined by ELISA were plotted against those for ToBI, and results are shown in Figures 3a-3g for Lab.□1 to Lab. 8, respectively (apart from Lab. 6). Correlation coefficients and slopes of the line of agreement are summarised in Table 15.

Table 15 — *Correlation coefficients (Pearson) and slopes of line of agreement between ELISA and ToBI results for the individual serum samples*

Laboratory	Correlation ELISA-ToBI	Slope
1	0.903	0.855
2	0.913	0.756
3	0.937	0.816
4	0.916	0.740
5	0.945	0.806
7	0.876	0.714
8	0.966	0.744

Figure 3a — *Correlation ELISA-ToBI - Laboratory 1*

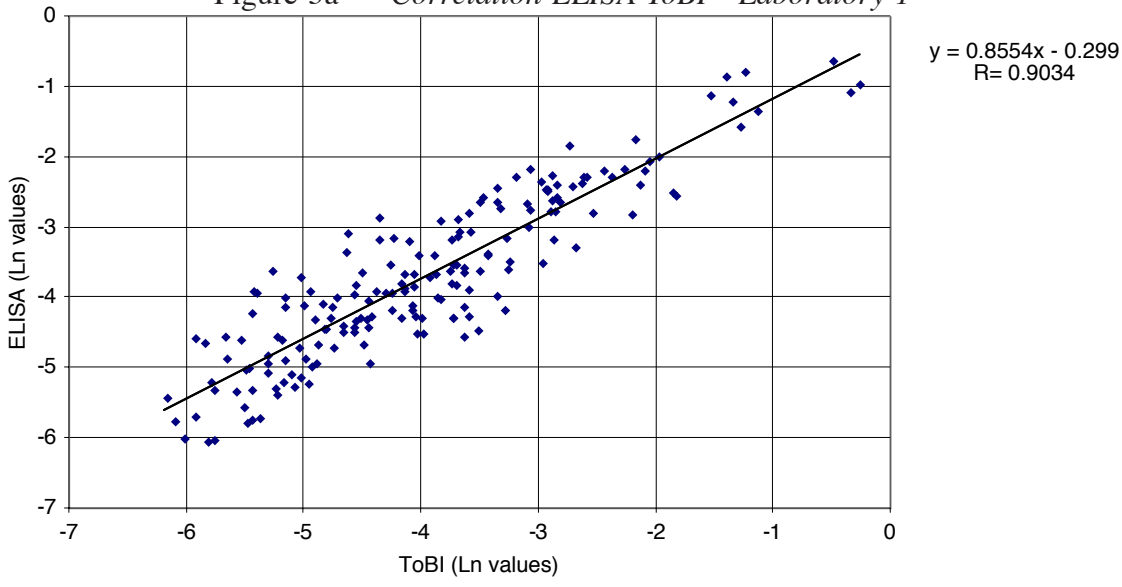


Figure 3b — *Correlation ELISA-ToBI - Laboratory 2*

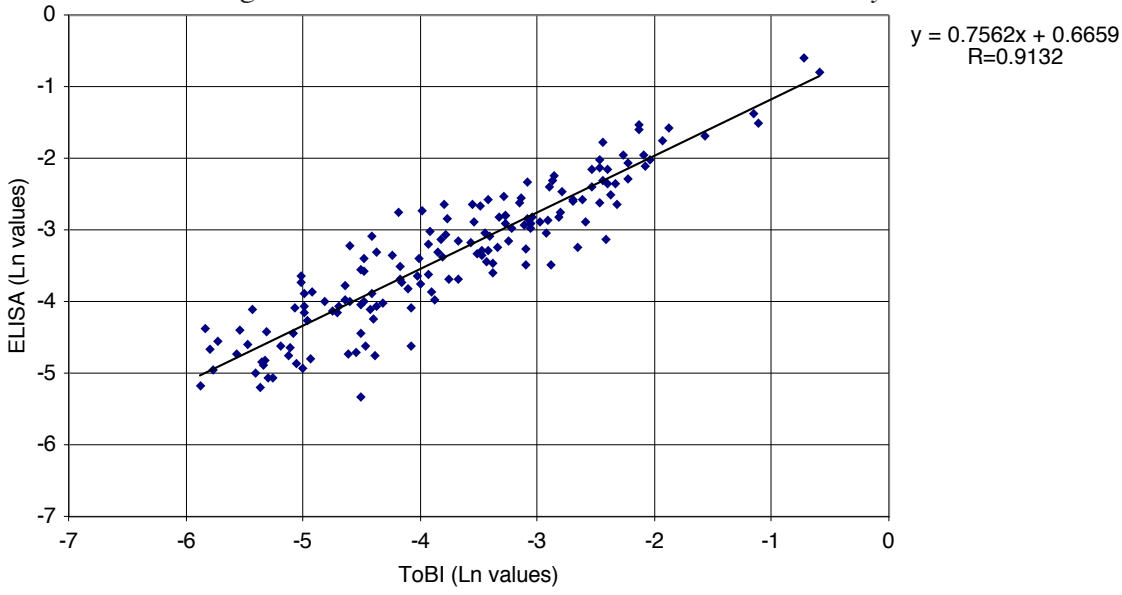


Figure 3c — *Correlation ELISA-ToBI - Laboratory 3*

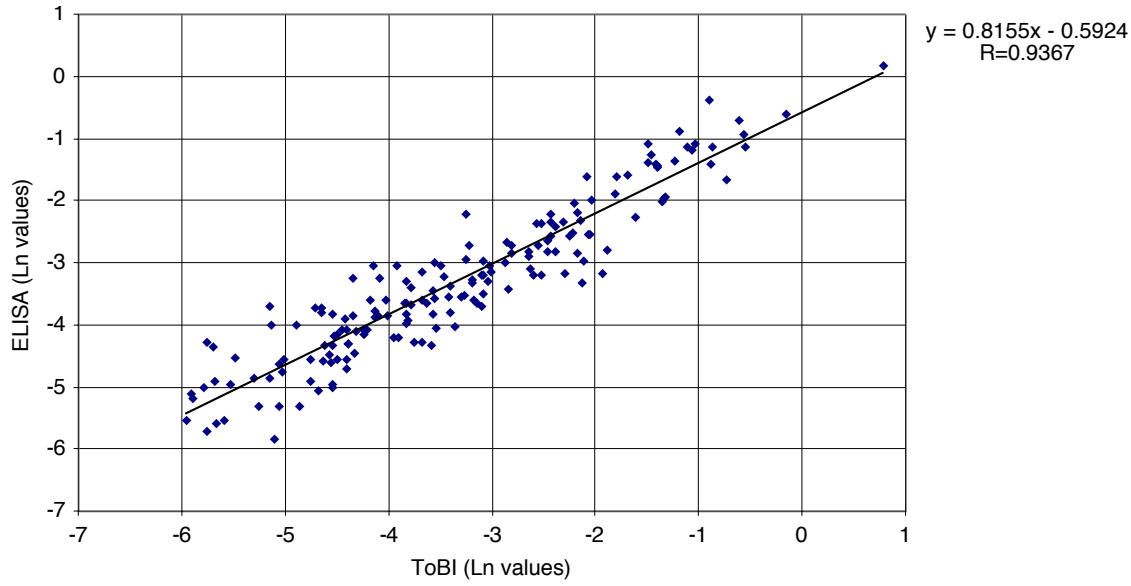


Figure 3d — *Correlation ELISA-ToBI - Laboratory 4*

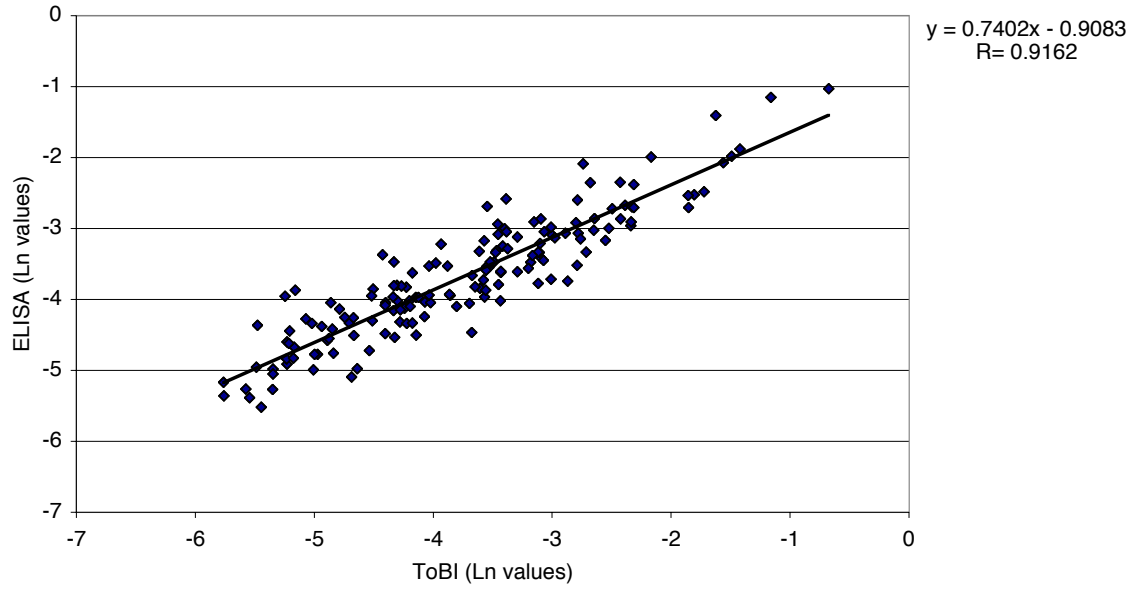


Figure 3e — *Correlation ELISA-ToBI - Laboratory 5*

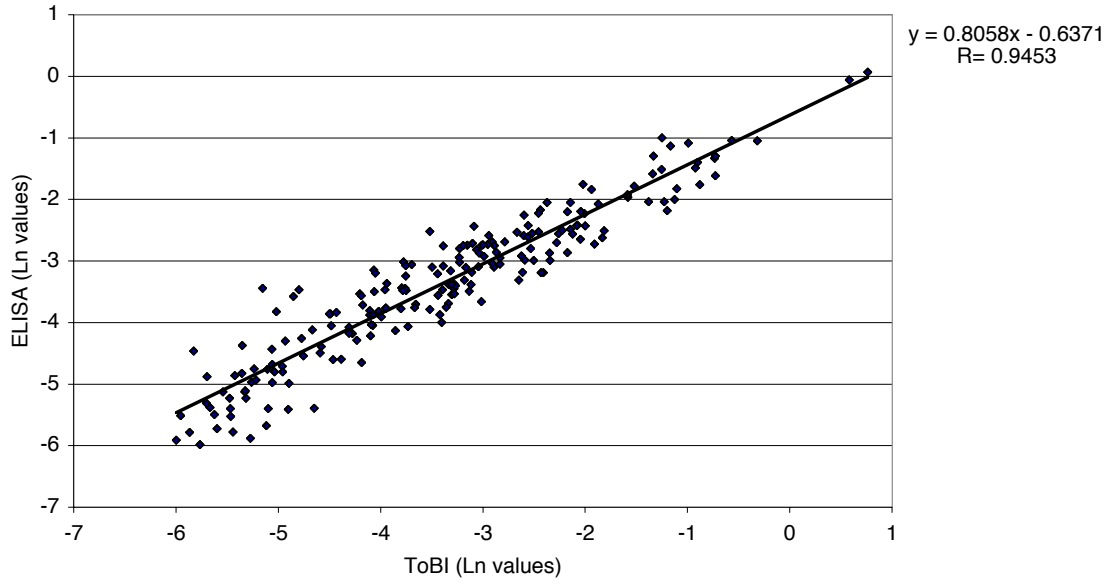


Figure 3f — *Correlation ELISA-ToBI - Laboratory 7*

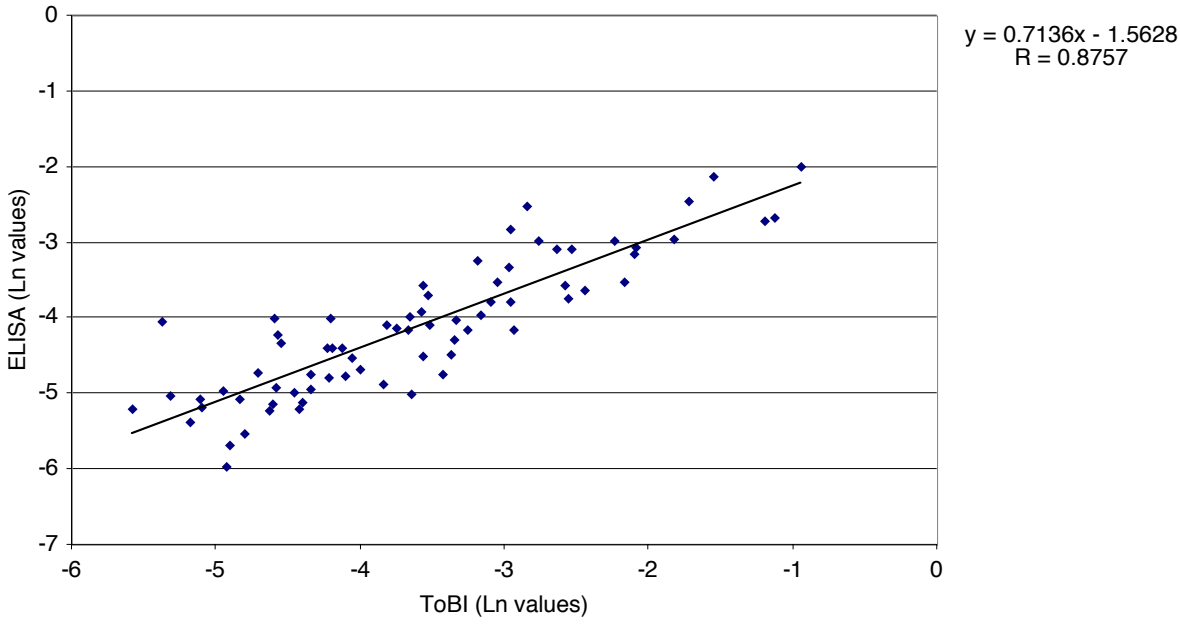
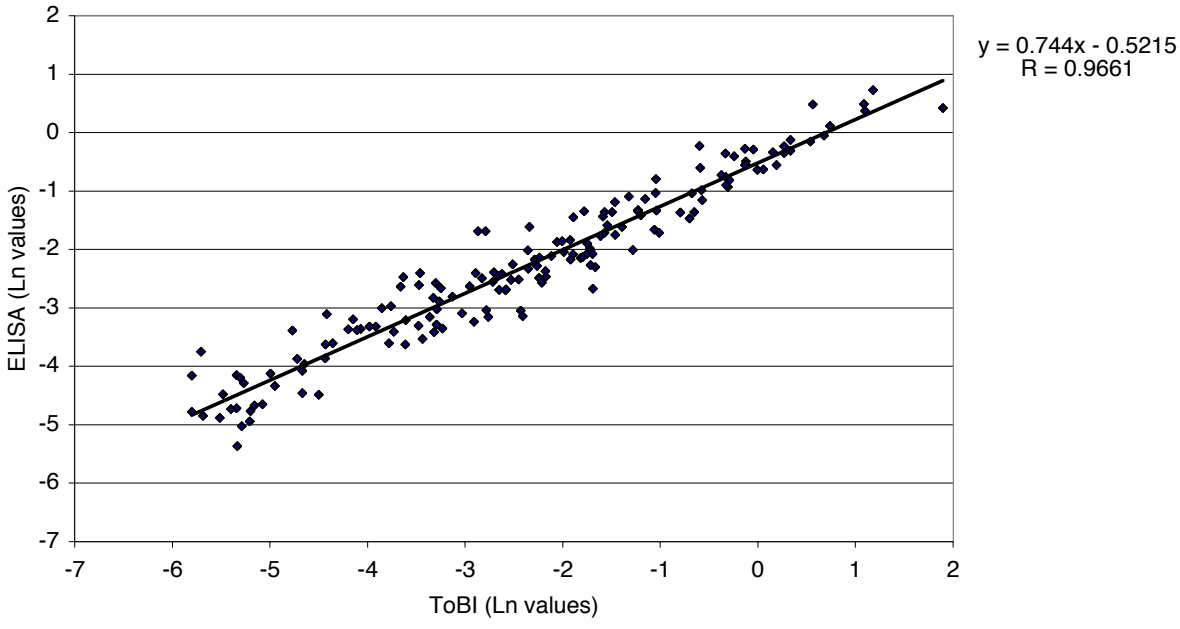


Figure 3g — *Correlation ELISA-ToBI - Laboratory 8*



As the slopes were below 1, it can be concluded that there is no 1:1 relationship over the whole range of titres measured. The absence of the 1:1 relation particularly occurs in the lower antitoxin range (antitoxin titre for ELISA and ToBI smaller than e^{-6}, which is about 0.0025□IU/ml).

Data, specified per vaccine dilution, were analysed by the Sign test to explore trends in differences between ELISA and ToBI results. It should be noted that as ln-transformed antitoxin concentrations were used, only a limited number of data from the lower dilution groups was available. Compared to ELISA, the ToBI tends to give higher responses for high-titre sera in Lab. 1 and Lab. 4 (Table 16). The opposite trend can be observed for the low-titre sera. For Lab.□2 and Lab. 3 higher responses are observed for ELISA, both in the high- and low-titre serum samples.

Table 16 — *Comparison of ToBI - versus ELISA results in the estimation of individual antitoxin titres using the Sign test (lnAVG# ToBI minus lnAVG ELISA)*

	Laboratory 1			Laboratory 2			Laboratory 3			Laboratory 4		
Sign test	Nega-tive	Posi-tive	p-value (<0.05)	Nega-tive	Posi-tive	p-value (<0.05)	Nega-tive	Posi-tive	p-value (<0.05)	Nega-tive	Posi-tive	p-value (<0.05)
Overall*	100	90	0.514	134	29	0	148	65	0	112	79	0.021
Dilution 1**	23	48	0.004	56	16	0	54	35	0.056	28	41	0.149
Dilution 2**	30	30	1	50	7	0	54	22	0	44	17	0.001
Dilution 3**	28	11	0.01	26	5	0	30	7	0	28	10	0.006
Dilution 4**	19	1	0	2	1	1	10	1	0.012	12	11	1

* ln-transformed data of all vaccine dilutions tested.

** ln-transformed data of nth vaccine dilution of all vaccines tested.

AVG: average.

When the 5-parameter fit results were analysed (data not shown), it could be concluded that ToBI tends to give higher values for high-titre sera for Lab. 2 and Lab. 3, but not for Lab. 1 and that ELISA gives higher results for low-titre sera for all laboratories.

5.7. TNT, ELISA AND TOBI RESULTS AND CORRELATION BETWEEN *IN VITRO* ASSAYS AND TNT (PHASE I STUDY ONLY)

Pooled serum sample and serum samples of the 13th guinea pigs were titrated once in TNT and in triplicate in ToBI and ELISA. Results of the *in vitro* tests and the *in vivo* TNT of 13th guinea pig serum samples are presented in Table 17. Antitoxin concentrations of the pooled serum samples obtained by TNT and ELISA and ToBI are shown in Table 18.

TNT titres were in the range of below 0.0020 to 0.703 IU/ml for the 13th guinea pig serum samples and in the range of below 0.0009 to 0.460 IU/ml for the pooled serum samples. It should be noted that results are presented as below values for a number of samples because antitoxin concentrations were below the LOD in TNT. Because of the limited set of data available, no statistical analysis could be performed on the 13th animal TNT results. Nevertheless, TNT generally demonstrates a good reproducibility between the laboratories. The same is true for the average of ToBI and ELISA. The comparison between TNT data of the 13th guinea pigs with average ELISA and ToBI data demonstrates that TNT almost consistently produces antitoxin concentrations which are lower than the average ELISA and ToBI concentrations. Correlations between TNT and *in vitro* tests are very good for the pooled serum samples (Table 19) and although more serum samples exhibit a slight overestimation of antitoxin titres in the *in vitro* tests, the opposite effect can also be observed.

Table 17 — *Tetanus antitoxin concentrations of 13th animal serum samples obtained in ELISA, ToBI and TNT (RIVM calculations)*

				Laboratory 1			Laboratory 2			Laboratory 3		
13 th guinea pig serum Serum nr.		Vaccine	Dose µl	ELISA* AU/ml	ToBI* AU/ml	TNT IU/ml	ELISA* AU/ml	ToBI* AU/ml	TNT IU/ml	ELISA* AU/ml	ToBI* AU/ml	TNT IU/ml
451	13	ERTA	15.625	0.0841	0.0534	0.0453	0.0789	0.0456	2)	0.0383	0.0402	0.038
451	26	ERTA	7.813	0.0617	0.0582	0.0218	0.0800	0.0315	<0.0343	0.0451	0.0393	0.019
451	39	ERTA	3.906	0.0000	0.0000	<0.0039	n.t.	n.t.	n.t.	0.0010	0.0000	<0.001
451	65	C	8.032	0.0769	0.0544	0.0236	0.0998	0.0299	0.0477	0.0590	0.0402	0.029
451	78	C	4.016	0.0558	0.0098	0.0108	0.0481	n.t.	<0.0094	0.0240	0.0079	0.0075 1)
451	91	C	2.008	0.0000	0.0000	<0.0020	n.t.	n.t.	n.t.	0.0008	0.0000	n.t.
451	104	C	1.004	0.0000	0.0000	<0.0020	n.t.	n.t.	n.t.	0.0008	0.0000	n.t.
451	117	D	10.309	0.0058	0.0066	0.0059	0.0108	0.0073	0.0045	n.s.a.	n.s.a.	n.s.a.
451	130	D	5.155	0.0141	0.0176	0.0055	0.0129	0.0136	<0.0076	0.0101	0.0151	0.0075 1)
451	143	D	2.577	0.0000	0.0000	<0.0048	n.t.	n.t.	n.t.	0.0003	0.0000	n.t.
451	169	E	11.173	0.0262	0.0305	0.0103	0.0626	0.0187	<0.0162	0.0341	0.0216	0.011
451	182	E	5.587	0.0062	0.0050	0.0047	n.s.a.	n.s.a.	n.s.a.	0.0050	0.0053	0.004 1)
451	195	E	2.793	0.0009	0.0000	n.t.	n.t.	n.t.	n.t.	0.0005	0.0000	n.t.
497	13	ERTA	15.625	0.0266	0.0387	0.0118	0.0412	n.t.	<0.0187	0.0239	0.0314	0.012
497	26	ERTA	7.813	0.0584	0.0275	0.0430	0.0461	0.0190	<0.012	0.0269	0.0203	0.017
497	39	ERTA	3.906	0.0190	0.0046	<0.0074	0.0185	0.0010	<0.0034	n.s.a.	n.s.a.	n.s.a.
497	65	F	4.95	0.0088	0.0088	0.0069	n.t.	n.t.	0.0032	0.0085	0.0098	0.005
497	78	F	2.475	0,0057	0.0063	0.0042	n.s.a.	n.s.a.	n.s.a.	0.0029	0.0044	0.0075 1)
497	91	F	1.238	0.0000	0.0000	n.t.	n.t.	n.t.	n.t.	0.0011	0.0000	n.t.
497	117	H	30.075	0.0572	0.1115	0.0703	0.0992	0.0654	0.0341	0.0527	0.0749	0.034
497	130	H	15.038	0.0199	0.0144	0.0059	n.t.	n.t.	<0.0035	0.0184	0.0101	<0.0063
497	143	H	7.519	0.0000	0.0000	<0.0020	n.t.	n.t.	n.t.	0.0005	0.0000	n.t.

*: average values. 1): estimated value.
n.t. = not tested. 2): cannot be calculated.
n.s.a. = no serum available.

Table 18 — *Tetanus antitoxin concentrations of pooled serum samples obtained in ELISA, ToBI and TNT (RIVM calculations)*

Guinea-pigs serumpools		Laboratory 1			Laboratory 2			Laboratory 3		
Vaccine	Dose µl	TNT IU/ml 1 test	ToBI* AU/ml AVG	ELISA* AU/ml AVG	TNT IU/ml 1 test	ToBI# AU/ml AVG	ELISA# AU/ml AVG	TNT IU/ml 1 test	ToBI AU/ml AVG	ELISA** AU/ml AVG
ERTA	15.625	0.0536	0.0859	0.0742	0.0309	0.0479	0.0620	0.1720	0.1804	0.1075
ERTA	7.813	0.0242	0.0358	0.0359	0.0041	0.0060	0.0054	0.0150	0.0175	0.0188
ERTA	3.906	<0.0059	0.0073	0.0124	<0.0009	0.0000	0.0006	<0.005	0.0022	0.0041
ERTA	1.953	n.t.	0.0000	0.0007	<0.0009	0.0000	0.0000	<0.01	0.0020	0.0031
Vac.C	8.032	0.0278	0.0375	0.0454	0.0672	0.0391	0.0936	0.0860	0.0606	0.0531
Vac.C	4.016	0.0097	0.0151	0.0125	0.1120	n.t.	0.0686	0.0140	0.0165	0.0213
Vac.C	2.008	<0.0039	0.0032	0.0056	0.0090	0.0116	0.0150	0.0070	0.0079	0.0135
Vac.C	1.004	<0.0037	0.0003	0.0027	<0.0014	0.0007	0.0026	<0.003	0.0000	0.0030
Vac.D	10.309	0.0098	0.0088	0.0083	0.0255	0.0369	0.0503	0.0680	0.0622	0.0411
Vac.D	5.155	0.0035	0.0059	0.0041	0.0208	n.t.	0.0131	0.004-0.006	0.0073	0.0069
Vac.D	2.577	<0.0081	0.0000	0.0010	<0.0016	0.0000	0.0028	<0.003	0.0021	0.0028
Vac.D	1.289	<0.0055	0.0000	0.0001	<0.0009	n.t.	0.0000	<0.003	0.0000	0.0011
Vac.E	11.173	0.0147	0.05112	0.0253	0.1328	0.0527	0.0731	0.0350	0.0658	0.0446
Vac.E	5.587	0.0111	0.0170	0.0113	0.0196	0.0321	0.0373	0.0130	0.0333	0.0216
Vac.E	2.793	<0.0020	0.0004	0.0011	>0.004	n.t.	0.0024	0.0040	0.0042	0.0053
Vac.E	1.397	<0.0020	0.0000	0.0007	<0.0009	0.0000	0.0010	<0.003	0.0000	0.0010
Vac.F	4.95	0.0075	0.0132	0.0114	0.0112	0.0330	0.0306	0.1550	0.2388	0.1197
Vac.F	2.475	<0.0029	0.00504	0.0035	<0.0034	n.t.	n.t.	0.0400	0.0545	0.0548
Vac.F	1.238	<0.0020	0.0000	0.0015	<0.0014	0.0044	0.0044	0.0170	0.0189	0.0247
Vac.F	0.619	<0.0020	0.0000	0.0007	<0.0014	0.0000	0.0013	<0.006	0.0026	0.0071
Vac.H	30.075	0.1721	0.2083	0.1731	0.4600	0.1601	0.1873	0.3750	0.5127	0.3741
Vac.H	15.038	0.0275	0.0490	0.0384	0.1160	n.t.	n.t.	0.1800	0.2201	0.1640
Vac.H	7.519	0.033	0.0368	0.0313	>0.0158	n.t.	0.0337	0.0500	0.0684	0.0512
Vac.H	3.759	<0.0020	0.0000	0.0009	<0.0014	0.0000	0.0029	0.0160	0.0067	0.0069
ERTA	15.625	0.0616	0.0481	0.0626						
ERTA	7.813	0.0057	0.0099	0.0063						
ERTA	3.906	<0.0042	0.0064	0.0041						
ERTA	1.953	<0.0020	0.0000	0.0000						

* Average of three tests.
** Average of three tests; cut-off value = 2 times the average of negative sera.
#: Average values.

Table 19 — *Correlation coefficients (Pearson) between TNT and ELISA and ToBI results for the pooled serum samples*

	Correlation coefficient (Pearson)		
Test systems	**Laboratory 1**	**Laboratory 2**	**Laboratory 3**
ELISA/TNT	0.986	0.925	0.977
ToBI/TNT	0.968	0.970	0.985

5.8. INTER-LABORATORY VARIATION FOR ELISA AND TOBI

Results of inter-laboratory variation of ELISA and ToBI in the titration of the 13th guinea pig serum samples are presented in Table 20. RSDs are within the range of 10□% to 50□%, excluding data for samples 39 and 91 (due to 0 values). In addition, c.i. for the mean antitoxin concentrations obtained in the participating laboratories overlap in all cases (data not shown). As intra-laboratory RSDs are also in the same range (data not shown), it might be concluded that the inter-laboratory variation of the *in vitro* tests is acceptable. However, as data were available for only a limited number of serum samples (shortage of serum or responses below the cut-off value), this conclusion should be reconfirmed in the Phase III study.

Table 20 — *Inter-laboratory variation for ELISA and ToBI in the titration of the 13th□guinea pig serum samples*

ELISA	**Laboratory 1**		**Laboratory 2**		**Laboratory 3**			
Serum No.	Mean IU/ml	Std. Error IU/ml	Mean IU/ml	Std. Error IU/ml	Mean IU/ml	Std. Error IU/ml	Average IU/ml	RSD %
13	0.084	0.008	0.079	0.006	0.038	0.004	0.067	37.4
26	0.061	0.008	0.077	0.006	0.045	0.004	0.061	26.5
39	0.000	0.013	0.088	0.011	0.002	0.006	0.030	168.8
65	0.094	0.009	0.100	0.008	0.055	0.004	0.083	29.5
78	0.046	0.008	0.066	0.006	0.024	0.004	0.045	46.4
91	0.000	0.008	0.048	0.006	0.001	0.004	0.016	168.8
130	0.014	0.008	0.013	0.006	0.010	0.004	0.012	16.0
169	0.026	0.008	0.064	0.006	0.034	0.004	0.041	47.8
1013	0.027	0.008	0.041	0.006	0.024	0.004	0.031	29.9
1026	0.061	0.008	0.046	0.006	0.027	0.004	0.045	38.2
1117	0.059	0.008	0.104	0.006	0.053	0.004	0.072	38.6
							overall*	34.5

*without samples 39 and 91

ToBI	**Laboratory 1**		**Laboratory 2**		**Laboratory 3**			
Serum No.	Mean IU/ml	Std. Error IU/ml	Mean IU/ml	Std. Error IU/ml	Mean IU/ml	Std. Error IU/ml	Average IU/ml	RSD %
13	0.05336	0.003	0.0456	0.002	0.04021	0.003	0.046	14.2
26	0.05813	0.003	0.03152	0.002	0.03925	0.003	0.043	31.9
65	0.0544	0.003	0.02987	0.002	0.04021	0.003	0.041	29.7
130	0.01592	0.004	0.0136	0.003	0.01628	0.003	0.015	9.5
169	0.03045	0.003	0.01867	0.002	0.0216	0.003	0.024	26.0
1026	0.02752	0.003	0.01899	0.002	0.02032	0.003	0.022	20.6
1117	0.1115	0.003	0.06165	0.002	0.07485	0.003	0.083	31.2
							overall	23.3

6. DISCUSSION

In order to refine the Ph.□Eurpotency test for vaccines containing tetanus toxoid for the sake of animal welfare, and to reduce the number of animals used, the EDQM, in collaboration with the ECVAM/IHCP/JRC, commissioned a collaborative study as part of the Biological Standardisation Programme, on the evaluation of alternative assay methods for batch consistency testing.

In laboratories obtaining valid results, vaccine potencies estimated by the challenge test were in agreement with potencies estimated by the *in vitro* serological tests, also for a borderline vaccine. The 95□% c.i. of potencies estimated by ELISAand ToBI testing were slightly smaller than those estimated by challenge test. A similar magnitude of the c.i. ranges in per cent was to be expected since potencies were calculated by probit analysis after dichotomisation of the antitoxin data. However, similar ranges were observed also by parallel line assay, calculated due to non-optimal antitoxin concentrations in relation to the dose response curve. The tetanus toxoid found to have borderline potency in the Ph.□Eurdirect challenge test, in mice and guinea pigs, was identified as a borderline product also by ELISA and ToBI.

Potencies obtained sometimes differed substantially between the laboratories, both in the challenge test and in the *in vitro* serological tests. This might be related to the guinea pig strain, as it was observed in mice (Huet 1981, Hardegree et al. 1972, Lyng and Nyerges 1984), the immunological status and health condition of the animals, or diet (Knight 1996) and environment, which have been reported to have great impact on induction of antibody-response. Laboratories were in close agreement when rank orders of potencies of the test vaccines, estimated by challenge, ELISA and ToBI methods, were compared. For individual serum samples, a good correlation was seen between the predictive value of ELISA antitoxin concentration and survival after challenge test (90.53-94.56□%) and between the predictive value of ToBI antitoxin concentration and survival after challenge (91.84-96.97□%). For the pooled serum samples, a good correlation was seen between antitoxin concentrations obtained by TNT and by ELISA (r = 0.925-0.986) and between antitoxin concentrations obtained by TNT and ToBI (r = 0.968-0.985), as it was previously reported for tetanus vaccines for veterinary use (Hendriksen et al. 1994).

Although no correlation coefficient could be calculated for individual serum samples between TNT and ELISA and between TNT and ToBI, due to the large number of samples with an antitoxin concentration below the LOD in TNT, it appeared that the *in vitro* serological tests tend to overestimate antitoxin concentrations, in particular in the lower antitoxin range (antitoxin titres < 0.3 IU/ml), as it has also been observed by others (Gupta and Siber 1994, Hagenaars et al. 1984, Simonsen et al. 1986). An explanation might be that ELISA and ToBI might detect and quantitate both neutralising and non-neutralising antibodies. However, overestimation was not seen for the pooled serum samples. This phenomenon may be explained by the presence, in the pooled sera, of antibodies bearing different epitope-specificity, in sufficient number to compensate low affinity and enabling efficient masking of the binding and/or the toxic sites of tetanus toxin.

The good correlation between the individual serum samples in the direct challenge test and *in vitro* serological assays may be explained by similar magnitude of the contributions of non-neutralising antibodies to the *in vitro* serological assays and of cellular immunity to the direct challenge test.

The cut-off values for the antitoxin concentration to be protective for the tetanus toxin challenge in 50□% of the guinea pigs (the PC_{50}) and in 99□% of the animals (the PC_{99}) were at about the same level (0.0075 IU/ml and 0.0400 IU/ml) in the laboratories of Phase I and

Phase IIa studies. In all the participating laboratories, the PC_{50} value was about in the same range as the lowest antitoxin concentration (0.01 IU/ml) which may be protective in humans (Galaska 1993).

Information on intra-laboratory variation of the *in vitro* serological tests was based on the assessment of test repeatability (RSD of antitoxin concentrations) and on assessment of the distribution of intra-laboratory precision (relative width of c.i. from individual triplicate assays). In general, RSD and precision were within 20-50 % and are considered to be acceptable.

Information on inter-laboratory variation of the *in vitro* serological tests was based on the assessment of RSD. As intra-laboratory and inter-laboratory RSDs are in the same range, inter-laboratory variation is considered to be acceptable. However, the volume of data available on inter-laboratory variation is too limited for a final conclusion.

For all types of tetanus vaccines investigated, a good agreement was demonstrated between potencies estimated by challenge and serology in guinea pigs. It is thus concluded that both ELISA and ToBI should provide the same information as challenge when used for batch consistency control of tetanus vaccines. Data on intra-laboratory precision and inter-laboratory variation suggest that ELISA is more robust and superior to ToBI. Additional data will be required for final conclusion on robustness and inter-laboratory variation. Therefore both ELISA and ToBI testing of a panel of test sera in a large number of laboratories, using standardised procedures, protocols, materials and reagents will be performed, in parallel with ELISA and ToBI testing with in-house materials, reagents and protocols. This part of the collaborative study, referred to as "Phase III" will take place in the first part of year 2000.

Finally, it should be emphasised that *in vitro* serological assays are important to guarantee batch consistency. However, they cannot be used to replace the animal challenge assays in mice or guinea pigs as "golden" standards for the licensing of new vaccines or for confirmation of potency after significant modification of manufacturing processes.

7. CONCLUSION

According to the Ph. Eur monograph *Tetanus vaccine (adsorbed) (0452)*, assessment of potency is based on a direct challenge test in guinea pigs or mice, with the end-point paralysis or death. The test requires a large number of animals and causes severe distress. The aim of the present study was to refine the test, and reduce the number of animals needed, for batch release purposes. Serological assays having the potential of being internationally accepted, have been compared with Ph. Eur assays. The study included 7 tetanus vaccines of various combinations, produced by different manufacturers, calibrated against the Ph. Eur BRP for Tetanus vaccine (adsorbed).

Results from individual measurements on animals indicated a good correlation between ELISA and the direct challenge test (predictive value = 92 %, range 91-95 %, for six participating laboratories), as well as between ToBI and the direct challenge test (predictive value = 94 %, range 92-97 %, for 6 participating laboratories) and between ELISA and ToBI ($r = 0.92$, range 0.88-0.97 for 7 participating laboratories).

The slope of line of agreement between ELISA and ToBI results differed from 1 for all laboratories indicating that there is no 1 to 1 relationship over the whole range of titres measured. This was particularly noticeable for titres below 0.0025 IU/ml. Antitoxin concentrations determined by ELISA and ToBI were generally in the same range. An overall

excellent correlation was seen for serum pools of the guinea pigs injected with equal vaccine doses, between TNT and ELISA (r = 0.96, range 0.925-0.986 for 3 laboratories) as well as between TNT and ToBI (r = 0.97, range 0.968-0.985 for 3□laboratories).

The good correlation observed between ToBI/ELISA and the challenge test results justifies the extension of this project to Phase III, in which intra- and inter-laboratory variation of the *in vitro* serological assays will be studied in more than 20 laboratories. In the future, it should also be investigated whether tetanus and diphtheria components of combined vaccines could be assayed using the same test sera.

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Collaborative Study for the Validation of Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use Part 2

Collaborative Study for the Validation of Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use – Part 2

Project leaders: Randi Winsnes[1], Coenraad Hendriksen[2]
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1. INTRODUCTION

A collaborative study, consisting of one prevalidation and three study phases, was initiated by the European Directorate for the Quality of Medicines (EDQM)[4] to assess the relevance and reliability of the *in vitro* serological assays Enzyme-Linked Immunosorbent Assay (ELISA) and Toxin Binding Inhibition test (ToBI) for replacing the direct challenge assay in animals [European Pharmacopoeia (Ph. Eur.) Chapter 2.7.8. *Assay of tetanus vaccine (adsorbed)*]. The serological assays are intended both for consistency of production control (multi-dilution assay) and routine batch release control (single-dilution assay).

Results of phase I-II of this collaborative study were published in Pharmeuropa (BIO 2000-1, August 2000, pp. 85-124 and Special Issue October 2000, pp. 29-61) and are also included in this issue (pp.□3-44). For background information, see the summary of the 3 study phases, published in this issue (pp. 73-78).

2. MAIN CONCLUSIONS OF THE PREVIOUS PHASES

The prevalidation study showed that prolongation of the time interval between immunisation and bleeding from four to six weeks improved the correlation between the toxin neutralisation test in mice (TNT) and ELISA and ToBI. From the results of the Phase I and II studies, it was concluded that both ELISA and ToBI may be acceptable methods to replace the challenge procedure. For all types of products tested (including a borderline product) a good agreement was demonstrated between the direct challenge results and the potencies as estimated by ELISA and ToBI. Furthermore, a good prediction of survival of individual animals after tetanus toxin challenge could be established based on antitoxin concentrations obtained in ELISA and ToBI. Intra-laboratory variations of both ELISA and ToBI are acceptable, but more extensive examination of intra- and inter-laboratory variation were needed to confirm the acceptability of the methods for routine use.

3. PHASE III STUDY

3.1. Objectives

In the Phase III study a panel of serum samples, covering a wide range of antitoxin titres, were titrated in ELISA and ToBI in 23 laboratories with the following objectives:

- to transfer ELISA and ToBI technology for the titration of tetanus antitoxin.
- to evaluate intra- and inter-laboratory variation of ELISA and ToBI titration. Essential materials and reagents were provided.
- to evaluate the robustness of ELISA and ToBI test by using in-house materials and reagents.

3.2. Participants

Twenty-five laboratories, all familiar and experienced in the field of vaccine potency testing, were formally invited by Division IV (Biological Standardisation Programme) of the EDQM to participate

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[4]Abbreviations: **ABTS**: 2,2 Azino-di-ethylbenzthiazoline sulphonate, **AP**: Acellular pertussis, **BRP**: European Pharmacopoeia Biological Reference Preparation, **c**: Candidate, **c.i.**: Confidence interval, **c.l.**: Confidence limit, **Cl.**: Clostridium, **D**: Diphtheria, **EDQM**: European Directorate for the Quality of Medicines, **ELISA**: Enzyme-linked immunosorbent assay, **GSK Bio** : Glaxo Smithkline Biologicals, **IS**: International standard, **IU**: International unit, **Lab**: Laboratory, $\mathbf{LD_{50}}$: The statistically determined quantity of toxin that, when administered by the specified route, may be expected to cause the death of 50 per cent of the test animals within a given period, **ln**: Logarithm, **NIBSC**: National Institute for Biological Standards and Control, **OD**: Optical density, **OMCL**: Official Medicines Control Laboratory, **P**: Polio, **PBS**: Phosphate buffered saline, **PBST**: Phosphate buffered saline with Tween, **Ph. Eur.**: European Pharmacopoeia, **PS** : Polystyrene, **RIVM**: Rijksinstituut voor Volksgezondheid en Milieu, **SD**: Standard deviation, **SDS**: Sodium dodecyl sulfate, **SLV**: Statens legemiddelverk, **SOP** : Standardised operating procedures, **T**: Tetanus, **ToBI**: Toxin binding inhibition test, **TMB**: Tetramethylbenzidine, **TNT**:Toxin neutralisation test in mice, **TT**: Tetanus toxin, **WHO**: World Health Organization.

in Phase III of the collaborative study. These laboratories included both Official Medicines Control Laboratories (OMCLs) and manufacturers. Two laboratories had to withdraw at a later stage. Throughout this report, the laboratories are referred to by their code numbers (1 to 23), allocated at random and not necessarily corresponding to the order of appearance on the list of participants.

3.3. Serum samples

A total of 28 serum sample pools were prepared, covering a wide range of tetanus antitoxin titres and produced at different locations, in different strains of guinea pigs and using different vaccines and different vaccine dilutions (Table 1). Some of the serum samples were obtained from the participants of the Phase I and Phase IIB study and included serum samples from animals immunised with the tetanus vaccine (adsorbed) *Ph. Eur.* Biological Reference Preparation (BRP) Batch 1, a T (monovalent tetanus) borderline vaccine, a DTaP (diphtheria-tetanus-acellular pertussis) and a DTP (diphtheria-tetanus-whole cell pertussis) vaccine, respectively. In addition, serum samples were obtained from guinea pigs immunised with in-house T vaccines at two private sector laboratories.

The serum samples were prepared according to the immunisation schedule used in the phase I and phase II study, that is by immunisation of guinea pigs (250-350g) and bleeding at day 40 to 42. Blood was processed according to the standard procedure, and serum samples per vaccine and vaccine

Table 1. Samples specifications

No.	Sample	Vaccine – Origin	Producer
1	A	BRP Batch 1 tetanus vaccine-IIb-pool 1	RIVM
2	B	BRP Batch 1 tetanus vaccine-IIb-pool 2	RIVM
3	C	F-DTP-IIb-pool 5	RIVM
4	D	F-DTP-IIb-pool 6	RIVM
5	E	I-T border-IIb-pool 9	RIVM
6	F	I-T border-IIb-pool 10	RIVM
7	G	K-DTaP-IIb-pool 13	RIVM
8	H	K-DTaP-IIb-pool 14	RIVM
9	I	Neg	RIVM
10	K	Serum pool 1	SLV
11	L	Serum pool 2	SLV
12	M	Serum pool 3	SLV
13	N	Serum pool 4	SLV
14	O	DTP-Impstoff	Chiron Behring
15	P	DTP-HIB Impstoff	Chiron Behring
16	Q	Pentacoq	Aventis Pasteur
17	R	Tetravac	Aventis Pasteur
18	S	DTPa	GSK Bio
19	T	DTPwHB	GSK Bio
20	U	Negative controls	GSK Bio
21	V	BRP Batch 2/3rdWHO IS tetanus vaccine	NIBSC
22	W	BRP Batch 2/3rdWHO IS tetanus vaccine	NIBSC
23	X	BRP Batch 2/3rdWHO IS tetanus vaccine	NIBSC
24	Y	*Cl. tetani* guinea pig antiserum (human) BRP starting material (liquid undiluted)	RIVM
25	Z	Pool 1 (phase IIb)	RIVM
26	α	Pool 2 (phase IIb)	RIVM
27	β	Pool 3 (phase IIb)	RIVM
28	ε	BRP Batch 2/3rdWHO IS tetanus vaccine	NIBSC

dilution were pooled, respectively, to a total volume of about 15 to 20 ml. For the purpose of the inter-laboratory study, serum samples were aliquoted to volumes of 0.25 ml and each participant of the study received 2 coded vials, thus preventing freezing and thawing in duplicate tests.

The *Clostridium (Cl.) tetani* guinea pig antiserum (human) BRP batch 1[5] (freeze-dried) was used as the reference preparation (assigned potency 0.20 IU/vial).

3.4. Design

Each participant was provided with two vials of each of 28 code-labelled serum samples and with 10 vials of the *Cl. tetani* guinea pig antiserum (human) BRP batch 1. Participants were requested to perform two independent assays on separate days; titrating the tetanus antitoxin content of the 28 serum samples provided against the *Cl. tetani* guinea pig antiserum (human) BRP batch 1, using ELISA and ToBI. A testing scheme, shown in Table 2, was recommended. Tests were performed according to standard operating procedures (SOPs) provided by the project leaders (referred to as standardised *Ph. Eur.* ELISA and *Ph. Eur.* ToBI), using standardised and centrally provided materials and reagents. In addition, participants were allowed to perform in parallel to the standardised tests, ELISA and ToBI using their in-house protocol, reagents and materials.

The raw data of both the standardised tests and the in-house tests were forwarded to EDQM, using the provided data recording sheets for elaboration and statistical analysis.

3.5. Statistical analysis

The assay-data were screened for suitability for analysis using some standard checks: Optical densities (OD) exceeding 1000 were divided by 1000; frequently observed ODs that coincided with the maximum observed OD were considered to be limit-values and replaced by "not available"; values that did not represent a real number were replaced by a meaningful entry, e.g. ">4" was replaced by "not available" and non-numbers like "0.0.354" were replaced by the value that was possibly intended, in this case "0.354", etc.

The raw data of the standardised ELISA and ToBI assays were analysed by fitting logistic curves to the data using non linear least squares techniques (PROC NLIN, The SAS System). Four parameters were estimated to characterise the standard curve, and one parameter per sample to characterise the horizontal distance between the curves appearing on the same plate. The goodness of fit was characterised by the correlation coefficient (r^2). In cases where the algorithm failed to converge it was first attempted to force convergence by selecting an optimal convergence path by eye. If this still did not work, and this was clearly due to one sample being on the edge of the space of convergence (e.g. close to 0), this parameter was eliminated, and the procedure repeated with the remaining parameters. If this still did not work, the outcome was set to "no convergence". In no case have individual ODs been excluded, even when of doubtful quality, in order to maintain information on the robustness of the methods with respect to outlying observations. Titres calculated by the participants were only used as a backup to avoid misinterpretation of the raw data, but were not used in further evaluations.

Table 2. Testing scheme

Day	Test	SOPs	Test samples
Day 1	ELISA	Ph. Eur.	Vial 1 of each test serum
Day 1	ELISA	In-house	Vial 1 of each test serum
Day 2	ToBI	Ph. Eur.	Vial 1 of each test serum
Day 2	ToBI	In-house	Vial 1 of each test serum
Day Y	ELISA	Ph. Eur.	Vial 2 of each test serum
Day Y	ELISA	In-house	Vial 2 of each test serum
Day Y + 1	ToBI	Ph. Eur.	Vial 2 of each test serum
Day Y + 1	ToBI	In-house	Vial 2 of each test serum

[5] Catalog No. C2424550

Raw data of in-house ELISA and ToBI assays were not evaluated at the EDQM since the in-house calculations are supposed to be an integral part of the procedure in place at the laboratory. An exception has been made for laboratories 3 and 17 which used an in-house method so similar to the standardised procedures, but without calculations, that the titres were calculated at the EDQM using the same methods as for the standardised assays. Laboratory 4 was not able to provide calculated titres for the in-house assays. Since the raw data could clearly not be treated in the same way as those from the standardised assays, the in-house assays from this laboratory had to be excluded from further evaluations.

4. RESULTS AND DISCUSSION

All 23 laboratories submitted results of the standardised ELISA and 21 of them submitted results of the standardised ToBI. Laboratory 18 did not perform the ToBI because of lack of time. Laboratory 22 tried to run the standardised ToBI, but failed on 2 attempts.

Comments and deviations from the protocol are listed in Tables 3a and 3b. It can be seen that not all laboratories strictly adhered to the protocol: some laboratories performed more than 2 assays, some laboratories provided readings after different time intervals and some laboratories changed various parameters throughout the assays. In one case, the samples were received thawed. In another case there was insufficient material to test all samples twice.

Table 3a. Comments and deviations from protocol (ELISA)

Lab	Comments
1	Performed 3 assays. Adapted predilutions in assays 2 and 3
3	Assay 1: The enzymatic reaction is measured after 30 minutes at 405 nm
	Assay 2: The enzymatic reaction is stopped after 30 minutes by addition of 2M sulfuric acid after which the blue-green colour is measured at 405 nm
	Assay 3: The enzymatic reaction is measured after 15 minutes at 405 nm
	Assay 4: The enzymatic reaction is stopped after 15 minutes by addition of 1% sodium dodecyl sulfate (SDS) after which the blue-green colour is measured at 405 nm
9	Reported readings after 10, 15 and 30 minutes
10	cBRP (GPTA-1) : Reconstituted with 0.5 ml of sterile water for injections. I. Sera dilutions: 40 µl serum + 360 µl diluent = 1:10. Test protocol is followed as per supplied. Plate washing was done with Wash Buffer, for 3, 3, 4 & 4 times respectively. Serum working dilutions were 1:10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120. Composition of diluent: PBST + 2.5% skimmed milk. Readings were taken at 405 nm
12	Blocking reagent modified: 3% BSA has been used instead of skimmed milk. Reading at 405 nm, 12 minutes after addition of ABTS substrate. Absorbance data = OD - mean blank value
13	Reported readings after 10, 15 and 30 minutes
14	Reported readings after 15 and 30 minutes
15	Readings after 30 minutes. Wrong application of substrate on plate 1 in assay 1
16	Performed 3 assays
17	In assay 2 accidentally column 12 has been coated with antigen resulting in extremely high OD's. Sample K not included
18	In general, the Nag background is much higher than some sample/reference dilutions (due to edge-effect?)
19	All –20°C reagents were received thawed, and stored immediately at 4°C. After 5 days storage at 4°C, and following consultation with EDQM, all the serum samples (excluding the lyophilised cBRP) were transferred to -20°C and kept at that temperature until use
21	Readings also reported after 14 and 30 minutes in assay 2
23	Incubation time with ABTS substrate 15 minutes

Table 3b. Comments and deviations from protocol (ToBI)

Lab	Comments
7	Did not use the standard TMB for substrate reaction, but used the substrate in-house TMB-combination
8	Substrate: The reaction was stopped after 10 minutes.
9	Performed 3 assays, but provided for assay 2 only results of plate 3
10	Sera dilutions : Double dilution scheme was used as per protocol, in PS plates. Test protocol is followed as per supplied. Plate washing was done with Wash Buffer, for 4, 3, 4 & 4 times respectively. Substrate incubation was done for 10 min. Readings were taken at 450 nm
12	Absorbance data = OD – mean blank value
14	Assay 3, plates 1 to 4 respectively: Stop after 15 minutes. Coating: overnight at 4°C. Mixture antitoxin + toxin overnight at 4°C Stop after 13 minutes. Coating: overnight at 37°C. Mixture antitoxin + toxin overnight at 4°C Stop after 13 minutes. Coating: overnight at 4°C. Mixture antitoxin + toxin overnight at 4°C Stop after 13 minutes. Coating: overnight at 37°C. Mixture toxin + antitoxin overnight at 4°C
15	Sample U: only 50 µl were available
16	Assay 3, plate 4: Tetanus toxin has been added to column 12 by mistake
17	Assay 1, plate 2 was lost due to a technical error Assay 2: samples K and U were omitted due to insufficient material
19	All –20°C reagents were received thawed, and stored immediately at 4°C. After 5 days storage at 4°C, and following consultation with EDQM, all the serum samples (excluding the lyophilised cBRP) were transferred to –20°C and kept at that temperature until use
23	Assay 2, plate 4: Problem with substrate distribution on position E7

Four laboratories (1, 4, 5 and 17) also submitted results of their in-house ELISA assays, and five laboratories (2, 3, 4, 17 and 22) submitted results of their in-house ToBI assays.

A complete overview of calculated titres per sample and per assay is given in Tables 4a (ELISA) and 4b (ToBI) (see end of text for Tables and Figures). Results where the correlation coefficient was less than 0.980 are printed on a grey background. Considering the fact that many laboratories have used these techniques for the first time, the tables reveal that the number of assays with a correlation coefficient below 0.980 is not excessive and that the reproducibility is in general very satisfactory for both techniques.

Tables 5a and b lists for each laboratory the ranks of the samples within that laboratory. For example, Laboratory 1 found sample M to be the 17th in both ELISA-assays. The plots at the bottom of these tables are helpful to judge if inversions should be considered important. For example, an inversion between sample T and W is more important than an inversion between L and T which are practically equipotent. The samples are also presented in ranked order in Tables 6a and 6b. The ranking within the laboratories is in general fairly reproducible and satisfactory for both assay techniques.

A convenient way to get an impression of the inter-laboratory variation (reproducibility) and the differences between both assay techniques is offered by Figures 1.1 and 1.2. These figures show for each sample histograms in which the black bars represent the ELISA assays, and the dashed bars represent the ToBI assays. The titres are shown on a logarithmic scale (ln). The histograms are based on the mean geometric titre per laboratory (in cases where more than 2 assays were reported by one laboratory, or when titres are calculated after different time intervals, the overall mean of all titres was used). The histograms show that the reproducibility is in general very satisfactory: the difference between any two laboratories is generally less than 2-fold and only rarely more than 3-fold. However, these histograms also show a striking difference between the ToBI and ELISA results depending on the origin of the sample. For example: serum A gives a significantly higher titre in the ToBI assay than in ELISA. The opposite is true for Sample B. Serum E shows no significant differences. Serum Q shows a highly significant difference.

A 3-dimensional representation of the histograms for all sera is shown in Figures 2.1 (ELISA) and 2.2 (ToBI). These figures show the ability of the laboratories to discriminate between different sera, provided the titre is not too close to zero. In general, any pair of laboratories should be able to discriminate between a 2-fold difference.

The differences in outcome between ELISA and ToBI are summarised in Table 7. For each sample the median potencies are listed (median of the geometric means per laboratory). The sign-test was used to determine whether the differences are significant. It can be seen that only 7 samples do not show a significant difference. Samples A, D, G, K, O, V, W, X, α, β and ε gave a significantly higher titre in the ToBI assays than in the ELISA assays, whereas samples B, C, F, H, M, P, Q, R, S and U gave a significantly lower titre in the ToBI assays than in the ELISA assays.

Although there is a 7-fold difference for serum U, this is considered irrelevant since the titre is approximately zero. More important is the almost 2.5-fold difference for serum Q (1.35 IU/ml for ELISA vs. 0.57 IU/ml for ToBI). The importance of this observation is best demonstrated by comparing sample Q (Pentacoq produced by Aventis Pasteur) with sample V [3rd WHO IS/BRP Batch 2 tetanus vaccine (adsorbed)]. Both samples give practically the same titre in the ELISA assay (1.312 and 1.425 IU/ml, respectively) but very different titres in the ToBI assay (0.533 and 2.332 IU/ml respectively).

In order to investigate the relationship between the ToBI and the ELISA results, respectively, of sample A, B, Q and V, to a functional antibody test, TNT was carried out once by one of the participating laboratories. The results, given in Table 8, indicate that ToBI may have overestimated the tetanus antitoxin content of sample A, B and V and underestimated sample Q, whereas ELISA has overestimated sample B and Q and underestimated sample A and V. Inversions do not only occur for antisera obtained from completely different vaccines (Table 8). Sera A and B, for example, were raised in the same strain of animals against the same vaccine, the vaccine preparations injected differing only by their dilution level. As the amount and type of diluent may influence the degree of adsorption of the tetanus toxoid to the aluminium compound, antibodies to partly different epitopes, and of different avidity, may be elicited, which would have an impact on the test results since tetanus toxin (TT) is used in the ToBI while tetanus toxoid is used in the ELISA. Furthermore, different incubation periods are used in ELISA and ToBI. Also the TT dose chosen for the ToBI might play a role as is seen in the TNT.

In general, however, it can be seen that the high serum titre results give a higher response in ToBI than in ELISA, and that the low serum titre results give a higher response in ELISA than in ToBI. The correlation-plots in Figure 3 show that the slopes are less than one.

The correlation coefficient between ELISA and ToBI test was 0.90 which is comparable to the correlation coefficients found in the Phase I and Phase II studies (0.918, 0.913, 0.928, 0.885 and 0.953 in the five participating laboratories, respectively).

Another representation of the inter-laboratory variation is given in Figures 4.1 and 4.2. These figures show for each serum and each method the inter-laboratory standard deviation (SD) (on ln-scale). Reproducibility was established by calculating the standard deviations of the sample estimates. This was done including all results, the results with $r^2 \geq 0.99$ and the results with $r^2 \geq 0.98$, respectively. The reproducibility can be markedly improved when assays with a correlation coefficient below 0.98 are excluded, especially for the ToBI assays. There is no substantial gain in reproducibility if assays with a correlation coefficient below 0.99 and ≥ 0.98 are also excluded. The large SD visualised for samples I and U is expected since they are negative controls.

Based on these results individual laboratory titres are expected to vary within a range of approximately 60 to 160 per cent of the mean titre for ELISA, and between 65 to 150 per cent for ToBI as evident from Table 9a and 9b.

The intra-laboratory SD is on average 0.14 for ELISA and 0.20 for ToBI, and for both methods does usually not exceed 0.50. In practice this means that repeated assays within a laboratory should usually stay within a range of 65 to 150 per cent of the mean titre and only seldom show a difference of more than 2-fold. This means that both methods are almost as reproducible as repeatable, which is noteworthy.

Tables 10a and 10b show the results from the in-house methods. Although not many laboratories carried out an in-house method, it is possible to compare Table 10 with Table 4. Intra-laboratory variation within Laboratory 1 is worse with the in-house method (compare notably samples K, Q and W). Laboratory 17 found a fairly high titre for sample Y. Laboratory 3 had a poor correlation in the ToBI assays. Laboratory 22 found very high titres for samples K and V (4 IU/ml compared to 2.9 and

2.4 IU/ml, respectively, for the standardised method). It would seem that the standardised protocol has improved the reproducibility, but due to the limited number of laboratories having carried out an in-house method, a firm conclusion cannot be drawn.

5. CONCLUSION

This collaborative study was carried out to validate two *in vitro*/serological methods (ELISA and ToBI) for potency testing of tetanus toxoid components of vaccines for human use. This report describes the results of the final phase of this study (Phase III). The objectives of Phase III were to assess intra- and inter-laboratory variations (repeatability and reproducibility, respectively) in ELISA and ToBI and to evaluate protocol transfer. To this end, 28 serum samples, produced at different locations, in different strains of guinea pigs and using different vaccines and different vaccine dilutions, were titrated in duplicate in 23 laboratories. The antitoxin titres of the serum samples covered a range of at least 100-fold as was recommended for validation of serological methods (WHO, 1997). Tests were performed according to SOPs provided by the project leaders, using standardised and centrally provided materials and reagents. In addition, participants were allowed to perform in parallel to the standardised tests, ELISA and ToBI using their in-house procedure. Only the data of the standard ELISA and ToBI were statistically evaluated at one of the participating laboratories.

Intra-laboratory variation was considered to be acceptable for ELISA and ToBI test (on average 0.14 and 0.20, respectively), and generally did not exceed 0.50. The somewhat higher intra-laboratory variation for ToBI test might be due to the fact that most of the participating laboratories did not have previous experience with the ToBI test and to the more complex technical steps.

Inter-laboratory variation was generally very satisfactory, differences between two laboratories were normally less than 2-fold and only rarely more than 3-fold.

From the results of the study it can be concluded that test reliability (repeatability and reproducibility) of both techniques is acceptable.

The results of the few laboratories that performed in-house methods in parallel to the standardised methods might indicate that standardisation of the test protocol is an essential prerequisite for the implementation of serological techniques.

As regards the comparability of ELISA and ToBI potency results for antisera, it could be seen, as in Phases I and II of the study, that the ELISA/ToBI ratio deviates from 1 and that a statistically significant difference in antitoxin titre may be obtained by ELISA and ToBI. Divergence in titres particularly occurred in the low antitoxin range where ELISA titres tended to be higher than ToBI titres. In the high antitoxin range ToBI titres tended to be higher than ELISA titres, although some opposite examples were also noted (e.g. samples A and Q).

Inversions of ELISA and ToBI titres were also seen when using different dilutions of the same vaccine as immunising preparations (e.g. samples A and B). The degree of dilution of adsorbed vaccines, and the composition of the diluent are also known to have an impact on the amount and nature of the anti-toxin antibodies induced in direct challenge assays in animals. Such qualitative and quantitative differences in antisera may result in different specific antibody levels measured in ELISA and ToBI.

However, the differences observed were usually very small between the results of the 2 assays for most antisera tested in this study. Furthermore, in the Phase I and II studies, no differences were seen in estimated vaccine potencies obtained by ELISA and ToBI, although ELISA/ToBI ratios deviated from 1.

The main conclusions arising from phase III are that ELISA and ToBI are both considered as satisfactory and appropriate methods for the monitoring of tetanus anti-toxin levels in guinea pig sera, obtained from multi-dilution vaccine potency assays. As using either indirect ELISA or ToBI in tetanus vaccine potency testing may lead to statistically different titres in some cases, it is recommended to choose only one of these methods for the purpose of batch consistency and routine batch release monitoring. The method must be properly standardised and the variability of the *in vitro* part of the potency test be monitored by the use of a positive and a negative run control.

For the estimation of potency, a vaccine of similar composition, manufactured by the same procedure as the test vaccine and calibrated against the current tetanus vaccine (adsorbed) Ph. Eur. BRP[6] must be included in the assay as the reference preparation and used for the production of positive serum samples. A pool of such positive sera should be calibrated against the *Cl. tetani* guinea pig reference antiserum Ph. Eur. BRP Batch□1 and subsequently used as the positive run control in routine titrations.

Recommendations based on the outcome of the two projects run in the framework of the Biological Standardisation Programme (Phases I and II, i.e. BSP019 and Phase III, i.e. BSP035) are published in this issue (pp. 73-78). The latter publication is summarising the results of all three phases, including simulation studies on the suitability of the single dose assay (Akkermans, 2000; Daas, 2000).

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Table 4a. Titres of the samples (ELISA)

Lab	Rep	Time	Plate 1							Plate 2							Plate 3							Plate 4						
			A	B	C	D	E	F	G	H	I	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	e
1	1	.	0.183	0.090	0.035	0.013	0.033	0.027	0.122	0.018	0.003	2.106	0.400	0.247	0.068	0.150	0.093	1.317	0.046	0.412	0.428	0.012	1.566	0.616	0.262	0.795	0.380	0.086	0.278	0.150
	2	.	0.189	0.081	0.038	0.015	0.041	0.030	0.119	0.019	0.002	2.316	0.451	0.297	0.076	0.154	0.081	1.396	0.036	0.352	0.414	0.008	1.519	0.736	0.391	0.991	0.442	0.114	0.328	0.193
	3	.	0.179	0.077	0.035	0.013	0.034	0.029	0.118	0.021	0.002	2.955	0.447	0.288	0.070	0.144	0.090	1.415	0.038	0.385	0.429	0.008	1.614	0.681	0.341	0.984	0.440	0.106	0.319	0.180
2	1	.	0.223	0.094	0.040	0.015	0.042	0.029	0.120	0.019	0.002	2.351	0.436	0.293	0.068	0.118	0.136	2.028	0.065	0.671	0.866	0.013	1.838	0.700	0.364	0.932	0.457	0.122	0.334	0.184
	2	.	0.188	0.086	0.036	0.013	0.033	0.028	0.117	0.013	0.001	2.111	0.374	0.183	0.058	0.106	0.082	1.300	0.046	0.396	0.474	0.012	1.406	0.696	0.305	0.928	0.404	0.087	0.244	0.137
3	1	30	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	0.038	0.039	2.073	0.427	0.327	0.112	0.222	0.127	1.381	0.159	0.356	0.522	0.081	1.341	0.660	0.397	0.612	0.475	0.189	0.399	0.295
	2	30	0.148	0.089	0.053	0.044	0.061	0.051	0.111	0.035	0.038	1.775	0.341	0.272	0.089	0.178	0.127	1.313	0.150	0.332	0.483	0.076	1.167	0.687	0.407	0.655	0.448	0.173	0.387	0.288
	3	15	0.195	0.085	0.049	0.021	0.054	0.043	0.146	0.018	0.002	3.375	0.457	0.294	0.061	0.140	0.071	1.102	0.047	0.316	0.355	0.011	1.369	0.888	0.472	1.349	0.753	0.142	0.531	0.298
	4	15	0.134	0.055	0.029	0.012	0.030	0.025	0.084	0.019	0.003	2.580	0.415	0.330	0.077	0.156	0.084	1.030	0.051	0.336	0.382	0.014	1.422	0.624	0.292	0.778	0.381	0.109	0.305	0.217
4	1	.	0.186	0.066	0.034	0.010	0.036	0.025	0.109	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	0.088	0.969	0.078	0.319	0.362	0.017	1.029	0.429	0.320	0.761	0.334	0.126	0.533	0.673
	2	.	0.400	0.080	0.038	0.027	0.061	0.023	0.188	0.023	0.007	1.730	0.416	0.390	0.098	0.230	0.236	1.782	0.194	0.766	1.392	0.000	10.119	0.624	0.448	0.724	0.450	0.159	0.404	0.393
5	1	.	0.230	0.099	0.044	0.018	0.039	0.036	0.139	0.020	0.004	1.965	0.400	0.249	0.067	0.178	0.076	1.085	0.034	0.292	0.311	0.011	1.557	0.521	0.327	0.783	0.350	0.093	0.281	0.152
	2	.	0.165	0.077	0.034	0.013	0.034	0.026	0.117	0.017	0.002	1.897	0.335	0.198	0.058	0.151	0.071	1.225	0.033	0.331	0.351	0.009	1.416	0.571	0.282	0.735	0.336	0.082	0.286	0.153
6	1	.	0.169	0.081	0.037	0.015	0.034	0.029	0.110	0.025	0.006	2.057	0.449	0.300	0.072	0.160	0.115	1.415	0.067	0.453	0.429	0.015	1.322	0.607	0.312	0.806	0.381	0.102	0.258	0.159
	2	.	0.178	0.077	0.034	0.013	0.030	0.024	0.098	0.023	0.005	1.991	0.386	0.268	0.067	0.130	0.085	1.157	0.047	0.329	0.374	0.010	1.139	0.590	0.266	0.802	0.349	0.093	0.297	0.153
7	1	.	0.173	0.077	0.039	0.013	0.040	0.027	0.120	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	0.143	1.876	0.087	0.405	0.505	n.c.	1.229	0.814	0.495	1.714	0.622	0.162	0.416	0.150
	2	.	0.152	0.063	0.039	0.014	0.033	0.026	0.102	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	1.969	0.131	0.382	0.471	n.c.	1.797	0.907	1.315	0.445	0.545	0.164	0.438	0.248
8	1	.	0.205	0.099	0.035	0.017	0.044	0.065	0.162	0.027	0.010	2.969	0.608	0.712	0.104	0.230	0.068	1.068	0.036	0.309	0.291	0.015	1.429	0.921	0.404	1.355	0.490	0.115	0.390	0.206
	2	.	0.259	0.089	0.047	0.023	0.048	0.038	0.131	0.025	0.002	2.242	0.469	0.318	0.076	0.175	0.091	1.251	0.042	0.397	0.435	0.013	1.921	0.667	0.358	0.923	0.420	0.093	0.271	0.162
9	1	10	0.134	0.067	0.016	0.007	0.024	0.017	0.054	0.029	0.009	2.787	0.626	0.394	0.090	0.184	0.107	2.171	0.044	0.563	0.652	0.023	2.791	0.700	0.339	0.929	0.463	0.127	0.351	0.187
	1	15	0.124	0.083	0.022	0.008	0.027	0.021	0.098	0.031	0.007	4.594	0.743	0.488	0.104	0.200	0.093	2.020	0.037	0.512	0.487	0.018	1.241	0.609	0.315	0.997	0.428	0.100	0.295	0.166
	1	30	0.160	0.095	0.035	0.014	0.044	0.028	0.106	0.026	0.006	2.631	0.519	0.405	0.085	0.187	0.085	1.811	0.032	0.499	0.476	0.017	1.571	0.738	0.286	1.074	0.434	0.112	0.269	0.180
	2	10	0.162	0.065	0.029	0.010	0.028	0.018	0.089								0.077	1.753	0.033	0.370	0.597	0.006	1.037	0.785	0.302	0.962	0.443	0.105	0.341	0.184
	2	15	0.172	0.070	0.030	0.010	0.029	0.021	0.107	0.020	0.002	2.282	0.643	0.368	0.094	0.257	0.090	1.549	0.042	0.405	0.480	0.009	1.543	0.794	0.332	1.125	0.495	0.112	0.351	0.185
	2	30	0.201	0.079	0.034	0.012	0.035	0.025	0.112	0.018	0.002	2.206	0.551	0.392	0.087	0.211	0.078	1.298	0.035	0.353	0.426	0.007	1.245	0.818	0.298	1.060	0.524	0.116	0.384	0.189
10	1	30	0.232	0.105	0.049	0.017	0.049	0.039	0.149	0.019	0.002	2.641	0.431	0.299	0.072	0.159	0.075	1.261	0.033	0.410	0.434	0.013	1.530	0.627	0.358	0.978	0.431	0.106	0.356	0.153
	2	10	0.183	0.077	0.036	0.013	0.035	0.026	0.110	0.023	0.002	3.253	0.687	0.443	0.111	0.247	0.084	1.407	0.042	0.408	0.485	0.016	2.497	0.685	0.370	0.950	0.519	0.141	0.455	0.234
	2	15	0.203	0.097	0.048	0.019	0.050	0.040	0.171	0.024	0.002	2.781	0.637	0.436	0.113	0.239	0.086	1.465	0.043	0.424	0.536	0.018	2.255	0.543	0.297	0.733	0.375	0.104	0.285	0.174
	2	30	0.188	0.079	0.038	0.014	0.037	0.028	0.116	0.022	0.002	2.717	0.541	0.356	0.083	0.169	0.078	1.382	0.038	0.419	0.428	0.015	1.516	0.645	0.326	0.967	0.466	0.117	0.327	0.179
	3	10	0.152	0.068	0.030	0.011	0.031	0.021	0.102	0.015	0.001	1.957	0.460	0.322	0.072	0.163	0.073	1.606	0.038	0.461	0.574	0.014	1.722	0.584	0.300	0.860	0.425	0.103	0.353	0.180
	3	15	0.167	0.078	0.035	0.014	0.037	0.027	0.105	0.016	0.001	1.896	0.410	0.261	0.062	0.132	0.070	1.399	0.035	0.367	0.426	0.011	1.526	0.600	0.309	0.851	0.444	0.115	0.384	0.190
	3	30	0.158	0.073	0.031	0.012	0.034	0.023	0.114	0.015	0.001	2.011	0.404	0.246	0.056	0.125	0.063	1.326	0.032	0.350	0.404	0.011	1.368	0.594	0.300	0.917	0.428	0.096	0.302	0.157
11	1	.	0.165	0.061	0.026	0.010	0.027	0.019	0.073	0.019	0.002	1.901	0.385	0.254	0.075	0.122	0.079	0.962	0.045	0.308	0.320	0.009	1.059	0.702	0.299	0.834	0.397	0.103	0.287	0.179
	2	.	0.216	0.068	0.011	0.013	0.031	0.032	0.080	0.017	0.004	1.689	0.465	0.267	0.069	0.168	0.097	1.414	0.058	0.395	0.399	0.009	1.224	0.777	0.442	0.873	0.339	0.114	0.272	0.132
12	1	12	0.135	0.052	0.031	0.009	0.025	0.025	0.093	0.014	0.012	2.690	0.450	0.300	0.079	0.189	0.071	1.253	0.045	0.291	0.395	0.017	1.673	0.591	0.292	0.880	0.349	0.094	0.341	0.170
	2	12	0.141	0.055	0.015	0.004	0.014	0.010	0.063	0.013	0.019	2.297	0.397	0.169	0.071	0.150	0.060	1.241	0.031	0.287	0.349	0.015	1.645	0.523	0.286	0.816	0.348	0.087	0.254	0.168
13	1	10															0.088	1.813	0.042	0.396	0.505	0.012	1.138	0.912	0.371	0.840	0.379	0.077	0.296	0.136
	1	15															0.086	1.682	0.042	0.397	0.512	0.013	1.195	0.893	0.363	0.827	0.389	0.077	0.305	0.134
	1	30	0.195	0.076	0.038	0.015	0.037	0.026	0.103	0.023	0.007	1.966	0.414	0.247	0.055	0.108	0.088	1.630	0.042	0.416	0.526	0.013	1.243	0.838	0.371	0.821	0.421	0.079	0.346	0.140
	2	10	0.156	0.041	0.032	0.008	0.024	0.021	0.069	0.018	0.002	2.723	0.347	0.222	0.054	0.084	0.084	1.420	0.037	0.292	0.310	0.000	1.241	0.727	0.363	1.047	0.377	0.102	0.256	0.163
	2	15	0.166	0.048	0.035	0.008	0.026	0.022	0.074	0.018	0.001	2.700	0.350	0.238	0.052	0.092	0.087	1.575	0.034	0.339	0.345	0.000	1.242	0.837	0.428	1.290	0.526	0.131	0.326	0.187
	2	30	0.183	0.051	0.034	0.009	0.026	0.023	0.094	0.018	0.002	2.967	0.393	0.259	0.059	0.107	0.096	2.143	0.037	0.403	0.368	0.000	1.382	0.677	0.283	1.047	0.484	0.118	0.341	0.185
14	1	15	0.327	0.120	0.057	0.026	0.055	0.039	0.170	0.029	0.000	2.878	0.514	0.359	0.089	0.201	0.101	3.043	0.051	0.483	0.687	0.008	2.435	0.838	0.384	0.912	0.379	0.079	0.305	0.195
	1	30	0.162	0.067	0.033	0.012	0.030	0.021	0.085	0.019	0.001	2.659	0.443	0.266	0.061	0.123	0.069	1.796	0.036	0.395	0.428	0.007	1.819	0.732	0.342	1.102	0.429	0.092	0.280	0.147
	2	15	0.180	0.059	0.030	0.010	0.028	0.021	0.091	0.017	0.001	3.142	0.488	0.281	0.071	0.156	0.062	1.948	0.030	0.394	0.466	0.006	1.659	0.729	0.318	1.080	0.420	0.094	0.297	0.172
	2	30	0.174	0.058	0.030	0.012	0.028	0.023	0.093	0.017	0.000	2.898	0.473	0.275	0.065	0.138	0.061	1.670	0.030	0.395	0.418	0.007	1.545	0.703	0.311	1.016	0.415	0.085	0.303	0.167
15	1	30	0.194	0.079	0.040	0.010	0.031	0.019	0.115	0.018	0.001	2.461	0.417	0.288	0.054	0.151	0.078	1.514	0.062	0.684	0.737	0.009	1.400	0.691	0.311	1.107	0.389	0.091	0.284	0.183
	2	30	0.184	0.075	0.027	0.009	0.031	0.021	0.128	0.014	0.001	2.802	0.468	0.294	0.061	0.173	0.060	0.981	0.030	0.352	0.326	0.006	1.450	0.503	0.251	0.933	0.361	0.078	0.306	0.179
16	1	.	0.197	0.086	0.040	0.014	0.044	0.028	0.132	0.023	0.002	2.356	0.493	0.317	0.087	0.186	0.091	1.384	0.045	0.344	0.436	0.011	1.397	0.677	0.349	0.942	0.403	0.106	0.315	0.179
	2	.	0.207	0.094	0.045	0.016	0.042	0.034	0.129	0.022	0.002	2.618	0.515	0.322	0.107	0.193	0.101	1.311	0.057	0.399	0.475	0.015	1.458	0.823	0.416	1.190	0.477	0.133	0.393	0.220
	3	.	0.204	0.101	0.053	0.018	0.049	0.031	0.130	0.026	0.004	2.251	0.498	0.319	0.073	0.128	0.110	1.243	0.070	0.330	0.461	0.015	1.353	0.697	0.343	0.926	0.375	0.108	0.300	0.186
17	1	.	0.222	0.116	0.049	0.022	0.048	0.040	0.147	0.027	0.006	2.811	0.505	0.405	0.108	0.236	0.103	2.143	0.086	0.668	0.908	0.019	3.489	0.707	0.341	0.953	0.404	0.120	0.368	0.213
	2	.	0.251	0.092	0.044	0.017	0.042	0.033	0.134	0.021	0.004		0.403	0.302	0.071	0.158	0.099	0.916	0.053	0.452	0.557	0.013	1.788	0.685	0.343	1.018	0.432	0.115	0.336	0.218
18	1	.	0.171	0.073	0.034	0.043	0.021	0.037	0.165	0.021	0.008	2.441	0.396	0.301	0.100	0.116	0.017	0.918	0.037	0.327	0.406	0.014	0.762	0.625	0.282	1.013	0.437	0.149	0.368	0.204
	2	.	0.180	0.081	0.039	0.016	0.046	0.034	0.130	0.020	0.003	2.567	0.494	0.233	0.091	0.143	0.099	1.904	0.049	0.442	0.513	0.010	1.855	0.631	0.341	0.737	0.222	0.106	0.372	0.179
19	1	.	0.173	0.072	0.030	0.010	0.028	0.021	0.092	0.017	0.002	2.330	0.433	0.271	0.070	0.142	0.096	1.837	0.044	0.447	0.535	0.011	1.858	0.804	0.388	1.059	0.465	0.111	0.353	0.177
	2	.	0.213	0.086	0.041	0.014	0.037	0.024	0.119	0.020	0.003	2.641	0.495	0.348	0.085	0.194	0.094	1.681	0.045	0.447	0.504	0.013	1.854	0.700	0.358	1.015	0.386	0.102	0.313	0.178
20	1	.	0.213	0.111	0.065	0.032	0.061	0.048	0.137	0.074	0.079	3.346	0.568	0.411	0.148	0.261	0.143	1.997	0.111	0.413	0.548	0.061	1.771	0.709	0.381	0.882	0.443	0.120	0.321	0.194
	2	.	0.217	0.096	0.067	0.037	0.065	0.053	0.145	0.036	0.026	3.052	0.502	0.315	0.087	0.193	0.119	1.804	0.091	0.390	0.513	0.044	1.808	0.692	0.313	0.960	0.380	0.086	0.258	0.158
21	1	.	0.209	0.104	0.055	0.025	0.050	0.039	0.118	0.045	0.005	1.805	0.427	0.247	0.088	0.200	0.115	2.554	0.080	0.476	0.621	0.013	2.377	0.946	0.406	1.150	0.528	0.123	0.343	0.285
	2	14	0.179	0.078	0.037	0.015	0.039	0.031	0.141	0.017	0.002	2.651	0.559	0.416	0.088	0.228	0.560	1.549	0.042	0.410	0.473	0.008	2.757	0.071	0.311	0.954	0.401	0.121	0.508	0.265
	2	30	0.170	0.082	0.038	0.016	0.038	0.029	0.127	0.019	0.002	2.172	0.430	0.343	0.069	0.186	0.691	1.698	0.046	0.372	0.427	0.010	2.110	0.070	0.345	0.816	0.383	0.096	0.366	0.199
22	1	.	0.191	0.084	0.060	0.034	0.062	0.079	0.215	0.022	0.006	1.612	0.470	0.233	0.094	0.236	0.130	0.790	0.050	0.290	0.250	0.015	0.771	0.634	0.267	0.963	0.401	0.099	0.360	0.297
	2	.	0.150	0.076	0.058	0.032	0.062	0.047	0.178	0.028	0.004	1.905	0.431	0.283	0.081	0.222	0.054	0.781	0.035	0.220	0.277	0.014	1.093	0.482	0.209	0.364	0.253	0.100	0.204	0.169
23	1	15	0.170	0.070	0.037	0.013	0.035	0.026	0.103	0.016	0.002	2.051	0.404	0.251	0.071	0.141	0.073	1.469	0.036	0.356	0.428	0.007	1.331	0.663	0.317	0.842	0.374	0.094	0.276	0.164
	2	15	0.166	0.071	0.036	0.014	0.033	0.027	0.085	0.022	0.007	1.865	0.380	0.246	0.069	0.131	0.078	1.239	0.039	0.324	0.361	0.007	1.138	0.748	0.378	1.000	0.447	0.113	0.292	0.171

Titres are expressed in IU/ml
n.c. = no convergence. (The calculation method failed to converge)
Times are only indicated if this was stated explicitly on the reporting sheets.
If a sample was not tested, the cell is crossed out.
Results from plates with a correlation coefficient below 0.98 are printed on a grey background.

Table 4b. Titres of the samples (ToBI)

Lab	Rep	Time	Plate 1							Plate 2							Plate 3							Plate 4						
			A	B	C	D	E	F	G	H	I	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	e
1	1	.	0.338	0.044	0.017	0.016	0.031	0.019	0.160	0.019	0.003	2.632	0.511	0.251	0.078	0.202	0.056	0.614	0.029	0.346	0.490	0.000	2.086	1.095	0.479	0.831	0.434	0.143	0.519	0.232
	2	.	0.339	0.055	0.024	0.019	0.038	0.026	0.185	0.018	0.002	2.581	0.424	0.224	0.068	0.167	0.052	0.557	0.029	0.323	0.450	0.001	2.049	1.267	0.566	0.908	0.467	0.157	0.517	0.271
2	1	.	0.278	0.043	0.016	0.015	0.033	0.022	0.149	0.017	0.002	2.221	0.413	0.189	0.059	0.179	0.061	0.538	0.030	0.318	0.444	0.001	2.388	0.911	0.425	0.736	0.429	0.150	0.452	0.280
	2	.	0.262	0.038	0.015	0.012	0.026	0.016	0.117	0.014	0.001	2.155	0.384	0.218	0.065	0.135	0.056	0.613	0.037	0.308	0.447	0.001	1.844	1.058	0.520	0.917	0.493	0.220	0.529	0.240
3	1	.	0.338	0.043	0.019	0.016	0.032	0.020	0.163	0.015	0.000	2.928	0.627	0.233	0.066	0.177	0.054	0.564	0.022	0.479	0.537	0.000	1.916	1.493	0.571	0.831	0.565	0.134	0.538	0.215
	2	.	0.282	0.041	0.014	0.017	0.060	0.024	0.165	0.013	0.002	5.567	0.640	0.237	0.070	0.193	0.071	0.987	0.024	0.306	0.398	0.000	4.032	1.757	0.710	0.999	0.478	0.179	1.002	0.408
4	1	.	0.387	0.050	0.017	0.016	0.036	0.020	0.137	0.019	0.008	3.520	0.571	0.218	0.078	0.218	0.096	0.850	0.050	0.456	0.637	0.000	3.741	1.996	0.964	1.670	0.833	0.249	0.801	0.407
	2	.	0.425	0.073	0.032	0.029	0.060	0.047	0.277	0.020	0.000	5.883	1.045	0.331	0.105	0.414	0.101	1.483	0.109	0.577	0.884	0.033	6.228	1.316	0.660	0.954	0.628	0.224	0.715	0.414
5	1	.	0.355	0.064	0.022	0.026	0.042	0.029	0.163	0.020	0.008	3.632	0.585	0.248	0.090	0.223	0.084	0.717	0.038	0.452	0.620	0.000	2.898	2.351	0.668	1.028	0.494	0.189	0.867	0.331
	2	.	0.338	0.058	0.025	0.018	0.042	0.026	0.169	0.020	0.003	3.090	0.621	0.246	0.094	0.248	0.070	0.533	0.035	0.375	0.768	0.001	2.549	1.366	0.639	1.004	0.491	0.203	0.676	0.378
6	1	.	0.354	0.053	0.020	0.017	0.037	0.023	0.209	0.019	0.000	3.329	0.605	0.291	0.091	0.251	0.063	0.760	0.034	0.442	0.634	0.001	3.411	1.602	0.710	1.211	0.578	0.198	0.777	0.328
	2	.	0.294	0.051	0.019	0.014	0.033	0.021	0.166	0.021	0.002	3.461	0.586	0.292	0.082	0.223	0.061	0.657	0.036	0.385	0.512	0.003	2.421	1.428	0.652	1.155	0.584	0.178	0.679	0.314
7	1	.	0.290	0.047	0.019	0.016	0.031	0.021	0.156	0.015	0.002	2.750	0.475	0.222	0.067	0.175	0.054	0.710	0.026	0.351	0.501	0.001	2.655	1.301	0.592	0.981	0.513	0.151	0.607	0.263
	2	.	0.342	0.058	0.013	0.013	0.025	0.014	0.149	0.019	0.002	3.031	0.514	0.283	0.082	0.158	0.064	0.859	0.045	0.562	0.728	0.003	2.234	1.353	1.247	0.645	0.641	0.166	0.540	0.266
8	1	10	0.350	0.060	0.020	0.017	0.035	0.022	0.158	0.016	0.005	2.637	0.494	0.240	0.074	0.204	0.059	0.523	0.028	0.342	0.465	0.004	2.099	0.916	0.459	0.743	0.349	0.129	0.390	0.198
	2	10	0.353	0.058	0.022	0.017	0.037	0.022	0.153	0.021	0.007	2.713	0.556	0.242	0.076	0.206	0.061	0.501	0.033	0.374	0.509	0.005	2.373	1.137	0.568	0.954	0.425	0.157	0.544	0.268
9	1	.	0.327	0.059	0.022	0.017	0.035	0.022	0.168	0.017	0.002	3.080	0.469	0.223	0.070	0.175	0.052	0.546	0.027	0.327	0.419	0.001	2.074	1.049	0.498	0.915	0.473	0.174	0.550	0.295
	2	.															0.081	0.768	0.034	0.386	0.563	0.000	2.375							
	3	.	0.300	0.048	0.018	0.012	0.032	0.020	0.160	0.015	0.001	2.492	0.378	0.190	0.060	0.168	0.047	0.481	0.025	0.269	0.605	0.002	3.657	1.407	0.361	0.486	0.355	0.180	0.558	0.429
10	1	10	0.309	0.048	0.017	0.018	0.036	0.026	0.173	0.013	0.000	2.941	0.502	0.208	0.072	0.209	0.050	0.548	0.029	0.309	0.497	0.001	3.108	1.325	0.529	0.773	0.330	0.112	0.612	0.738
	2	10	0.345	0.051	0.025	0.030	0.035	0.026	0.175	0.016	0.015	2.741	0.566	0.229	0.100	0.206	0.059	0.929	0.031	0.348	0.585	0.001	3.393	1.293	0.670	1.010	0.476	0.171	0.589	0.328
	3	10	0.322	0.048	0.018	0.015	0.029	0.020	0.147	0.014	0.003	3.268	0.607	0.223	0.076	0.199	0.049	0.597	0.028	0.359	0.483	0.001	2.600	1.253	0.592	1.012	0.505	0.158	0.549	0.359
11	1	.	0.138	0.028	0.039	0.020	0.021	0.013	0.090	0.003	0.000	6.619	0.803	0.175	0.054	0.196	0.032	0.251	0.022	0.165	0.260	0.000	0.959	8.494	4.457	8.265	3.571	1.136	3.860	1.875
	2	.	0.338	0.061	0.030	0.025	0.047	0.035	0.151	0.018	0.000	2.817	0.599	0.290	0.087	0.206	0.072	0.535	0.040	0.714	0.433	0.000	1.875	0.973	0.603	1.094	0.586	0.191	0.493	0.294
12	1	.	0.064	0.005	0.002	0.002	0.015	0.003	0.014	0.004	0.000	0.398	0.078	0.046	0.010	0.027	0.014	0.186	0.006	0.335	0.148	0.000	0.511	0.296	0.152	0.193	0.210	0.053	0.102	0.047
	2	.	0.352	0.068	0.031	0.036	0.071	0.039	0.170	0.009	0.000	2.240	0.350	0.158	0.056	0.124	0.049	0.462	0.022	0.235	0.442	0.000	1.774	0.858	0.398	0.521		0.101	0.260	0.168
13	1	.	0.407	0.054	0.021	0.019	0.038	0.025	0.192	0.024	0.006	2.844	0.383	0.167	0.033	0.139	0.056	0.571	0.026	0.347	0.541	0.000	2.940	1.190	0.605	0.934	0.569	0.182	0.567	0.272
	2	.	0.319	0.068	0.020	0.028	0.044	0.026	0.245	0.016	0.001	2.940	0.390	0.136	0.043	0.153	0.078	0.960	0.048	0.439	0.650	0.001	3.167	1.517	0.753	1.178	0.561	0.192	0.641	0.333
14	1	.	0.309	0.047	0.018	0.015	0.032	0.025	0.182	0.017	0.003	3.065	0.478	0.261	0.079	0.231	0.050	0.527	0.026	0.247	0.427	0.002	2.432	1.410	0.691	0.913	0.412	0.186	0.592	0.293
	2	.	0.336	0.047	0.022	0.021	0.040	0.030	0.200	0.017	0.003	3.328	0.490	0.246	0.085	0.219	0.056	0.600	0.038	0.278	0.416	0.005	2.729	1.338	0.590	0.988	0.476	0.170	0.593	0.308
	3	15	0.327	0.057	0.032	0.029	0.046	0.040	0.202	0.020	0.004	2.859	0.567	0.253	0.106	0.226	0.069	0.570	0.046	0.311	0.550	0.006	2.537	1.238	0.651	0.928	0.573	0.199	0.580	0.337
15	1	.	0.380	0.049	0.019	0.019	0.044	0.024	0.199	0.019	0.012	2.842	0.498	0.223	0.069	0.155	0.052	0.687	0.027	0.390	0.538	0.003	3.717	1.638	0.778	1.281	0.660	0.152	0.771	0.458
	2	.	0.309	0.040	0.014	0.012	0.024	0.014	0.120	0.013	0.000	2.441	0.465	0.193	0.050	0.266	0.032	0.411	0.015	0.215	0.246	0.001	2.594	0.970	0.477	0.753	0.349	0.108	0.414	0.407
16	1	.	0.364	0.058	0.027	0.017	0.043	0.028	0.201	0.018	0.003	2.287	0.515	0.243	0.085	0.236	0.061	0.477	0.033	0.331	0.472	0.002	2.283	1.035	0.490	0.602	0.355	0.152	0.503	0.350
	2	.	0.279	0.044	0.018	0.015	0.034	0.021	0.141	0.007	0.001	0.840	0.170	0.085	0.029	0.065	0.072	0.470	0.024	0.306	0.543	0.000	1.537	0.904	0.436	0.558	0.307	0.115	0.301	0.199
	3	.	0.301	0.047	0.018	0.016	0.032	0.020	0.151	0.015	0.000	2.463	0.499	0.216	0.075	0.220	0.059	0.521	0.031	0.334	0.495	0.003	2.028	1.047	0.595	0.802	0.446	0.185	0.533	0.279
17	1	.	0.343	0.060	0.028	0.017	0.034	0.023	0.160								0.067	0.441	0.030	0.401	0.531	0.004	2.167	1.177	0.545	0.939	0.452	0.176	0.523	0.261
	2	.	0.398	0.060	0.018	0.016	0.040	0.029	0.197	0.019	0.008		0.721	0.285	0.085	0.262	0.069	0.388	0.035	0.285	0.436		2.022	1.512	0.734	1.204	0.621	0.209	0.760	0.378
19	1	.	0.286	0.044	0.016	0.014	0.030	0.016	0.126	0.017	0.007	2.901	0.589	0.264	0.076	0.231	0.054	0.729	0.028	0.329	0.547	0.000	2.638	1.009	0.305	0.487	0.253	0.102	0.281	0.164
	2	.	0.249	0.036	0.013	0.010	0.020	0.018	0.120	0.012	0.000	3.314	0.489	0.213	0.072	0.258	0.047	0.562	0.033	0.311	0.407	0.000	2.072	1.253	0.651	1.234	0.509	0.208	0.562	0.284
20	1	.	0.312	0.061	0.024	0.019	0.037	0.030	0.175	0.023	0.006	2.631	0.471	0.219	0.084	0.214	0.064	0.463	0.031	0.303	0.415	0.007	2.107	1.052	0.521	0.912	0.406	0.155	0.576	0.260
	2	.	0.423	0.065	0.022	0.025	0.049	0.034	0.188	0.018	0.005	3.121	0.521	0.261	0.087	0.210	0.058	0.482	0.030	0.261	0.383	0.005	2.279	1.407	0.664	0.837	0.483	0.169	0.676	0.264
21	1	.	0.298	0.048	0.021	0.017	0.035	0.018	0.175	0.015	0.001	2.676	0.507	0.246	0.070	0.218	0.058	0.551	0.025	0.368	0.450	0.000	2.257	1.473	0.652	1.042	0.719	0.160	0.547	0.301
	2	.	0.306	0.051	0.029	0.017	0.046	0.035	0.229	0.016	0.000	3.714	0.601	0.300	0.088	0.302	0.088	0.561	0.038	0.361	0.573	0.006	3.961	1.461	0.966	1.620	0.613	0.167	0.698	0.368
23	1	.	0.328	0.079	0.037	0.019	0.043	0.030	0.208	0.026	0.005	1.816	0.428	0.240	0.069	0.156	0.073	0.608	0.042	0.396	0.403	0.062	1.934	0.824	0.445	0.744	0.377	0.125	0.381	0.221
	2	.	0.286	0.037	0.019	0.020	0.032	0.042	0.136	0.015	0.005	2.786	0.452	0.211	0.071	0.186	0.065	0.498	0.037	0.327	0.471	0.026	2.623	1.192	0.603	0.892	0.545	0.203	0.541	0.352

Titres are expressed in IU/ml
n.c. = no convergence. (The calculation method failed to converge.
Times are only indicated if this was stated explicitly on the reporting sheets.
If a sample was not tested, the cell is crossed out.
Results from plates with a correlation coefficient below 0.98 are printed on a grey background.

Table 5a. Ranks of the samples (ELISA)

Lab	Rep	Time	Plate 1							Plate 2							Plate 3							Plate 4						
			A	B	C	D	E	F	G	H	I	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	e
1	1	.	16	11	7	3	6	5	13	4	1	28	21	17	9	14	12	26	8	22	23	2	27	24	18	25	20	10	19	15
	2	.	15	10	7	3	8	5	13	4	1	28	23	17	9	14	11	26	6	19	21	2	27	24	20	25	22	12	18	16
	3	.	15	10	7	3	6	5	13	4	1	28	23	17	9	14	11	26	8	20	21	2	27	24	19	25	22	12	18	16
2	1	.	16	10	6	3	7	5	12	4	1	28	20	17	9	11	14	27	8	22	24	2	26	23	19	25	21	13	18	15
	2	.	17	11	7	3	6	5	14	4	1	28	20	16	9	13	10	26	8	21	23	2	27	24	19	25	22	12	18	15
3	1	30	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	5	6	28	21	17	10	15	11	27	12	18	23	8	26	25	19	24	22	14	20	16
	2	30	12	9	5	3	6	4	10	1	2	28	19	16	8	15	11	27	13	18	23	7	26	25	21	24	22	14	20	17
	3	15	15	11	7	4	8	5	14	3	1	28	20	16	9	12	10	25	6	18	19	2	27	24	21	26	23	13	22	17
	4	15	14	9	6	2	7	5	11	4	1	28	23	19	10	15	12	26	8	20	22	3	27	24	17	25	21	13	18	16
4	1	.	15	8	6	2	7	5	12	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	11	26	10	17	20	3	27	21	18	25	19	13	23	24
	2	.	17	8	6	5	7	4	11	3	2	26	19	15	9	13	14	27	12	24	25	1	28	22	20	23	21	10	18	16
5	1	.	16	12	8	3	7	6	13	4	1	28	23	17	9	15	10	26	5	19	20	2	27	24	21	25	22	11	18	14
	2	.	16	11	8	3	7	5	13	4	1	28	21	17	9	14	10	26	6	20	23	2	27	24	18	25	22	12	19	15
6	1	.	16	10	7	2	6	5	12	4	1	28	22	18	9	15	13	27	8	23	21	3	26	24	19	25	20	11	17	14
	2	.	16	10	7	3	6	5	13	4	1	28	23	18	9	14	11	27	8	20	22	2	26	24	17	25	21	12	19	15
7	1	.	16	9	6	3	7	5	11	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	12	27	10	18	22	n.c.	25	24	21	26	23	15	19	13
	2	.	13	8	7	3	6	5	11	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	27	12	18	22	n.c.	26	24	25	21	23	15	19	16
8	1	.	14	10	5	3	7	8	13	4	1	28	22	23	11	16	9	25	6	18	17	2	27	24	20	26	21	12	19	15
	2	.	16	10	7	3	8	5	13	4	1	28	23	18	9	15	11	26	6	20	22	2	27	24	19	25	21	12	17	14
9	1	10	14	10	3	1	6	4	9	7	2	27	22	19	11	15	12	26	8	21	23	5	28	24	17	25	20	13	18	16
	1	15	14	9	5	2	6	4	11	7	1	28	24	21	13	16	10	27	8	22	20	3	26	23	18	25	19	12	17	15
	1	30	14	11	7	2	8	5	12	4	1	28	23	19	10	16	9	27	6	22	21	3	26	24	18	25	20	13	17	15
	2	10	15	9	7	3	6	4	12								11	27	8	20	23	2	26	24	18	25	21	13	19	16
	2	15	14	9	7	3	6	5	12	4	1	28	23	19	11	16	10	27	8	20	21	2	26	24	17	25	22	13	18	15
	2	30	15	10	6	3	7	5	12	4	1	28	23	20	11	16	9	27	8	18	21	2	26	24	17	25	22	13	19	14
10	1	30	16	11	7	3	8	6	13	4	1	28	21	17	9	15	10	26	5	20	23	2	27	24	19	25	22	12	18	14
	2	10	14	9	7	2	6	5	11	4	1	28	24	19	12	16	10	26	8	18	21	3	27	23	17	25	22	13	20	15
	2	15	15	10	7	3	8	5	13	4	1	28	24	21	12	16	9	26	6	20	22	2	27	23	18	25	19	11	17	14
	2	30	16	10	7	2	6	5	12	4	1	28	23	19	11	14	9	26	8	20	21	3	27	24	17	25	22	13	18	15
	3	10	14	9	6	2	7	5	12	4	1	28	21	18	10	15	11	26	8	22	23	3	27	24	17	25	20	13	19	16
	3	15	15	11	7	3	8	5	12	4	1	28	21	17	9	14	10	26	6	19	22	2	27	24	18	25	23	13	20	16
	3	30	16	11	6	3	8	5	13	4	1	28	22	17	9	14	10	26	7	20	21	2	27	24	18	25	23	12	19	15
11	1	.	15	9	6	3	7	5	10	4	1	28	22	17	11	14	12	26	8	20	21	2	27	24	19	25	23	13	18	16
	2	.	16	9	3	4	6	7	11	5	1	28	23	17	10	15	12	27	8	20	21	2	26	24	22	25	19	13	18	14
12	1	12	14	9	7	1	5	6	12	3	2	28	23	19	11	16	10	26	8	17	22	4	27	24	18	25	21	13	20	15
	2	12	14	9	6	1	4	2	11	3	7	28	23	17	12	15	10	26	8	20	22	5	27	24	19	25	21	13	18	16
13	1	10															12	27	8	21	23	2	26	25	19	24	20	11	18	14
	1	15															12	27	8	21	23	3	26	25	19	24	20	10	18	14
	1	30	16	10	7	3	6	5	13	4	1	28	20	17	9	14	12	27	8	21	23	2	26	25	19	24	22	11	18	15
	2	10	15	9	7	3	6	5	11	4	2	28	21	17	10	12	13	27	8	19	20	1	26	24	22	25	23	14	18	16
	2	15	15	9	8	3	6	5	11	4	2	28	21	17	10	13	12	27	7	19	20	1	25	24	22	26	23	14	18	16
	2	30	15	9	7	3	6	5	11	4	2	28	21	17	10	13	12	27	8	22	20	1	26	24	18	25	23	14	19	16
14	1	15	17	12	8	3	7	5	13	4	1	27	22	18	10	15	11	28	6	21	23	2	26	24	20	25	19	9	16	14
	1	30	16	10	7	3	6	5	12	4	1	28	23	17	9	14	11	26	8	20	21	2	27	24	19	25	22	13	18	15
	2	15	16	9	7	3	6	5	12	4	1	28	23	17	11	14	10	27	8	20	22	2	26	24	19	25	21	13	18	15
	2	30	16	9	7	3	6	5	13	4	1	28	23	17	11	14	10	27	8	20	22	2	26	24	19	25	21	12	18	15
15	1	30	16	11	7	3	6	5	13	4	1	28	21	18	8	14	10	27	9	22	24	2	26	23	19	25	20	12	17	15
	2	30	16	11	6	3	8	5	13	4	1	28	23	18	10	14	9	26	7	21	20	2	27	24	17	25	22	12	19	15
16	1	.	16	9	6	3	7	5	13	4	1	28	23	18	10	15	11	26	8	19	22	2	27	24	20	25	21	12	17	14
	2	.	15	9	7	3	6	5	12	4	1	28	23	17	11	14	10	26	8	19	21	2	27	24	20	25	22	13	18	16
	3	.	16	10	7	3	6	5	14	4	1	28	23	18	9	13	12	26	8	19	22	2	27	24	20	25	21	11	17	15
17	1	.	15	11	7	3	6	5	13	4	1	27	21	20	10	16	9	26	8	22	24	2	28	23	17	25	19	12	18	14
	2	.	16	10	7	3	6	5	13	4	1		20	17	9	14	11	25	8	22	23	2	27	24	19	26	21	12	18	15
18	1	.	15	10	6	9	4	8	14	5	1	28	21	18	11	12	3	26	7	19	22	2	25	24	17	27	23	13	20	16
	2	.	16	9	6	3	7	5	13	4	1	28	22	18	10	14	11	27	8	21	23	2	26	24	19	25	17	12	20	15
19	1	.	15	10	7	2	6	5	11	4	1	28	20	17	9	14	12	26	8	21	23	3	27	24	19	25	22	13	18	16
	2	.	16	10	7	3	6	5	13	4	1	28	22	18	9	15	11	26	8	21	23	2	27	24	19	25	20	12	17	14
20	1	.	15	9	5	1	4	2	11	6	7	28	23	19	13	16	12	27	8	20	22	3	26	24	18	25	21	10	17	14
	2	.	16	11	7	3	6	5	13	2	1	28	22	19	9	15	12	26	10	21	23	4	27	24	18	25	20	8	17	14
21	1	.	15	10	7	3	6	4	12	5	1	26	20	16	9	14	11	28	8	21	23	2	27	24	19	25	22	13	18	17
	2	14	14	10	6	3	7	5	13	4	1	27	23	20	11	15	24	26	8	19	21	2	28	9	17	25	18	12	22	16
	2	30	14	11	6	3	7	5	13	4	1	28	23	17	9	15	24	26	8	20	22	2	27	10	18	25	21	12	19	16
22	1	.	13	9	6	4	7	8	14	3	1	28	23	15	10	16	12	26	5	19	17	2	25	24	18	27	22	11	21	20
	2	.	13	10	8	4	9	6	15	3	1	28	24	22	11	19	7	26	5	18	21	2	27	25	17	23	20	12	16	14
23	1	15	16	9	8	3	6	5	13	4	1	28	22	17	10	14	11	27	7	20	23	2	26	24	19	25	21	12	18	15
	2	15	15	10	7	3	6	5	12	4	1	28	22	17	9	14	11	27	8	19	20	2	26	24	21	25	23	13	18	16

Listed are the ranknumbers of the samples within each laboratory (see text for details)
n.c. = no convergence. (The calculation method failed to converge)
Times are only indicated if this was stated explicitly on the reporting sheets.
If a sample was not tested, the cell is crossed out.
Ranks from plates with a correlation coefficient below 0.98 are printed on a grey background.

Use this plot to judge if inversions are important

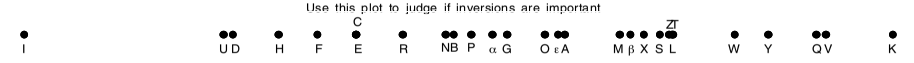


Table 5b. Ranks of the samples (ToBl)

Lab	Rep	Time	Plate 1							Plate 2							Plate 3							Plate 4						
			A	B	C	D	E	F	G	H	I	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	e
1	1	.	17	9	4	3	8	6	13	5	2	28	22	16	11	14	10	24	7	18	21	1	27	26	20	25	19	12	23	15
	2	.	18	10	5	4	8	6	14	3	2	28	19	15	11	13	9	23	7	17	20	1	27	26	24	25	21	12	22	16
2	1	.	16	9	4	3	8	6	12	5	2	27	19	15	10	14	11	24	7	18	22	1	28	26	20	25	21	13	23	17
	2	.	17	9	5	3	7	6	12	4	1	28	19	14	11	13	10	24	8	18	20	2	27	26	22	25	21	15	23	16
3	1	.	17	9	5	4	8	6	13	3	2	28	24	16	11	14	10	21	7	18	19	2	27	26	23	25	22	12	20	15
	2	.	16	8	4	5	9	6	12	3	2	28	21	15	10	14	11	23	7	17	18	1	27	26	22	24	20	13	25	19
4	1	.	16	8	4	3	7	6	12	5	2	27	19	13	10	14	11	23	9	18	20	1	28	26	24	25	22	15	21	17
	2	.	17	8	4	3	7	6	13	2	1	27	24	14	10	16	9	26	11	18	22	5	28	25	20	23	19	12	21	15
5	1	.	17	9	4	5	8	6	12	3	1	28	21	15	11	14	10	25	7	18	19	2	26	27	22	24	20	13	23	16
	2	.	16	9	5	3	8	6	12	4	2	28	21	14	11	15	10	20	7	17	24	1	27	26	22	25	19	13	23	18
6	1	.	17	9	5	3	8	6	13	4	1	27	20	15	11	14	10	23	7	18	21	2	28	26	22	25	19	12	24	16
	2	.	16	9	4	3	7	6	12	5	1	28	21	15	11	14	10	23	8	18	19	2	27	26	22	25	20	13	24	17
7	1	.	17	9	5	4	8	6	13	3	2	28	19	15	11	14	10	24	7	18	20	1	27	26	22	25	21	12	23	16
	2	.	17	9	4	3	7	5	12	6	1	28	18	16	11	13	10	24	8	20	23	2	27	26	25	22	21	14	19	15
8	1	10	19	10	5	4	8	6	13	3	2	28	23	16	11	15	9	24	7	17	22	1	27	26	21	25	18	12	20	14
	2	10	17	9	5	3	8	6	12	4	2	28	23	15	11	14	10	20	7	18	21	1	27	26	24	25	19	13	22	16
9	1	.	17	10	5	3	8	6	12	4	2	28	20	15	11	14	9	23	7	18	19	1	27	26	22	25	21	13	24	16
	2	.															11	25	7	20	23	1	28							
	3	.	17	10	5	3	8	6	12	4	1	27	20	15	11	13	9	22	7	16	25	2	28	26	19	23	18	14	24	21
10	1	10	16	9	4	5	8	6	13	3	1	27	20	14	11	15	10	22	7	17	19	2	28	26	21	25	18	12	23	24
	2	10	17	9	4	6	8	5	13	3	2	27	20	15	11	14	10	24	7	18	21	1	28	26	23	25	19	12	22	16
	3	10	16	9	5	4	8	6	12	3	2	28	24	15	11	14	10	23	7	18	19	1	27	26	22	25	20	13	21	17
11	1	.	13	8	10	5	6	4	12	3	2	26	19	15	11	16	9	17	7	14	18	2	20	28	25	27	23	21	24	22
	2	.	17	9	5	4	8	6	12	3	2	28	22	15	11	14	10	20	7	24	18	2	27	25	23	26	21	13	19	16
12	1	.	17	7	3	4	12	5	11	6	1	27	18	14	9	13	10	22	8	26	20	2	28	25	21	23	24	16	19	15
	2	.	20	10	5	6	11	7	16	3	2	28	19	14	9	13	8	24	4	17	23	2	27	26	21	25		12	18	15
13	1	.	19	10	4	3	9	6	15	5	2	27	18	13	8	12	11	23	7	17	20	1	28	26	24	25	22	14	21	16
	2	.	16	10	4	6	8	5	15	3	2	27	18	12	7	13	11	24	9	19	22	1	28	26	23	25	20	14	21	17
14	1	.	18	9	5	3	8	6	12	4	2	28	21	16	11	14	10	22	7	15	20	1	27	26	24	25	19	13	23	17
	2	.	18	9	5	4	8	6	13	3	1	28	21	15	11	14	10	24	7	16	19	2	27	26	22	25	20	12	23	17
	3	15	17	9	5	4	8	6	13	3	1	28	20	15	11	14	10	21	7	16	19	2	27	26	24	25	22	12	23	18
15	1	.	16	9	4	5	8	6	14	3	2	27	19	15	11	13	10	22	7	17	20	1	28	26	24	25	21	12	23	18
	2	.	18	10	5	3	8	6	13	4	1	27	23	14	11	17	9	21	7	15	16	2	28	26	24	25	19	12	22	20
16	1	.	19	9	5	3	8	6	13	4	2	28	24	15	11	14	10	21	7	16	20	1	27	26	22	25	18	12	23	17
	2	.	18	10	5	4	9	6	15	3	2	26	16	13	8	11	12	23	7	20	24	1	28	27	22	25	21	14	19	17
	3	.	17	9	5	4	8	6	12	3	1	28	21	14	11	15	10	22	7	18	20	2	27	26	24	25	19	13	23	16
17	1	.	17	9	6	3	8	5	13								10	19	7	18	23	2	27	26	24	25	21	14	22	15
	2	.	20	9	4	3	8	6	13	5	1		23	17	11	15	10	19	7	16	21		28	27	24	26	22	14	25	18
19	1	.	19	9	5	3	8	4	13	6	2	28	24	17	11	15	10	25	7	21	23	1	27	26	20	22	16	12	18	14
	2	.	15	9	5	3	7	6	12	4	2	28	20	14	11	16	10	22	8	18	19	2	27	26	24	25	21	13	23	17
20	1	.	18	9	5	3	8	6	13	4	1	28	22	15	11	14	10	21	7	17	20	2	27	26	23	25	19	12	24	16
	2	.	19	10	4	5	8	7	13	3	2	28	22	16	11	14	9	20	6	15	18	1	27	26	23	25	21	12	24	17
21	1	.	16	9	6	4	8	5	13	3	2	28	20	15	11	14	10	22	7	18	19	1	27	26	23	25	24	12	21	17
	2	.	16	9	5	4	8	6	13	3	1	27	21	14	10	15	11	19	7	17	20	2	28	25	24	26	22	12	23	18
23	1	.	17	11	5	2	7	4	14	3	1	27	22	16	9	13	10	24	6	20	21	8	28	26	23	25	18	12	19	15
	2	.	16	8	3	4	6	9	12	2	1	28	19	15	11	13	10	21	7	17	20	5	27	26	24	25	23	14	22	18

Listed are the ranknumbers of the samples within each laboratory (see text for details)
n.c. = no convergence. (The calculation method failed to converge)
Times are only indicated if this was stated explicitly on the reporting sheets.
If a sample was not tested, the cell is crossed out.
Ranks from plates with a correlation coefficient below 0.98 are printed on a grey background.

Table 6a. Ranking of the samples (ELISA)

Lab	Rep	Time	Ranks																										
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	1	.	I	U	D	H	F	E	C	R	N	a	B	P	G	O	e	A	M	X	b	Z	L	S	T	W	Y	Q	V	K
	2	.	I	U	D	H	F	R	C	E	N	B	P	a	G	O	A	e	M	b	S	X	T	Z	L	W	Y	Q	V	K
	3	.	I	U	D	H	F	E	C	R	N	B	P	a	G	O	A	e	M	b	X	S	T	Z	L	W	Y	Q	V	K
2	1	.	I	U	D	H	F	C	E	R	N	B	O	G	a	P	e	A	M	b	X	L	Z	S	W	T	Y	V	Q	K
	2	.	I	U	D	H	F	E	C	R	N	P	B	a	O	G	e	M	A	b	X	L	S	Z	T	W	Y	Q	V	K
3	1	30	.	.	.	.	.	H	I	U	.	N	P	R	.	a	O	e	M	S	X	b	L	Z	T	Y	W	V	Q	K
	2	30	H	I	D	F	C	E	U	N	B	G	P	A	R	a	O	M	e	S	L	b	X	Z	T	Y	W	V	Q	K
	3	15	I	U	H	D	F	R	C	E	N	P	B	O	a	G	A	M	e	S	T	L	X	b	Z	W	Q	Y	V	K
	4	15	I	D	U	H	F	C	E	R	B	N	G	P	a	A	O	e	X	b	M	S	Z	T	L	W	Y	Q	V	K
4	1	.	.	D	U	.	F	C	E	B	.	R	P	G	a	.	A	.	S	X	Z	T	W	.	b	e	Y	Q	V	.
	2	.	U	I	H	F	D	C	E	B	N	a	G	R	O	P	M	e	A	b	L	X	Z	W	Y	S	T	K	Q	V
5	1	.	I	U	D	H	R	F	E	C	N	P	a	B	G	e	O	A	M	b	S	T	X	Z	L	W	Y	Q	V	K
	2	.	I	U	D	H	F	R	E	C	N	P	B	a	G	O	e	A	M	X	b	S	L	Z	T	W	Y	Q	V	K
6	1	.	I	D	U	H	F	E	C	R	N	B	a	G	P	e	O	A	b	M	X	Z	T	L	S	W	Y	V	Q	K
	2	.	I	U	D	H	F	E	C	R	N	B	P	a	G	O	e	A	X	M	b	S	Z	T	L	W	Y	V	Q	K
7	1	.	.	.	D	.	F	C	E	.	B	R	G	P	e	.	a	A	.	S	b	.	X	T	Z	W	V	Y	Q	.
	2	.	.	.	D	.	F	E	C	B	.	.	G	R	A	.	a	e	.	S	b	.	Y	T	Z	W	X	V	Q	.
8	1	.	I	U	D	H	C	R	E	F	P	B	N	a	G	A	e	O	T	S	b	X	Z	L	M	W	Q	Y	V	K
	2	.	I	U	D	H	F	R	C	E	N	B	P	a	G	e	O	A	b	M	X	S	Z	T	L	W	Y	Q	V	K
9	1	10	D	I	C	F	U	E	H	R	G	B	N	P	a	A	O	e	X	b	M	Z	S	L	T	W	Y	Q	K	V
	1	15	I	D	U	F	C	E	H	R	B	P	G	a	N	A	e	O	b	X	Z	T	M	S	W	L	Y	V	Q	K
	1	30	I	D	U	H	F	R	C	E	P	N	B	G	a	A	e	O	b	X	M	Z	T	S	L	W	Y	V	Q	K
	2	10	.	U	D	F	.	E	C	R	B	.	P	G	a	.	A	e	.	X	b	S	Z	.	T	W	Y	V	Q	.
	2	15	I	U	D	H	F	E	C	R	B	P	N	G	a	A	e	O	X	b	M	S	T	Z	L	W	Y	V	Q	K
	2	30	I	U	D	H	F	C	E	R	P	B	N	G	a	e	A	O	X	S	b	M	T	Z	L	W	Y	V	Q	K
10	1	30	I	U	D	H	R	F	C	E	N	P	B	a	G	e	O	A	M	b	X	S	L	Z	T	W	Y	Q	V	K
	2	10	I	D	U	H	F	E	C	R	B	P	G	N	a	A	e	O	X	S	M	b	T	Z	W	L	Y	Q	V	K
	2	15	I	U	D	H	F	R	C	E	P	B	a	N	G	e	A	O	b	X	Z	S	M	T	W	L	Y	Q	V	K
	2	30	I	D	U	H	F	E	C	R	P	B	N	G	a	O	e	A	X	b	M	S	T	Z	L	W	Y	Q	V	K
	3	10	I	D	U	H	F	C	E	R	B	N	P	G	a	A	O	e	X	M	b	Z	L	S	T	W	Y	Q	V	K
	3	15	I	U	D	H	F	R	C	E	N	P	B	G	a	O	A	e	M	X	S	b	L	T	Z	W	Y	Q	V	K
	3	30	I	U	D	H	F	C	R	E	N	P	B	a	G	O	e	A	M	X	b	S	T	L	Z	W	Y	Q	V	K
11	1	.	I	U	D	H	F	C	E	R	B	G	N	P	a	O	A	e	M	b	X	S	T	L	Z	W	Y	Q	V	K
	2	.	I	U	C	D	H	E	F	R	B	N	G	P	a	e	O	A	M	b	Z	S	T	X	L	W	Y	V	Q	K
12	1	12	D	I	H	U	E	F	C	R	B	P	N	G	a	A	e	O	S	X	M	b	Z	T	L	W	Y	Q	V	K
	2	12	D	F	H	E	U	C	I	R	B	P	G	N	a	A	O	e	M	b	X	S	Z	T	L	W	Y	Q	V	K
13	1	10	.	U	.	.	.	.	.	R	.	.	a	P	.	e	.	.	.	.	b	X	Z	S	T	Y	W	V	Q	.
	1	15	.	.	U	.	.	.	.	R	.	a	.	P	.	e	.	.	.	.	b	X	Z	S	T	Y	W	V	Q	.
	1	30	I	U	D	H	F	E	C	R	N	B	a	P	G	O	e	A	M	b	X	L	S	Z	T	Y	W	V	Q	K
	2	10	U	I	D	H	F	E	C	R	B	N	G	O	P	a	A	e	M	b	S	T	L	X	Z	W	Y	V	Q	K
	2	15	U	I	D	H	F	E	R	C	B	N	G	P	O	a	A	e	M	b	S	T	L	X	Z	W	V	Y	Q	K
	2	30	U	I	D	H	F	E	C	R	B	N	G	P	O	a	A	e	M	X	b	T	L	S	Z	W	Y	V	Q	K
14	1	15	I	U	D	H	F	R	E	C	a	N	P	B	G	e	O	b	A	M	Z	X	S	L	T	W	Y	V	K	Q
	1	30	I	U	D	H	F	E	C	R	N	B	P	G	a	O	e	A	M	b	X	S	T	Z	L	W	Y	Q	V	K
	2	15	I	U	D	H	F	E	C	R	B	P	N	G	a	O	e	A	M	b	X	S	Z	T	L	W	Y	V	Q	K
	2	30	I	U	D	H	F	E	C	R	B	P	N	a	G	O	e	A	M	b	X	S	Z	T	L	W	Y	V	Q	K
15	1	30	I	U	D	H	F	E	C	N	R	P	B	a	G	O	e	A	b	M	X	Z	L	S	W	T	Y	V	Q	K
	2	30	I	U	D	H	F	C	R	E	P	N	B	a	G	O	e	A	X	M	b	T	S	Z	L	W	Y	Q	V	K
16	1	.	I	U	D	H	F	C	E	R	B	N	P	a	G	e	O	A	b	M	S	X	Z	T	L	W	Y	Q	V	K
	2	.	I	U	D	H	F	E	C	R	B	P	N	G	a	O	A	e	M	b	S	X	T	Z	L	W	Y	Q	V	K
	3	.	I	U	D	H	F	E	C	R	N	B	a	P	O	G	e	A	b	M	S	X	Z	T	L	W	Y	Q	V	K
17	1	.	I	U	D	H	F	E	C	R	P	N	B	a	G	e	A	O	X	b	Z	M	L	S	W	T	Y	Q	K	V
	2	.	I	U	D	H	F	E	C	R	N	B	P	a	G	O	e	A	M	b	X	L	Z	S	T	W	Q	Y	V	.
18	1	.	I	U	P	E	H	C	R	F	D	B	N	O	a	G	A	e	X	M	S	b	L	T	Z	W	V	Q	Y	K
	2	.	I	U	D	H	F	C	E	R	B	N	P	a	G	O	e	A	Z	M	X	b	S	L	T	W	Y	V	Q	K
19	1	.	I	D	U	H	F	E	C	R	N	B	G	P	a	O	A	e	M	b	X	L	S	Z	T	W	Y	Q	V	K
	2	.	I	U	D	H	F	E	C	R	N	B	P	a	G	e	O	A	b	M	X	Z	S	L	T	W	Y	Q	V	K
20	1	.	D	F	U	E	C	H	I	R	B	a	G	P	N	e	A	O	b	X	M	S	Z	T	L	W	Y	V	Q	K
	2	.	I	H	D	U	F	E	C	a	N	R	B	P	G	e	O	A	b	X	M	Z	S	L	T	W	Y	Q	V	K
21	1	.	I	U	D	F	H	E	C	R	N	B	P	G	a	O	A	M	e	b	X	L	S	Z	T	W	Y	K	V	Q
	2	14	I	U	D	H	F	C	E	R	W	B	N	a	G	A	O	e	X	Z	S	M	T	b	L	P	Y	Q	K	V
	2	30	I	U	D	H	F	C	E	R	N	W	B	a	G	A	O	e	M	X	b	S	Z	T	L	P	Y	Q	V	K
22	1	.	I	U	H	D	R	C	E	F	B	N	a	P	A	G	M	O	T	X	S	e	b	Z	L	W	V	Q	Y	K
	2	.	I	U	H	D	R	F	P	C	E	B	N	a	A	e	G	b	X	S	O	Z	T	M	Y	L	W	Q	V	K
23	1	15	I	U	D	H	F	E	R	C	B	N	P	a	G	O	e	A	M	b	X	S	Z	L	T	W	Y	V	Q	K
	2	15	I	U	D	H	F	E	C	R	N	B	P	G	a	O	A	e	M	b	S	T	X	L	Z	W	Y	V	Q	K

The samples are sorted from left (low titres) to right (high titres)

Times are only indicated if this was stated explicitly on the reporting sheets.

Use this plot to judge if inversions are important

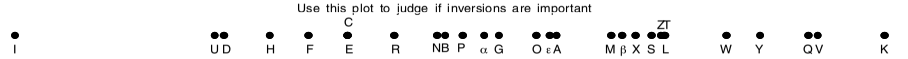


Table 6b. Ranking of the samples (ToBl)

Lab	Rep	Time	Ranks																											
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	1	.	U	I	D	C	H	F	R	E	B	P	N	a	G	O	e	M	A	S	Z	X	T	L	b	Q	Y	W	V	K
	2	.	U	I	H	D	C	F	R	E	P	B	N	a	O	G	M	e	S	A	L	T	Z	b	Q	X	Y	W	V	K
2	1	.	U	I	D	C	H	F	R	E	B	N	P	G	a	O	M	A	e	S	L	X	Z	T	b	Q	Y	W	K	V
	2	.	I	U	D	H	C	F	E	R	B	P	N	G	O	M	a	e	A	S	L	T	Z	X	b	Q	Y	W	V	K
3	1	.	I	U	H	D	C	F	R	E	B	P	N	a	G	O	e	M	A	S	T	b	Q	Z	X	L	Y	W	V	K
	2	.	U	I	H	C	D	F	R	B	E	N	P	G	a	O	M	A	S	T	e	Z	L	X	Q	Y	b	W	V	K
4	1	.	U	I	D	C	H	F	E	B	R	N	P	G	M	O	a	A	e	S	L	T	b	Z	Q	X	Y	W	K	V
	2	.	I	H	D	C	U	F	E	B	P	N	R	a	G	M	e	O	A	S	Z	X	b	T	Y	L	W	Q	K	V
5	1	.	U	I	H	C	D	F	R	E	B	P	N	G	a	O	M	e	A	S	Z	L	T	X	Q	b	Y	W	V	K
	2	.	U	I	D	H	C	F	R	E	B	P	N	G	a	M	O	A	S	e	Z	Q	L	X	b	T	Y	W	V	K
6	1	.	I	U	D	H	C	F	R	E	B	P	N	a	G	O	M	e	A	S	Z	L	T	X	Q	b	Y	W	K	V
	2	.	I	U	D	C	H	F	E	R	B	P	N	G	a	O	M	A	e	S	T	Z	L	X	Q	b	Y	W	V	K
7	1	.	U	I	H	D	C	F	R	E	B	P	N	a	G	O	M	e	A	S	L	T	Z	X	b	Q	Y	W	V	K
	2	.	I	U	D	C	F	H	E	R	B	P	N	G	O	a	e	M	A	L	b	S	Z	Y	T	Q	X	W	V	K
8	1	10	U	I	H	D	C	F	R	E	P	B	N	a	G	e	O	M	S	Z	A	b	X	T	L	Q	Y	W	V	K
	2	10	U	I	D	H	C	F	R	E	B	P	N	G	a	O	M	e	A	S	Z	Q	T	b	L	X	Y	W	V	K
9	1	.	U	I	D	H	C	F	R	E	P	B	N	G	a	O	M	e	A	S	T	L	Z	X	Q	b	Y	W	V	K
	2	.	U	.	.	.	.	.	R	.	.	.	P	.	.	.	.	.	.	S	.	.	.	T	.	Q	.	.	V	.
	3	.	I	U	D	H	C	F	R	E	P	B	N	G	O	a	M	S	A	Z	X	L	e	Q	Y	b	T	W	K	V
10	1	10	I	U	H	C	D	F	R	E	B	P	N	a	G	M	O	A	S	Z	T	L	X	Q	b	e	Y	W	K	V
	2	10	U	I	H	C	F	D	R	E	B	P	N	a	G	O	M	e	A	S	Z	L	T	b	X	Q	Y	W	K	V
	3	10	U	I	H	D	C	F	R	E	B	P	N	G	a	O	M	A	e	S	T	Z	b	X	Q	L	Y	W	V	K
11	1	.	I	U	H	F	D	E	R	B	P	C	N	G	A	S	M	O	Q	T	L	V	a	e	Z	b	X	K	Y	W
	2	.	I	U	H	D	C	F	R	E	B	P	N	G	a	O	M	e	A	T	b	Q	Z	L	X	S	W	Y	V	K
12	1	.	I	U	C	D	F	H	B	R	N	P	G	E	O	M	e	a	A	L	b	T	X	Q	Y	Z	W	S	K	V
	2	.	I	U	H	R	C	D	F	P	N	B	E	a	O	M	e	G	S	b	L	A	X	T	Q	.	Y	W	V	K
13	1	.	U	I	D	C	H	F	R	N	E	B	P	O	M	a	G	e	S	L	A	T	b	Z	Q	X	Y	W	K	V
	2	.	U	I	H	C	F	D	N	E	R	B	P	M	O	a	G	A	e	L	S	Z	b	T	X	Q	Y	W	K	V
14	1	.	U	I	D	H	C	F	R	E	B	P	N	G	a	O	S	M	e	A	Z	T	L	Q	b	X	Y	W	V	K
	2	.	I	U	H	D	C	F	R	E	B	P	N	a	G	O	M	S	e	A	T	Z	L	X	b	Q	Y	W	V	K
	3	15	I	U	H	D	C	F	R	E	B	P	N	a	G	O	M	S	A	e	T	L	Q	Z	b	X	Y	W	V	K
15	1	.	U	I	H	C	D	F	R	E	B	P	N	a	O	G	M	A	S	e	L	T	Z	Q	b	X	Y	W	K	V
	2	.	I	U	D	H	C	F	R	E	P	B	N	a	G	M	S	T	O	A	Z	e	Q	b	L	X	Y	W	K	V
16	1	.	U	I	D	H	C	F	R	E	B	P	N	a	G	O	M	S	e	Z	A	T	Q	X	b	L	Y	W	V	K
	2	.	U	I	H	D	C	F	R	N	E	B	O	P	M	a	G	L	e	A	b	S	Z	X	Q	T	Y	K	W	V
	3	.	I	U	H	D	C	F	R	E	B	P	N	G	a	M	O	e	A	S	Z	T	L	Q	b	X	Y	W	V	K
17	1	.	.	U	.	D	F	C	R	E	B	P	.	G	a	.	.	e	A	S	Q	Z	.	b	T	X	Y	W	V	.
	2	.	I	.	D	C	H	F	R	E	B	P	N	.	G	a	O	S	M	e	Q	A	T	Z	L	X	b	Y	W	V
19	1	.	U	I	D	F	C	H	R	E	B	P	N	a	G	e	O	Z	M	b	A	X	S	Y	T	L	Q	W	V	K
	2	.	I	U	D	H	C	F	E	R	B	P	N	G	a	M	A	O	e	S	T	L	Z	Q	b	X	Y	W	V	K
20	1	.	I	U	D	H	C	F	R	E	B	P	N	a	G	O	M	e	S	A	Z	T	Q	L	X	b	Y	W	V	K
	2	.	U	I	H	C	D	R	F	E	P	B	N	a	G	O	S	M	e	T	A	Q	Z	L	X	b	Y	W	V	K
21	1	.	U	I	H	D	F	C	R	E	B	P	N	a	G	O	M	A	e	S	T	L	b	Q	X	Z	Y	W	V	K
	2	.	I	U	H	D	C	F	R	E	B	N	P	a	G	M	O	A	S	e	Q	T	L	Z	b	X	W	Y	K	V
23	1	.	I	D	H	F	C	R	E	U	N	P	B	a	O	G	e	M	A	Z	b	S	T	L	X	Q	Y	W	K	V
	2	.	I	H	C	D	U	E	R	B	F	P	N	G	O	a	M	A	S	e	L	T	Q	b	Z	X	Y	W	V	K

The samples are sorted from left (low titres) to right (high titres)
Times are only indicated if this was stated explicitly on the reporting sheets.

Use this plot to judge if inversions are important

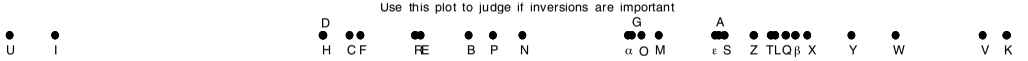
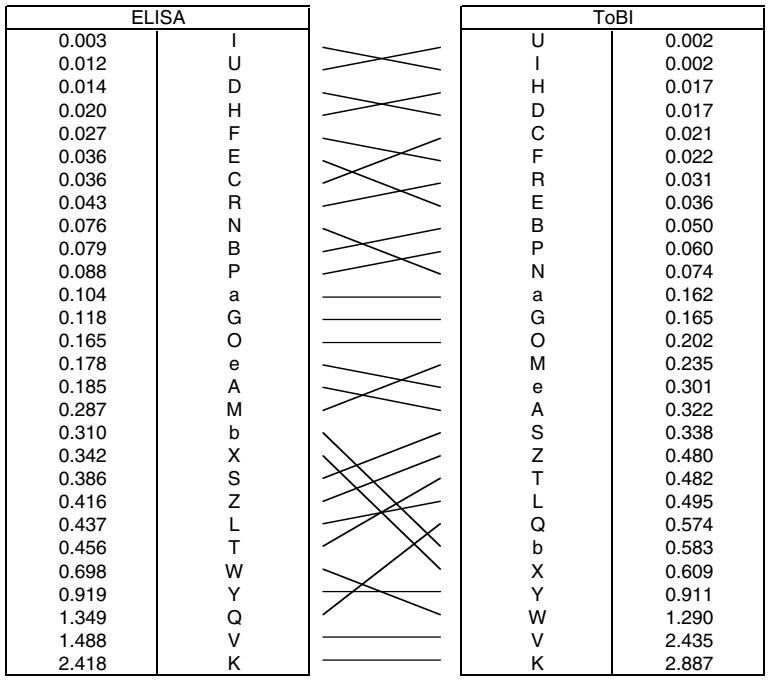


Table 7. Overall mean titres (in IU/ml)

Sample	ELISA	ToBI	ToBI / ELISA	ELISA / ToBI	Sign.
A	0.185	0.322	1.739	0.575	***
B	0.079	0.050	0.633	1.579	***
C	0.036	0.021	0.574	1.744	***
D	0.014	0.017	1.219	0.820	*
E	0.036	0.036	0.991	1.009	
F	0.027	0.022	0.837	1.195	**
G	0.118	0.165	1.389	0.720	***
H	0.020	0.017	0.837	1.195	***
I	0.003	0.002	0.747	1.339	
K	2.418	2.887	1.194	0.838	*
L	0.437	0.495	1.134	0.882	
M	0.287	0.235	0.817	1.223	***
N	0.076	0.074	0.971	1.030	
O	0.165	0.202	1.225	0.816	**
P	0.088	0.060	0.683	1.464	***
Q	1.349	0.574	0.426	2.349	***
R	0.043	0.031	0.714	1.401	***
S	0.386	0.338	0.877	1.140	*
T	0.456	0.482	1.055	0.948	
U	0.012	0.002	0.136	7.366	***
V	1.488	2.435	1.637	0.611	***
W	0.698	1.290	1.849	0.541	***
X	0.342	0.609	1.781	0.562	***
Y	0.919	0.911	0.992	1.008	
Z	0.416	0.480	1.152	0.868	
a	0.104	0.162	1.561	0.641	***
b	0.310	0.583	1.881	0.532	***
e	0.178	0.301	1.695	0.590	***



Stars indicate the level of significance of the difference between the two methods.
* = Significant ($p<0.05$), ** = Very significant ($p<0.01$), *** = Highly significant ($p<0.001$)

Table 8. The potency (IU/ml) of serum samples A, B, Q and V in Toxin neutralization test (TNT), Toxin Binding Inhibition test (ToBI) and Enzyme-Linked Immunosorbent Assay (ELISA)

Serum sample	TNT	ToBI	ELISA
A	0.2015	0.322	0.185
B	0.0336	0.050	0.079
Q	1.008	0.574	1.349
V	2.016	2.435	1.488

Table 9a. Reproducibility with regard to the correlation coefficient (ELISA)

Corr. (r^2)	Plate 1						
	A	B	C	D	E	F	G
0.999	0.183	0.090	0.035	0.013	0.033	0.027	0.122
0.999	0.188	0.079	0.038	0.014	0.037	0.028	0.116
0.999	0.213	0.086	0.041	0.014	0.037	0.024	0.119
0.999	0.167	0.078	0.035	0.014	0.037	0.027	0.105
0.999	0.180	0.081	0.039	0.016	0.046	0.034	0.130
0.999	0.203	0.097	0.048	0.019	0.050	0.040	0.171
0.998	0.158	0.073	0.031	0.012	0.034	0.023	0.114
0.998	0.148	0.089	0.053	0.044	0.061	0.051	0.111
0.998	0.223	0.094	0.040	0.015	0.042	0.029	0.120
0.998	0.166	0.071	0.036	0.014	0.033	0.027	0.085
0.998	0.170	0.070	0.037	0.013	0.035	0.026	0.103
0.998	0.197	0.086	0.040	0.014	0.044	0.028	0.132
0.998	0.152	0.068	0.030	0.011	0.031	0.021	0.102
0.998	0.180	0.059	0.030	0.010	0.028	0.021	0.091
0.998	0.172	0.070	0.030	0.010	0.029	0.021	0.107
0.998	0.189	0.081	0.038	0.015	0.041	0.030	0.119
0.998	0.195	0.076	0.038	0.015	0.037	0.026	0.103
0.998	0.173	0.072	0.030	0.010	0.028	0.021	0.092
0.998	0.174	0.058	0.030	0.012	0.028	0.023	0.093
0.998	0.170	0.082	0.038	0.016	0.038	0.029	0.127
0.998	0.183	0.077	0.036	0.013	0.035	0.026	0.110
0.998	0.201	0.079	0.034	0.012	0.035	0.025	0.112
0.998	0.207	0.094	0.045	0.016	0.042	0.034	0.129
0.998	0.184	0.075	0.027	0.009	0.031	0.021	0.128
0.997	0.179	0.077	0.035	0.013	0.034	0.029	0.118
0.997	0.186	0.066	0.034	0.010	0.036	0.025	0.109
0.997	0.165	0.061	0.026	0.010	0.027	0.019	0.073
0.997	0.222	0.116	0.049	0.022	0.048	0.040	0.147
0.996	0.179	0.078	0.037	0.015	0.039	0.031	0.141
0.996	0.152	0.063	0.039	0.014	0.033	0.026	0.102
0.996	0.230	0.099	0.044	0.018	0.039	0.036	0.139
0.996	0.160	0.095	0.035	0.014	0.044	0.028	0.106
0.996	0.204	0.101	0.053	0.018	0.049	0.031	0.130
0.995	0.178	0.077	0.034	0.013	0.030	0.024	0.098
0.995	0.162	0.067	0.033	0.012	0.030	0.021	0.085
0.994	0.195	0.085	0.049	0.021	0.054	0.043	0.146
0.994	0.169	0.081	0.037	0.015	0.034	0.029	0.110
0.993	0.165	0.077	0.034	0.013	0.034	0.026	0.117
0.993	0.188	0.086	0.036	0.013	0.033	0.028	0.117
0.992	0.209	0.104	0.055	0.025	0.050	0.039	0.118
0.992	0.162	0.065	0.029	0.010	0.028	0.018	0.089
0.991	0.134	0.055	0.029	0.012	0.030	0.025	0.084
0.991	0.135	0.052	0.031	0.009	0.025	0.025	0.093
0.989	0.141	0.055	0.015	0.004	0.014	0.010	0.063
0.989	0.251	0.092	0.044	0.017	0.042	0.033	0.134
0.989	0.213	0.111	0.065	0.032	0.061	0.048	0.137
0.989	0.194	0.079	0.040	0.010	0.031	0.019	0.115
0.988	0.150	0.076	0.058	0.032	0.062	0.047	0.178
0.988	0.232	0.105	0.049	0.017	0.049	0.039	0.149
0.987	0.217	0.096	0.067	0.037	0.065	0.053	0.145
0.987	0.156	0.041	0.032	0.008	0.024	0.021	0.069
0.984	0.166	0.048	0.035	0.008	0.026	0.022	0.074
0.979	0.124	0.083	0.022	0.008	0.027	0.021	0.098
0.978	0.327	0.120	0.057	0.026	0.055	0.039	0.170
0.976	0.183	0.051	0.034	0.009	0.026	0.023	0.094
0.969	0.134	0.067	0.016	0.007	0.024	0.017	0.054
0.969	0.216	0.068	0.011	0.013	0.031	0.032	0.080
0.950	0.400	0.080	0.038	0.027	0.061	0.023	0.188
0.949	0.191	0.084	0.060	0.034	0.062	0.079	0.215
0.946	0.259	0.089	0.047	0.023	0.048	0.038	0.131
0.934	0.173	0.077	0.039	0.013	0.040	0.027	0.120
0.898	0.171	0.073	0.034	0.043	0.021	0.037	0.165
0.779	0.205	0.099	0.035	0.017	0.044	0.065	0.162
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SD99	0.023	0.014	0.007	0.006	0.008	0.007	0.018
SD98	0.027	0.016	0.010	0.007	0.011	0.009	0.023
SD0	0.044	0.017	0.011	0.008	0.012	0.012	0.031

Corr. (r^2)	Plate 2						
	H	I	K	L	M	N	O
1.000	0.016	0.002	2.051	0.404	0.251	0.071	0.141
0.999	0.019	0.002	2.316	0.451	0.297	0.076	0.154
0.999	0.015	0.001	2.011	0.404	0.246	0.056	0.125
0.999	0.016	0.001	1.896	0.410	0.261	0.062	0.132
0.999	0.017	0.001	3.142	0.488	0.281	0.071	0.156
0.999	0.020	0.003	2.641	0.495	0.348	0.085	0.194
0.999	0.017	0.002	2.330	0.433	0.271	0.070	0.142
0.999	0.022	0.002	2.717	0.541	0.356	0.083	0.169
0.998	0.015	0.001	1.957	0.460	0.322	0.072	0.163
0.998	0.023	0.002	2.356	0.493	0.317	0.087	0.186
0.998	0.019	0.002	2.172	0.430	0.343	0.069	0.186
0.998	0.022	0.007	1.865	0.380	0.246	0.069	0.131
0.998	0.018	0.002	3.375	0.457	0.294	0.061	0.140
0.998	0.021	0.008	2.441	0.396	0.301	0.100	0.116
0.998	0.023	0.002	3.253	0.687	0.443	0.111	0.247
0.997	0.018	0.003	2.106	0.400	0.247	0.068	0.150
0.997	0.026	0.006	2.631	0.519	0.405	0.085	0.187
0.997	0.020	0.004	1.965	0.400	0.249	0.067	0.178
0.997	0.017	0.002	2.651	0.559	0.416	0.088	0.228
0.997	0.024	0.002	2.781	0.637	0.436	0.113	0.239
0.997	0.014	0.012	2.690	0.450	0.300	0.079	0.189
0.997	0.018	0.002	2.206	0.551	0.392	0.087	0.211
0.997	0.021	0.002	2.955	0.447	0.288	0.070	0.144
0.996	0.029	0.009	2.787	0.626	0.394	0.090	0.184
0.996	0.017	0.000	2.898	0.473	0.275	0.065	0.138
0.996	0.019	0.002	2.641	0.431	0.299	0.072	0.159
0.996	0.019	0.002	2.351	0.436	0.293	0.068	0.118
0.996	0.038	0.039	2.073	0.427	0.327	0.112	0.222
0.995	0.019	0.003	2.580	0.415	0.330	0.077	0.156
0.995	0.035	0.038	1.775	0.341	0.272	0.089	0.178
0.995	0.018	0.001	2.461	0.417	0.288	0.054	0.151
0.995	0.017	0.002	1.897	0.335	0.198	0.058	0.151
0.995	0.020	0.002	2.282	0.643	0.368	0.094	0.257
0.995	0.031	0.007	4.594	0.743	0.488	0.104	0.200
0.994	0.018	0.002	2.723	0.347	0.222	0.054	0.084
0.994	0.023	0.005	1.991	0.386	0.268	0.067	0.130
0.993	0.019	0.002	1.901	0.385	0.254	0.075	0.122
0.993	0.026	0.004	2.251	0.498	0.319	0.073	0.128
0.993	0.020	0.003	2.567	0.494	0.233	0.091	0.143
0.993	0.036	0.026	3.052	0.502	0.315	0.087	0.193
0.992	0.017	0.004	1.689	0.465	0.267	0.069	0.168
0.992	0.013	0.001	2.111	0.374	0.183	0.058	0.106
0.992	0.028	0.004	1.905	0.431	0.283	0.081	0.222
0.992	0.014	0.001	2.802	0.468	0.294	0.061	0.173
0.991	0.018	0.001	2.700	0.350	0.238	0.052	0.092
0.990	0.021	0.004	.	0.403	0.302	0.071	0.158
0.990	0.025	0.006	2.057	0.449	0.300	0.072	0.160
0.989	0.013	0.019	2.297	0.397	0.169	0.071	0.150
0.985	0.029	0.000	2.878	0.514	0.359	0.089	0.201
0.985	0.023	0.007	1.730	0.416	0.390	0.098	0.230
0.983	0.027	0.006	2.811	0.505	0.405	0.108	0.236
0.981	0.018	0.002	2.967	0.393	0.259	0.059	0.107
0.979	0.019	0.001	2.659	0.443	0.266	0.061	0.123
0.971	0.025	0.002	2.242	0.469	0.318	0.076	0.175
0.969	0.045	0.005	1.805	0.427	0.247	0.088	0.200
0.962	0.022	0.006	1.612	0.470	0.233	0.094	0.236
0.950	0.022	0.002	2.618	0.515	0.322	0.107	0.193
0.946	0.074	0.079	3.346	0.568	0.411	0.148	0.261
0.934	0.027	0.010	2.969	0.608	0.712	0.104	0.230
0.879	0.023	0.007	1.966	0.414	0.247	0.055	0.108
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SD99	0.006	0.009	0.532	0.082	0.060	0.014	0.038
SD98	0.006	0.008	0.523	0.078	0.063	0.015	0.039
SD0	0.009	0.012	0.529	0.078	0.083	0.018	0.042

Corr. (r^2)	Plate 3						
	P	Q	R	S	T	U	V
0.999	0.070	1.399	0.035	0.367	0.426	0.011	1.526
0.999	0.063	1.326	0.032	0.350	0.404	0.011	1.368
0.999	0.073	1.469	0.036	0.356	0.428	0.007	1.331
0.999	0.075	1.261	0.033	0.410	0.434	0.013	1.530
0.998	0.094	1.681	0.045	0.447	0.504	0.013	1.854
0.998	0.090	1.415	0.038	0.385	0.429	0.008	1.614
0.998	0.093	1.317	0.046	0.412	0.428	0.012	1.566
0.998	0.078	1.382	0.038	0.419	0.428	0.015	1.516
0.998	0.081	1.396	0.036	0.352	0.414	0.008	1.519
0.998	0.096	1.837	0.044	0.447	0.535	0.011	1.858
0.998	0.078	1.239	0.039	0.324	0.361	0.007	1.138
0.998	0.090	1.549	0.042	0.405	0.480	0.009	1.543
0.998	0.082	1.300	0.046	0.396	0.474	0.012	1.406
0.998	0.086	1.465	0.043	0.424	0.536	0.018	2.255
0.998	0.062	1.948	0.030	0.394	0.466	0.006	1.659
0.998	0.073	1.606	0.038	0.461	0.574	0.014	1.722
0.997	0.084	1.407	0.042	0.408	0.485	0.016	2.497
0.997	0.097	1.414	0.058	0.395	0.399	0.009	1.224
0.997	0.091	1.384	0.045	0.344	0.436	0.011	1.397
0.997	0.078	1.514	0.062	0.684	0.737	0.009	1.400
0.997	0.078	1.298	0.035	0.353	0.426	0.007	1.245
0.997	0.071	1.225	0.033	0.331	0.351	0.009	1.416
0.997	0.101	1.311	0.057	0.399	0.475	0.015	1.458
0.997	0.061	1.670	0.030	0.395	0.418	0.007	1.545
0.996	0.110	1.243	0.070	0.330	0.461	0.015	1.353
0.996	0.086	1.682	0.042	0.397	0.512	0.013	1.195
0.996	0.691	1.698	0.046	0.372	0.427	0.010	2.110
0.996	0.127	1.381	0.159	0.356	0.522	0.081	1.341
0.995	0.127	1.313	0.150	0.332	0.483	0.076	1.167
0.995	0.085	1.811	0.032	0.499	0.476	0.017	1.571
0.994	0.099	1.904	0.049	0.442	0.513	0.010	1.855
0.994	0.076	1.085	0.034	0.292	0.311	0.011	1.557
0.994	0.084	1.030	0.051	0.336	0.382	0.014	1.422
0.994	0.088	1.813	0.042	0.396	0.505	0.012	1.138
0.994	0.060	1.241	0.031	0.287	0.349	0.015	1.645
0.993	0.085	1.157	0.047	0.329	0.374	0.010	1.139
0.993	0.093	2.020	0.037	0.512	0.487	0.018	1.241
0.993	0.071	1.253	0.045	0.291	0.395	0.017	1.673
0.993	0.071	1.102	0.047	0.316	0.355	0.011	1.369
0.991	0.115	1.415	0.067	0.453	0.429	0.015	1.322
0.991	0.088	1.630	0.042	0.416	0.526	0.013	1.243
0.990	0.143	1.876	0.087	0.405	0.505	.	1.229
0.989	0.060	0.981	0.030	0.352	0.326	0.006	1.450
0.989	0.560	1.549	0.042	0.410	0.473	0.008	2.757
0.989	0.103	2.143	0.086	0.668	0.908	0.019	3.489
0.988	0.119	1.804	0.091	0.390	0.513	0.044	1.808
0.985	0.099	0.916	0.053	0.452	0.557	0.013	1.788
0.985	0.115	2.554	0.080	0.476	0.621	0.013	2.377
0.984	0.136	2.028	0.065	0.671	0.866	0.013	1.838
0.980	0.107	2.171	0.044	0.563	0.652	0.023	2.791
0.980	0.077	1.753	0.033	0.370	0.597	0.006	1.037
0.977	0.143	1.997	0.111	0.413	0.548	0.061	1.771
0.976	0.101	3.043	0.051	0.483	0.687	0.008	2.435
0.973	0.088	0.969	0.078	0.319	0.362	0.017	1.029
0.963	.	1.969	0.131	0.382	0.471	.	1.797
0.962	0.069	1.796	0.036	0.395	0.428	0.007	1.819
0.959	0.054	0.781	0.035	0.220	0.277	0.014	1.093
0.953	0.084	1.420	0.037	0.292	0.310	0.000	1.241
0.950	0.087	1.575	0.034	0.339	0.345	0.000	1.242
0.949	0.091	1.251	0.042	0.397	0.435	0.013	1.921
0.947	0.079	0.962	0.045	0.308	0.320	0.009	1.059
0.931	0.236	1.782	0.194	0.766	1.392	0.000	10.119
0.901	0.130	0.790	0.050	0.290	0.250	0.015	0.771
0.887	0.017	0.918	0.037	0.327	0.406	0.014	0.762
0.879	0.068	1.068	0.036	0.309	0.291	0.015	1.429
0.866	0.096	2.143	0.037	0.403	0.368	0.000	1.382
SD99	0.100	0.254	0.027	0.074	0.076	0.016	0.228
SD98	0.113	0.337	0.027	0.089	0.119	0.015	0.432
SD0	0.100	0.425	0.033	0.100	0.170	0.015	1.176

Corr. (r^2)	Plate 4						
	W	X	Y	Z	a	b	e
0.999	0.594	0.300	0.917	0.428	0.096	0.719	0.157
0.999	0.663	0.317	0.842	0.374	0.094	0.658	0.164
0.999	0.584	0.300	0.860	0.425	0.103	0.840	0.180
0.999	0.523	0.286	0.816	0.348	0.087	0.604	0.168
0.999	0.609	0.315	0.997	0.428	0.100	0.703	0.166
0.999	0.736	0.391	0.991	0.442	0.114	0.782	0.193
0.999	0.616	0.262	0.795	0.380	0.086	0.663	0.150
0.999	0.804	0.388	1.059	0.465	0.111	0.839	0.177
0.999	0.681	0.341	0.984	0.440	0.106	0.760	0.180
0.999	0.748	0.378	1.000	0.447	0.113	0.696	0.171
0.999	0.627	0.358	0.978	0.431	0.106	0.848	0.153
0.999	0.700	0.358	1.015	0.386	0.102	0.746	0.178
0.998	0.600	0.309	0.851	0.444	0.115	0.914	0.190
0.998	0.645	0.326	0.967	0.466	0.117	0.327	0.179
0.998	0.709	0.381	0.882	0.443	0.120	0.765	0.194
0.998	0.677	0.349	0.942	0.403	0.106	0.750	0.179
0.998	0.702	0.299	0.834	0.397	0.103	0.683	0.179
0.998	0.729	0.318	1.080	0.420	0.094	0.707	0.172
0.998	0.692	0.313	0.960	0.380	0.086	0.614	0.158
0.998	0.591	0.292	0.880	0.349	0.094	0.812	0.170
0.997	0.687	0.407	0.655	0.448	0.173	0.922	0.288
0.997	0.696	0.305	0.928	0.404	0.087	0.580	0.137
0.997	0.691	0.311	1.107	0.389	0.091	0.677	0.183
0.997	0.785	0.302	0.962	0.443	0.105	0.812	0.184
0.997	0.503	0.251	0.933	0.361	0.078	0.729	0.179
0.997	0.794	0.332	1.125	0.495	0.112	0.835	0.185
0.996	0.838	0.371	0.821	0.421	0.079	0.823	0.140
0.996	0.703	0.311	1.016	0.415	0.085	0.721	0.167
0.996	0.700	0.364	0.932	0.457	0.122	0.796	0.184
0.996	0.571	0.282	0.735	0.336	0.082	0.682	0.153
0.996	0.625	0.282	1.013	0.437	0.149	0.876	0.204
0.996	0.697	0.343	0.926	0.375	0.108	0.715	0.186
0.996	0.818	0.298	1.060	0.524	0.116	0.914	0.189
0.996	0.070	0.345	0.816	0.383	0.096	0.871	0.199
0.995	0.685	0.370	0.950	0.519	0.141	0.455	0.234
0.995	0.912	0.371	0.840	0.379	0.077	0.704	0.136
0.995	0.893	0.363	0.827	0.389	0.077	0.726	0.134
0.994	0.823	0.416	1.190	0.477	0.133	0.936	0.220
0.994	0.700	0.339	0.929	0.463	0.127	0.835	0.187
0.993	0.590	0.266	0.802	0.349	0.093	0.707	0.153
0.993	0.521	0.327	0.783	0.350	0.093	0.669	0.152
0.993	0.624	0.292	0.778	0.381	0.109	0.726	0.217
0.993	0.738	0.286	1.074	0.434	0.112	0.641	0.180
0.993	0.777	0.442	0.873	0.339	0.114	0.647	0.132
0.993	0.624	0.448	0.724	0.450	0.159	0.963	0.393
0.992	0.667	0.358	0.923	0.420	0.093	0.646	0.162
0.992	0.071	0.311	0.954	0.401	0.121	1.210	0.265
0.992	0.888	0.472	1.349	0.753	0.142	1.265	0.298
0.991	0.607	0.312	0.806	0.381	0.102	0.615	0.159
0.991	0.677	0.283	1.047	0.484	0.118	0.811	0.185
0.991	0.482	0.209	0.364	0.253	0.100	0.486	0.169
0.989	0.921	0.404	1.355	0.490	0.115	0.928	0.206
0.988	0.946	0.406	1.150	0.528	0.123	0.818	0.285
0.986	0.814	0.495	1.714	0.622	0.162	0.991	0.150
0.984	0.685	0.343	1.018	0.432	0.115	0.801	0.218
0.980	0.707	0.341	0.953	0.404	0.120	0.877	0.213
0.978	0.907	1.315	0.445	0.545	0.164	1.042	0.248
0.975	0.837	0.428	1.290	0.526	0.131	0.776	0.187
0.972	0.660	0.397	0.612	0.475	0.189	0.951	0.295
0.967	0.727	0.363	1.047	0.377	0.102	0.609	0.163
0.960	0.634	0.267	0.963	0.401	0.099	0.858	0.297
0.954	0.732	0.342	1.102	0.429	0.092	0.666	0.147
0.949	0.838	0.384	0.912	0.379	0.079	0.726	0.195
0.945	0.631	0.341	0.737	0.222	0.106	0.886	0.179
0.930	0.543	0.297	0.733	0.375	0.104	0.285	0.174
0.866	0.429	0.320	0.761	0.334	0.126	1.268	0.673
SD99	0.158	0.052	0.152	0.069	0.020	0.141	0.045
SD98	0.160	0.056	0.192	0.073	0.021	0.140	0.046
SD0	0.157	0.135	0.202	0.077	0.024	0.152	0.077

Explanations: Titres are sorted in order of decreasing correlation coefficient.

SD99 is the standard deviation excluding assays with a correlation coefficient (r^2) below 0.99

SD98 is the standard deviation excluding assays with a correlation coefficient (r^2) below 0.98

SD0 is the standard deviation including all assays

Table 9b. Reproducibility with regard to the correlation coefficient (ToBI)

Corr. (r^2)	Plate 1						
	A	B	C	D	E	F	G
0.999	0.183	0.090	0.035	0.013	0.033	0.027	0.122
0.999	0.188	0.079	0.038	0.014	0.037	0.028	0.116
0.999	0.213	0.086	0.041	0.014	0.037	0.024	0.119
0.999	0.167	0.078	0.035	0.014	0.037	0.027	0.105
0.999	0.180	0.081	0.039	0.016	0.046	0.034	0.130
0.999	0.203	0.097	0.048	0.019	0.050	0.040	0.171
0.998	0.158	0.073	0.031	0.012	0.034	0.023	0.114
0.998	0.148	0.089	0.053	0.044	0.061	0.051	0.111
0.998	0.223	0.094	0.040	0.015	0.042	0.029	0.120
0.998	0.166	0.071	0.036	0.014	0.033	0.027	0.085
0.998	0.170	0.070	0.037	0.013	0.035	0.026	0.103
0.998	0.197	0.086	0.040	0.014	0.044	0.028	0.132
0.998	0.152	0.068	0.030	0.011	0.031	0.021	0.102
0.998	0.180	0.059	0.030	0.010	0.028	0.021	0.091
0.998	0.172	0.070	0.030	0.010	0.029	0.021	0.107
0.998	0.189	0.081	0.038	0.015	0.041	0.030	0.119
0.998	0.195	0.076	0.038	0.015	0.037	0.026	0.103
0.998	0.173	0.072	0.030	0.010	0.028	0.021	0.092
0.998	0.174	0.058	0.030	0.012	0.028	0.023	0.093
0.998	0.170	0.082	0.038	0.016	0.038	0.029	0.127
0.998	0.183	0.077	0.036	0.013	0.035	0.026	0.110
0.998	0.201	0.079	0.034	0.012	0.035	0.025	0.112
0.998	0.207	0.094	0.045	0.016	0.042	0.034	0.129
0.998	0.184	0.075	0.027	0.009	0.031	0.021	0.128
0.997	0.179	0.077	0.035	0.013	0.034	0.029	0.118
0.997	0.186	0.066	0.034	0.010	0.036	0.025	0.109
0.997	0.165	0.061	0.026	0.010	0.027	0.019	0.073
0.997	0.222	0.116	0.049	0.022	0.048	0.040	0.147
0.996	0.179	0.078	0.037	0.015	0.039	0.031	0.141
0.996	0.152	0.063	0.039	0.014	0.033	0.026	0.102
0.996	0.230	0.099	0.044	0.018	0.039	0.036	0.139
0.996	0.160	0.095	0.035	0.014	0.044	0.028	0.106
0.996	0.204	0.101	0.053	0.018	0.049	0.031	0.130
0.995	0.178	0.077	0.034	0.013	0.030	0.024	0.098
0.995	0.162	0.067	0.033	0.012	0.030	0.021	0.085
0.994	0.195	0.085	0.049	0.021	0.054	0.043	0.146
0.994	0.169	0.081	0.037	0.015	0.034	0.029	0.110
0.993	0.165	0.077	0.034	0.013	0.034	0.026	0.117
0.993	0.188	0.086	0.036	0.013	0.033	0.028	0.117
0.992	0.209	0.104	0.055	0.025	0.050	0.039	0.118
0.992	0.162	0.065	0.029	0.010	0.028	0.018	0.089
0.991	0.134	0.055	0.029	0.012	0.030	0.025	0.084
0.991	0.135	0.052	0.031	0.009	0.025	0.025	0.093
0.989	0.141	0.055	0.015	0.004	0.014	0.010	0.063
0.989	0.251	0.092	0.044	0.017	0.042	0.033	0.134
0.989	0.213	0.111	0.065	0.032	0.061	0.048	0.137
0.989	0.194	0.079	0.040	0.010	0.031	0.019	0.115
0.988	0.150	0.076	0.058	0.032	0.062	0.047	0.178
0.988	0.232	0.105	0.049	0.017	0.049	0.039	0.149
0.987	0.217	0.096	0.067	0.037	0.065	0.053	0.145
0.987	0.156	0.041	0.032	0.008	0.024	0.021	0.069
0.984	0.166	0.048	0.035	0.008	0.026	0.022	0.074
0.979	0.124	0.083	0.022	0.008	0.027	0.021	0.098
0.978	0.327	0.120	0.057	0.026	0.055	0.039	0.170
0.976	0.183	0.051	0.034	0.009	0.026	0.023	0.094
0.969	0.134	0.067	0.016	0.007	0.024	0.017	0.054
0.969	0.216	0.068	0.011	0.013	0.031	0.032	0.080
0.950	0.400	0.080	0.038	0.027	0.061	0.023	0.188
0.949	0.191	0.084	0.060	0.034	0.062	0.079	0.215
0.946	0.259	0.089	0.047	0.023	0.048	0.038	0.131
0.934	0.173	0.077	0.039	0.013	0.040	0.027	0.120
0.898	0.171	0.073	0.034	0.043	0.021	0.037	0.165
0.779	0.205	0.099	0.035	0.017	0.044	0.065	0.162
.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.
SD99	0.023	0.014	0.007	0.006	0.008	0.007	0.018
SD98	0.027	0.016	0.010	0.007	0.011	0.009	0.023
SD0	0.044	0.017	0.011	0.008	0.012	0.012	0.031

Explanations: Titres are sorted in order of decreasing correlation coefficient.

Corr. (r^2)	Plate 2						
	H	I	K	L	M	N	O
1.000	0.016	0.002	2.051	0.404	0.251	0.071	0.141
0.999	0.019	0.002	2.316	0.451	0.297	0.076	0.154
0.999	0.015	0.001	2.011	0.404	0.246	0.056	0.125
0.999	0.016	0.001	1.896	0.410	0.261	0.062	0.132
0.999	0.017	0.001	3.142	0.488	0.281	0.071	0.156
0.999	0.020	0.003	2.641	0.495	0.348	0.085	0.194
0.999	0.017	0.002	2.330	0.433	0.271	0.070	0.142
0.999	0.022	0.002	2.717	0.541	0.356	0.083	0.169
0.998	0.015	0.001	1.957	0.460	0.322	0.072	0.163
0.998	0.023	0.002	2.356	0.493	0.317	0.087	0.186
0.998	0.019	0.002	2.172	0.430	0.343	0.069	0.186
0.998	0.022	0.007	1.865	0.380	0.246	0.069	0.131
0.998	0.018	0.002	3.375	0.457	0.294	0.061	0.140
0.998	0.021	0.008	2.441	0.396	0.301	0.100	0.116
0.998	0.023	0.002	3.253	0.687	0.443	0.111	0.247
0.997	0.018	0.003	2.106	0.400	0.247	0.068	0.150
0.997	0.026	0.006	2.631	0.519	0.405	0.085	0.187
0.997	0.020	0.004	1.965	0.400	0.249	0.067	0.178
0.997	0.017	0.002	2.651	0.559	0.416	0.088	0.228
0.997	0.024	0.002	2.781	0.637	0.436	0.113	0.239
0.997	0.014	0.012	2.690	0.450	0.300	0.079	0.189
0.997	0.018	0.002	2.206	0.551	0.392	0.087	0.211
0.997	0.021	0.002	2.955	0.447	0.288	0.070	0.144
0.996	0.029	0.009	2.787	0.626	0.394	0.090	0.184
0.996	0.017	0.000	2.898	0.473	0.275	0.065	0.138
0.996	0.019	0.002	2.641	0.431	0.299	0.072	0.159
0.996	0.019	0.002	2.351	0.436	0.293	0.068	0.118
0.996	0.038	0.039	2.073	0.427	0.327	0.112	0.222
0.995	0.019	0.003	2.580	0.415	0.330	0.077	0.156
0.995	0.035	0.038	1.775	0.341	0.272	0.089	0.178
0.995	0.018	0.001	2.461	0.417	0.288	0.054	0.151
0.995	0.017	0.002	1.897	0.335	0.198	0.058	0.151
0.995	0.020	0.002	2.282	0.643	0.368	0.094	0.257
0.995	0.031	0.007	4.594	0.743	0.488	0.104	0.200
0.994	0.018	0.002	2.723	0.347	0.222	0.054	0.084
0.994	0.023	0.005	1.991	0.386	0.268	0.067	0.130
0.993	0.019	0.002	1.901	0.385	0.254	0.075	0.122
0.993	0.026	0.004	2.251	0.498	0.319	0.073	0.128
0.993	0.020	0.003	2.567	0.494	0.233	0.091	0.143
0.993	0.036	0.026	3.052	0.502	0.315	0.087	0.193
0.992	0.017	0.004	1.689	0.465	0.267	0.069	0.168
0.992	0.013	0.001	2.111	0.374	0.183	0.058	0.106
0.992	0.028	0.004	1.905	0.431	0.283	0.081	0.222
0.992	0.014	0.001	2.802	0.468	0.294	0.061	0.173
0.991	0.018	0.001	2.700	0.350	0.238	0.052	0.092
0.990	0.021	0.004	.	0.403	0.302	0.071	0.158
0.990	0.025	0.006	2.057	0.449	0.300	0.072	0.160
0.989	0.013	0.019	2.297	0.397	0.169	0.071	0.150
0.985	0.029	0.000	2.878	0.514	0.359	0.089	0.201
0.985	0.023	0.007	1.730	0.416	0.390	0.098	0.230
0.983	0.027	0.006	2.811	0.505	0.405	0.108	0.236
0.981	0.018	0.002	2.967	0.393	0.259	0.059	0.107
0.979	0.019	0.001	2.659	0.443	0.266	0.061	0.123
0.971	0.025	0.002	2.242	0.469	0.318	0.076	0.175
0.969	0.045	0.005	1.805	0.427	0.247	0.088	0.200
0.962	0.022	0.006	1.612	0.470	0.233	0.094	0.236
0.950	0.022	0.002	2.618	0.515	0.322	0.107	0.193
0.946	0.074	0.079	3.346	0.568	0.411	0.148	0.261
0.934	0.027	0.010	2.969	0.608	0.712	0.104	0.230
0.879	0.023	0.007	1.966	0.414	0.247	0.055	0.108
.	.	.	.	.	.	.	.	.
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.	.	.	.	.	.	.	.	.
SD99	0.006	0.009	0.532	0.082	0.060	0.014	0.038
SD98	0.006	0.008	0.523	0.078	0.063	0.015	0.039
SD0	0.009	0.012	0.529	0.078	0.083	0.018	0.042

SD99 is the standard deviation excluding assays with a correlation coefficient (r^2) below 0.99

Corr. (r^2)	Plate 3						
	P	Q	R	S	T	U	V
0.999	0.070	1.399	0.035	0.367	0.426	0.011	1.526
0.999	0.063	1.326	0.032	0.350	0.404	0.011	1.368
0.999	0.073	1.469	0.036	0.356	0.428	0.007	1.331
0.999	0.075	1.261	0.033	0.410	0.434	0.013	1.530
0.998	0.094	1.681	0.045	0.447	0.504	0.013	1.854
0.998	0.090	1.415	0.038	0.385	0.429	0.008	1.614
0.998	0.093	1.317	0.046	0.412	0.428	0.012	1.566
0.998	0.078	1.382	0.038	0.419	0.428	0.015	1.516
0.998	0.081	1.396	0.036	0.352	0.414	0.008	1.519
0.998	0.096	1.837	0.044	0.447	0.535	0.011	1.858
0.998	0.078	1.239	0.039	0.324	0.361	0.007	1.138
0.998	0.090	1.549	0.042	0.405	0.480	0.009	1.543
0.998	0.082	1.300	0.046	0.396	0.474	0.012	1.406
0.998	0.086	1.465	0.043	0.424	0.536	0.018	2.255
0.998	0.062	1.948	0.030	0.394	0.466	0.006	1.659
0.998	0.073	1.606	0.038	0.461	0.574	0.014	1.722
0.997	0.084	1.407	0.042	0.408	0.485	0.016	2.497
0.997	0.097	1.414	0.058	0.395	0.399	0.009	1.224
0.997	0.091	1.384	0.045	0.344	0.436	0.011	1.397
0.997	0.078	1.514	0.062	0.684	0.737	0.009	1.400
0.997	0.078	1.298	0.035	0.353	0.426	0.007	1.245
0.997	0.071	1.225	0.033	0.331	0.351	0.009	1.416
0.997	0.101	1.311	0.057	0.399	0.475	0.015	1.458
0.997	0.061	1.670	0.030	0.395	0.418	0.007	1.545
0.996	0.110	1.243	0.070	0.330	0.461	0.015	1.353
0.996	0.086	1.682	0.042	0.397	0.512	0.013	1.195
0.996	0.691	1.698	0.046	0.372	0.427	0.010	2.110
0.996	0.127	1.381	0.159	0.356	0.522	0.081	1.341
0.995	0.127	1.313	0.150	0.332	0.483	0.076	1.167
0.995	0.085	1.811	0.032	0.499	0.476	0.017	1.571
0.994	0.099	1.904	0.049	0.442	0.513	0.010	1.855
0.994	0.076	1.085	0.034	0.292	0.311	0.011	1.557
0.994	0.084	1.030	0.051	0.336	0.382	0.014	1.422
0.994	0.088	1.813	0.042	0.396	0.505	0.012	1.138
0.994	0.060	1.241	0.031	0.287	0.349	0.015	1.645
0.993	0.085	1.157	0.047	0.329	0.374	0.010	1.139
0.993	0.093	2.020	0.037	0.512	0.487	0.018	1.241
0.993	0.071	1.253	0.045	0.291	0.395	0.017	1.673
0.993	0.071	1.102	0.047	0.316	0.355	0.011	1.369
0.991	0.115	1.415	0.067	0.453	0.429	0.015	1.322
0.991	0.088	1.630	0.042	0.416	0.526	0.013	1.243
0.990	0.143	1.876	0.087	0.405	0.505	.	1.229
0.989	0.060	0.981	0.030	0.352	0.326	0.006	1.450
0.989	0.560	1.549	0.042	0.410	0.473	0.008	2.757
0.989	0.103	2.143	0.086	0.668	0.908	0.019	3.489
0.988	0.119	1.804	0.091	0.390	0.513	0.044	1.808
0.985	0.099	0.916	0.053	0.452	0.557	0.013	1.788
0.985	0.115	2.554	0.080	0.476	0.621	0.013	2.377
0.984	0.136	2.028	0.065	0.671	0.866	0.013	1.838
0.980	0.107	2.171	0.044	0.563	0.652	0.023	2.791
0.980	0.077	1.753	0.033	0.370	0.597	0.006	1.037
0.977	0.143	1.997	0.111	0.413	0.548	0.061	1.771
0.976	0.101	3.043	0.051	0.483	0.687	0.008	2.435
0.973	0.088	0.969	0.078	0.319	0.362	0.017	1.029
0.963	.	1.969	0.131	0.382	0.471	.	1.797
0.962	0.069	1.796	0.036	0.395	0.428	0.007	1.819
0.959	0.054	0.781	0.035	0.220	0.277	0.014	1.093
0.953	0.084	1.420	0.037	0.292	0.310	0.000	1.241
0.950	0.087	1.575	0.034	0.339	0.345	0.000	1.242
0.949	0.091	1.251	0.042	0.397	0.435	0.013	1.921
0.947	0.079	0.962	0.045	0.308	0.320	0.009	1.059
0.931	0.236	1.782	0.194	0.766	1.392	0.000	10.119
0.901	0.130	0.790	0.050	0.290	0.250	0.015	0.771
0.887	0.017	0.918	0.037	0.327	0.406	0.014	0.762
0.879	0.068	1.068	0.036	0.309	0.291	0.015	1.429
0.866	0.096	2.143	0.037	0.403	0.368	0.000	1.382
SD99	0.100	0.254	0.027	0.074	0.076	0.016	0.228
SD98	0.113	0.337	0.027	0.089	0.119	0.015	0.432
SD0	0.100	0.425	0.033	0.100	0.170	0.015	1.176

SD98 is the standard deviation excluding assays with a correlation coefficient (r^2) below 0.98

Corr. (r^2)	Plate 4						
	W	X	Y	Z	a	b	e
0.999	0.594	0.300	0.917	0.428	0.096	0.719	0.157
0.999	0.663	0.317	0.842	0.374	0.094	0.658	0.164
0.999	0.584	0.300	0.860	0.425	0.103	0.840	0.180
0.999	0.523	0.286	0.816	0.348	0.087	0.604	0.168
0.999	0.609	0.315	0.997	0.428	0.100	0.703	0.166
0.999	0.736	0.391	0.991	0.442	0.114	0.782	0.193
0.999	0.616	0.262	0.795	0.380	0.086	0.663	0.150
0.999	0.804	0.388	1.059	0.465	0.111	0.839	0.177
0.999	0.681	0.341	0.984	0.440	0.106	0.760	0.180
0.999	0.748	0.378	1.000	0.447	0.113	0.696	0.171
0.999	0.627	0.358	0.978	0.431	0.106	0.848	0.153
0.999	0.700	0.358	1.015	0.386	0.102	0.746	0.178
0.998	0.600	0.309	0.851	0.444	0.115	0.914	0.190
0.998	0.645	0.326	0.967	0.466	0.117	0.327	0.179
0.998	0.709	0.381	0.882	0.443	0.120	0.765	0.194
0.998	0.677	0.349	0.942	0.403	0.106	0.750	0.179
0.998	0.702	0.299	0.834	0.397	0.103	0.683	0.179
0.998	0.729	0.318	1.080	0.420	0.094	0.707	0.172
0.998	0.692	0.313	0.960	0.380	0.086	0.614	0.158
0.998	0.591	0.292	0.880	0.349	0.094	0.812	0.170
0.997	0.687	0.407	0.655	0.448	0.173	0.922	0.288
0.997	0.696	0.305	0.928	0.404	0.087	0.580	0.137
0.997	0.691	0.311	1.107	0.389	0.091	0.677	0.183
0.997	0.785	0.302	0.962	0.443	0.105	0.812	0.184
0.997	0.503	0.251	0.933	0.361	0.078	0.729	0.179
0.997	0.794	0.332	1.125	0.495	0.112	0.835	0.185
0.996	0.838	0.371	0.821	0.421	0.079	0.823	0.140
0.996	0.703	0.311	1.016	0.415	0.085	0.721	0.167
0.996	0.700	0.364	0.932	0.457	0.122	0.796	0.184
0.996	0.571	0.282	0.735	0.336	0.082	0.682	0.153
0.996	0.625	0.282	1.013	0.437	0.149	0.876	0.204
0.996	0.697	0.343	0.926	0.375	0.108	0.715	0.186
0.996	0.818	0.298	1.060	0.524	0.116	0.914	0.189
0.996	0.070	0.345	0.816	0.383	0.096	0.871	0.199
0.995	0.685	0.370	0.950	0.519	0.141	0.455	0.234
0.995	0.912	0.371	0.840	0.379	0.077	0.704	0.136
0.995	0.893	0.363	0.827	0.389	0.077	0.726	0.134
0.994	0.823	0.416	1.190	0.477	0.133	0.936	0.220
0.994	0.700	0.339	0.929	0.463	0.127	0.835	0.187
0.993	0.590	0.266	0.802	0.349	0.093	0.707	0.153
0.993	0.521	0.327	0.783	0.350	0.093	0.669	0.152
0.993	0.624	0.292	0.778	0.381	0.109	0.726	0.217
0.993	0.738	0.286	1.074	0.434	0.112	0.641	0.180
0.993	0.777	0.442	0.873	0.339	0.114	0.647	0.132
0.993	0.624	0.448	0.724	0.450	0.159	0.963	0.393
0.992	0.667	0.358	0.923	0.420	0.093	0.646	0.162
0.992	0.071	0.311	0.954	0.401	0.121	1.210	0.265
0.992	0.888	0.472	1.349	0.753	0.142	1.265	0.298
0.991	0.607	0.312	0.806	0.381	0.102	0.615	0.159
0.991	0.677	0.283	1.047	0.484	0.118	0.811	0.185
0.991	0.482	0.209	0.364	0.253	0.100	0.486	0.169
0.989	0.921	0.404	1.355	0.490	0.115	0.928	0.206
0.988	0.946	0.406	1.150	0.528	0.123	0.818	0.285
0.986	0.814	0.495	1.714	0.622	0.162	0.991	0.150
0.984	0.685	0.343	1.018	0.432	0.115	0.801	0.218
0.980	0.707	0.341	0.953	0.404	0.120	0.877	0.213
0.978	0.907	1.315	0.445	0.545	0.164	1.042	0.248
0.975	0.837	0.428	1.290	0.526	0.131	0.776	0.187
0.972	0.660	0.397	0.612	0.475	0.189	0.951	0.295
0.967	0.727	0.363	1.047	0.377	0.102	0.609	0.163
0.960	0.634	0.267	0.963	0.401	0.099	0.858	0.297
0.954	0.732	0.342	1.102	0.429	0.092	0.666	0.147
0.949	0.838	0.384	0.912	0.379	0.079	0.726	0.195
0.945	0.631	0.341	0.737	0.222	0.106	0.886	0.179
0.930	0.543	0.297	0.733	0.375	0.104	0.285	0.174
0.866	0.429	0.320	0.761	0.334	0.126	1.268	0.673
SD99	0.158	0.052	0.152	0.069	0.020	0.141	0.045
SD98	0.160	0.056	0.192	0.073	0.021	0.140	0.046
SD0	0.157	0.135	0.202	0.077	0.024	0.152	0.077

SD0 is the standard deviation including all assays

Table 10a. Titres of the samples (ELISA)

Lab	Rep	Time	Plate 1							Plate 2							Plate 3							Plate 4						
			A	B	C	D	E	F	G	H	I	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	e
1	1		0.195	0.095	0.050	0.011	0.046	0.041	0.151	0.003	0.000	1.195	0.563	0.378	0.070	0.270	0.066	1.560	0.010	0.478	0.854	0.001	1.546	0.585	0.335	0.742	0.360	0.073	0.358	0.169
	2		0.214	0.097	0.063	0.013	0.064	0.045	0.176	0.004	0.000	2.647	0.469	0.371	0.074	0.258	0.087	2.193	0.022	0.380	0.557	0.002	1.849	0.807	0.397	0.915	0.472	0.101	0.356	0.218
	3		0.192	0.092	0.055	0.011	0.057	0.043	0.155	0.005	0.000	3.016	0.499	0.339	0.076	0.260	0.093	2.606	0.018	0.404	0.596	0.001	2.347	0.718	0.343	1.004	0.436	0.095	0.328	0.185
5	1		0.25	0.05	0.01	0.01	0.03	0.02	0.12	0.01	0.00	2.29	0.47	0.25	0.05	0.11	0.03	1.11	0.02	0.42	0.29	0.00	2.07	1.03	0.48	1.05	0.53	0.15	0.51	0.25
	2		0.21	0.05	0.02	0.01	0.03	0.02	0.09	0.02	0.00	2.28	0.56	0.30	0.06	0.14	0.03	1.09	0.02	0.48	0.34	0.00	2.07	0.66	0.37	0.79	0.44	0.14	0.39	0.20
17	1		0.172	0.095	0.037	0.016	0.038	0.027	0.146	0.028	0.013	2.074	0.457	0.308	0.088	0.203	0.069	1.435	0.118	0.484	0.560	0.036	1.601	0.780	0.409	1.272	0.526	0.134	0.415	0.259
	2		0.185	0.110	0.042	0.015	0.049	0.031	0.197	0.039	0.011		0.504	0.266	0.097	0.191	0.109	0.970	0.058	0.496	0.342	0.016	1.547	0.689	0.361	1.208	0.408	0.104	0.351	0.259

Titres are expressed in IU/ml
n.c. = no convergence. (The calculation method failed to converge)
Times are only indicated if this was stated explicitly on the reporting sheets.
If a sample was not tested, the cell is crossed out.
Results from plates with a correlation coefficient below 0.98 are printed on a grey background.

Table 10b. Titres of the samples (ToBl)

Lab	Rep	Time	Plate 1							Plate 2							Plate 3							Plate 4						
			A	B	C	D	E	F	G	H	I	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	e
2	1		0.24	0.07	0.05	0.03	0.05	0.00	0.15	0.03	0.00	3.18	0.65	0.37	0.07	0.14	0.09	1.65	0.58	0.72	0.69	0.00	1.76	0.91	0.61	0.95	0.66	0.19	0.47	0.29
	2		0.30	0.04	0.02	0.02	0.02	0.02	0.12	0.00	0.00	2.31	0.51	0.30	0.06	0.14	0.04	1.17	0.02	0.47	0.37	0.00	1.86	0.87	0.51	1.06	0.52	0.17	0.50	0.28
3	1		0.419	0.059	0.018	0.009	0.030	0.016	0.220	0.003	0.000	1.578	0.229	0.090	0.022	0.464	0.026	0.517	0.013	0.255	0.294	0.000	2.129	0.058	0.047	0.211	0.022	0.004	0.019	0.107
	2		0.320	0.059	0.019	0.040	0.064	0.046	0.158	0.006	0.000	1.301	0.570	0.145	0.036	0.231	0.022	0.221	0.005	0.157	0.207	0.000	1.708	1.586	0.547	0.526	0.481	0.152	0.471	0.624
17	1		0.405	0.081	0.030	0.022	0.064	0.026	0.190	0.046	0.012	4.208	1.161	0.657	0.150	0.303	0.094	1.063	0.059	0.696	0.846	0.001	2.212	1.151	0.573	1.070	0.564	0.206	0.590	0.238
	2		0.329	0.152	0.095	0.089	0.106	0.085	0.237	0.097	0.014	2.357	0.453	0.267	0.074	0.143	0.104	0.593	0.095	0.610	0.390	0.182	1.841	0.812	0.363	1.004	0.439	0.121	0.424	0.160
22	1		0.25	0.06	0.00	0.00	0.06	0.00	0.25	0.00	0.00	4.00	0.50	0.25	0.12	0.12	0.06	2.00	0.00	0.50	0.50	0.00	4.00	1.00	0.50	1.00	1.00	0.12	0.50	0.25

Titres are expressed in IU/ml
n.c. = no convergence. (The calculation method failed to converge.
Times are only indicated if this was stated explicitly on the reporting sheets.
If a sample was not tested, the cell is crossed out.
Results from plates with a correlation coefficient below 0.98 are printed on a grey background.

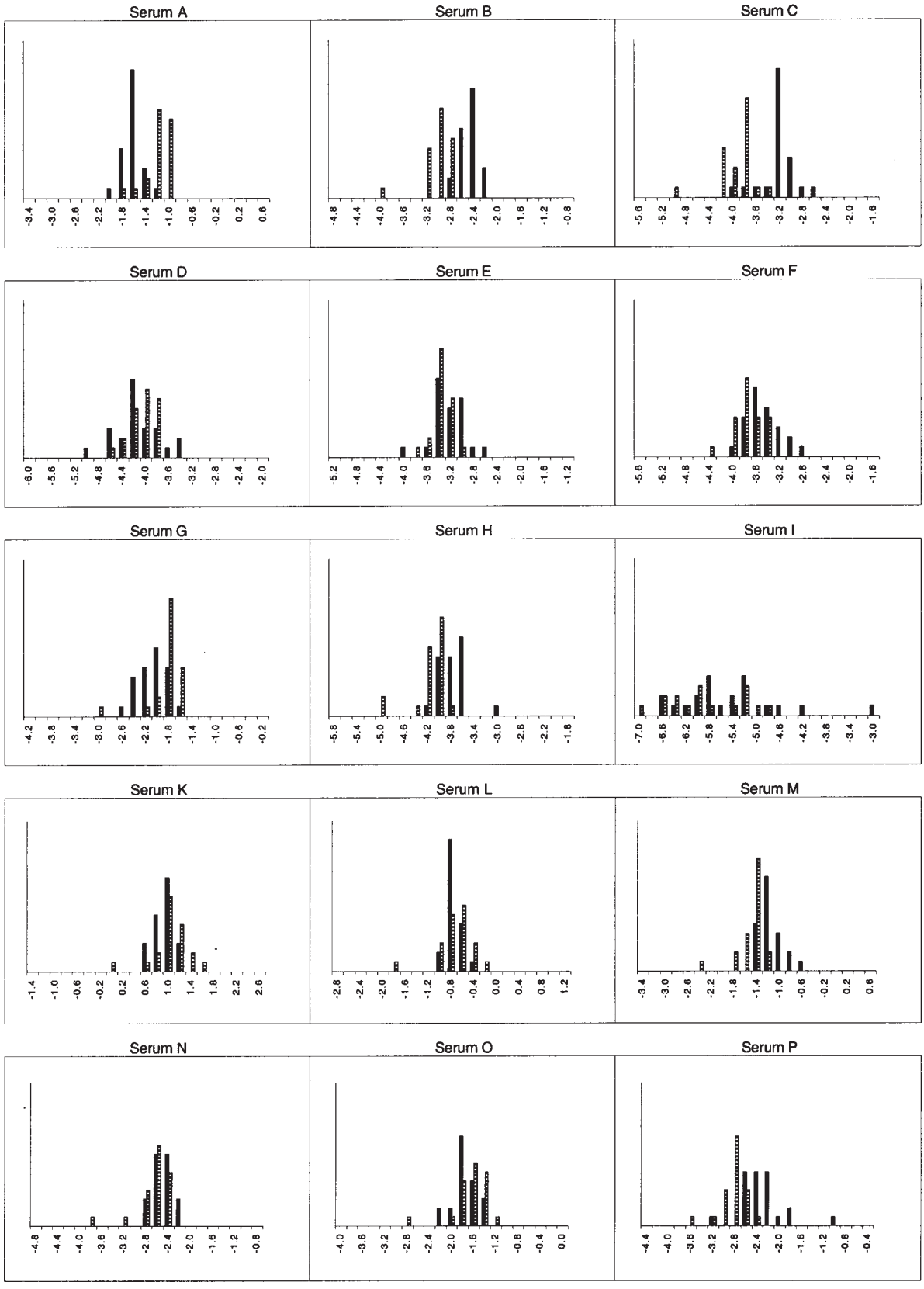


Figure 1.1. Histograms per sample

Titres are expressed as ln(titre).
Vertical bars represent the number of laboratories having found a specific titre (geometric mean of the repeated assays).
ELISA assays are represented by black bars. ToBI assays by dashed bars.

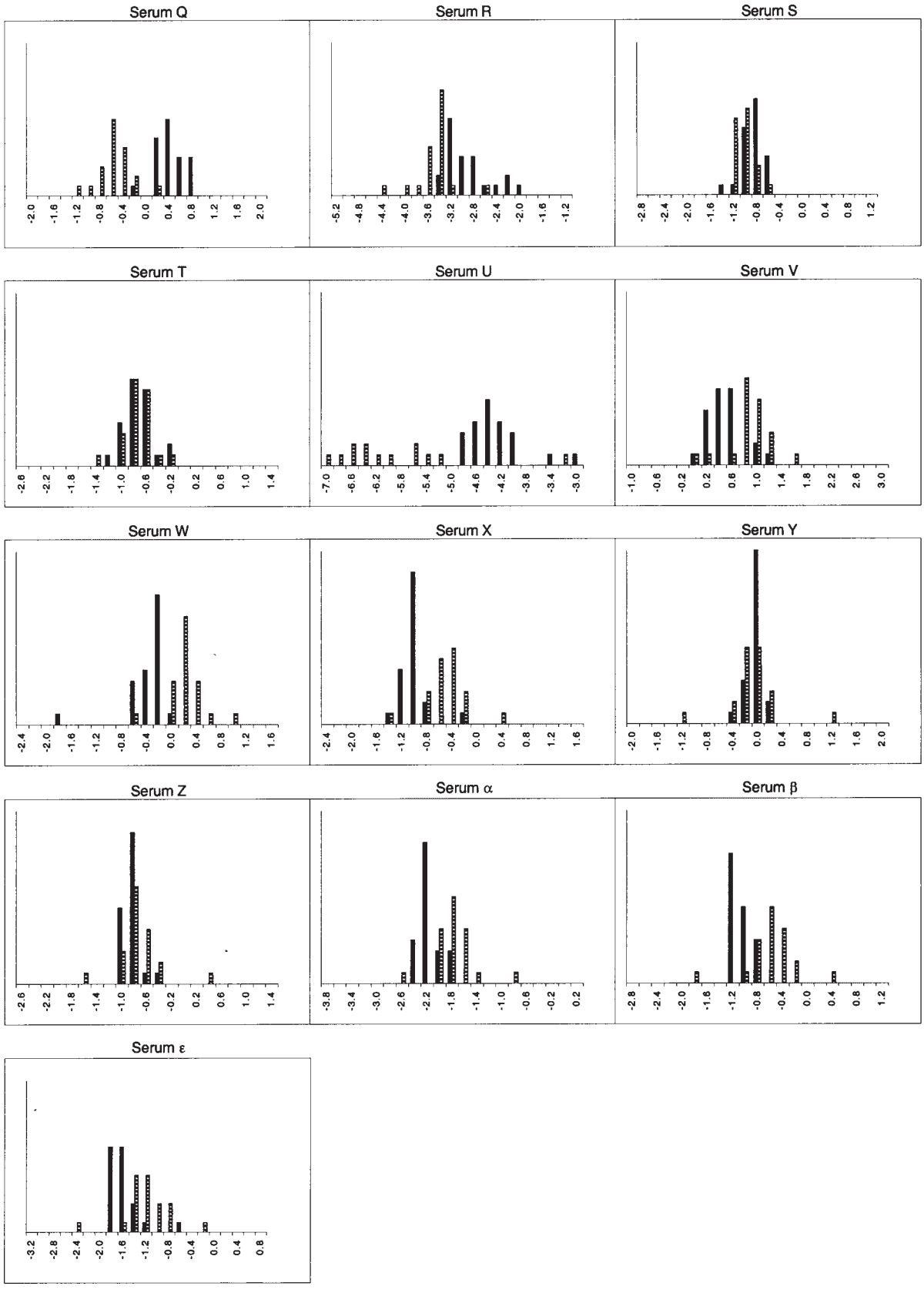


Figure 1.2. Histograms per sample

Titres are expressed as ln(titre).
Vertical bars represent the number of laboratories having found a specific titre (geometric mean of the repeated assays).
ELISA assays are represented by black bars. ToBI assays by dashed bars.

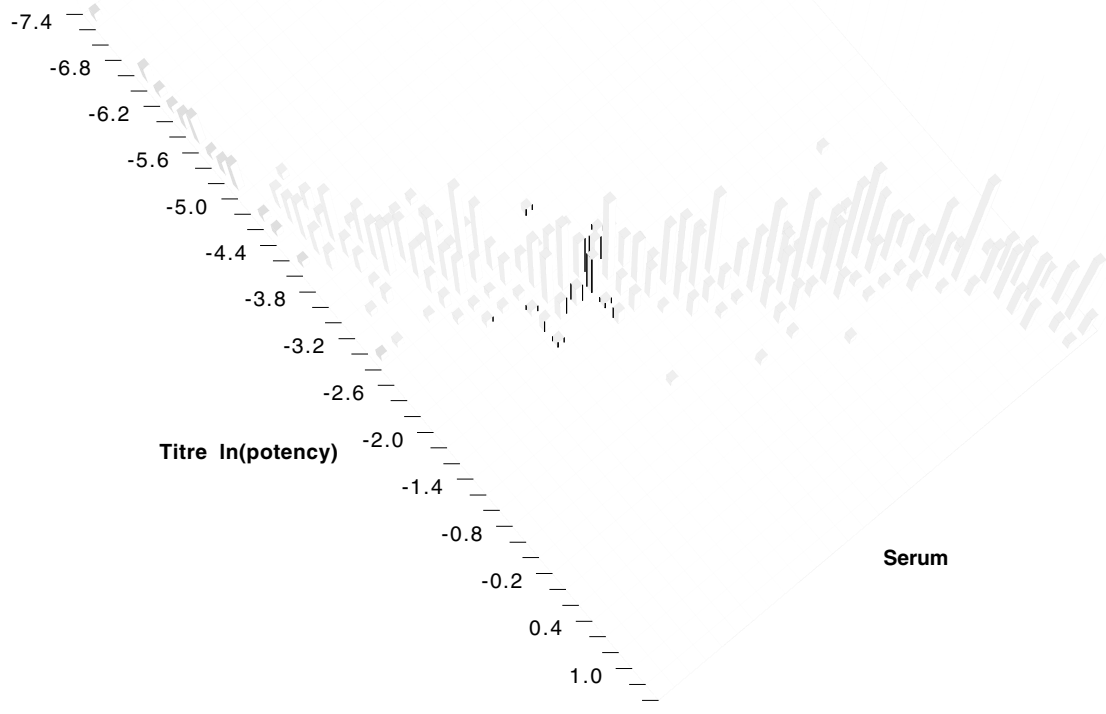


Figure 2.1 — Histograms of titres (ELISA)

This figure shows a 3-dimensional representation of the histograms for all sera in Figures 1.1 and 1.2 (ELISA).
The sera are ranked in increasing titres.

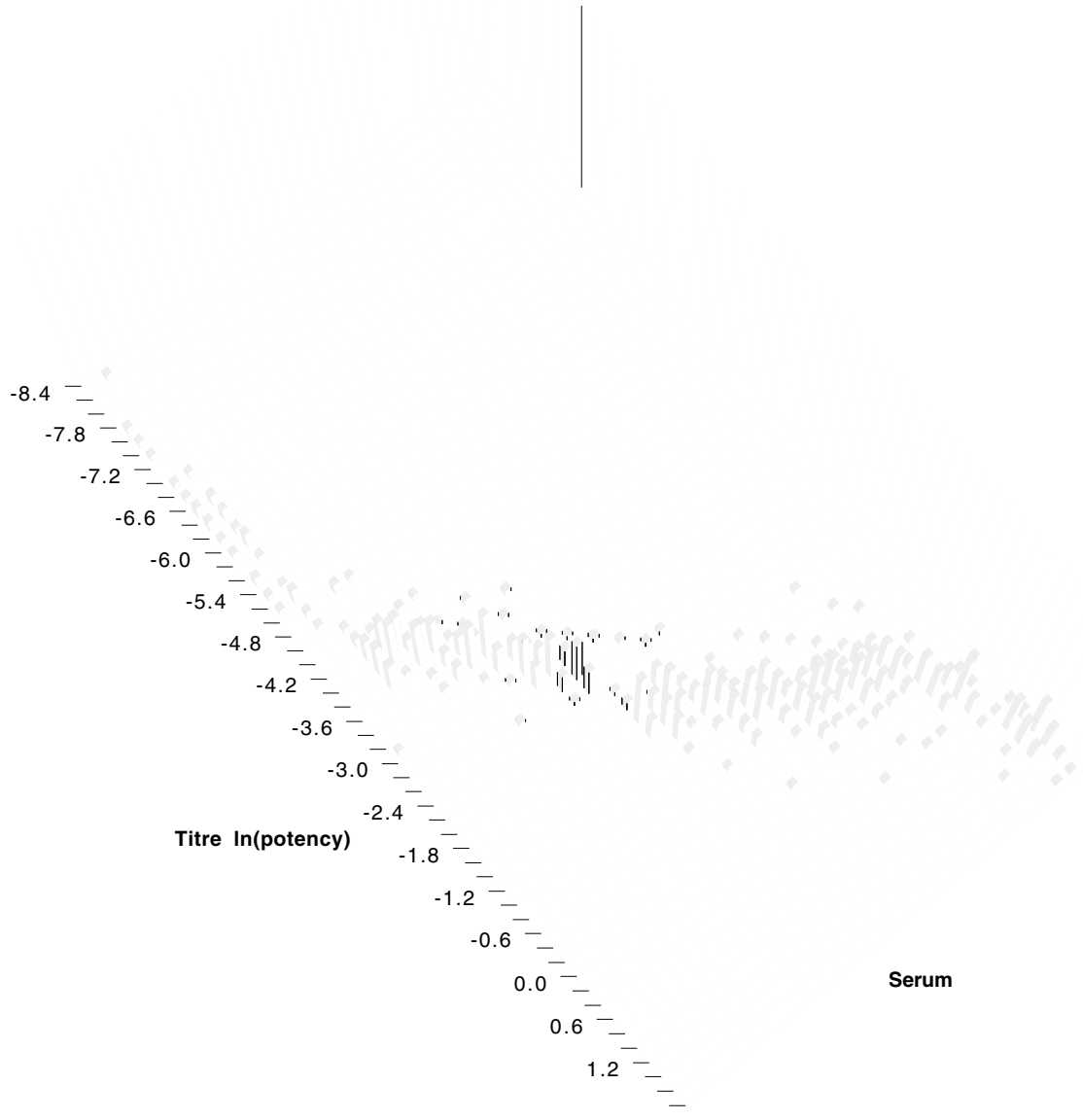
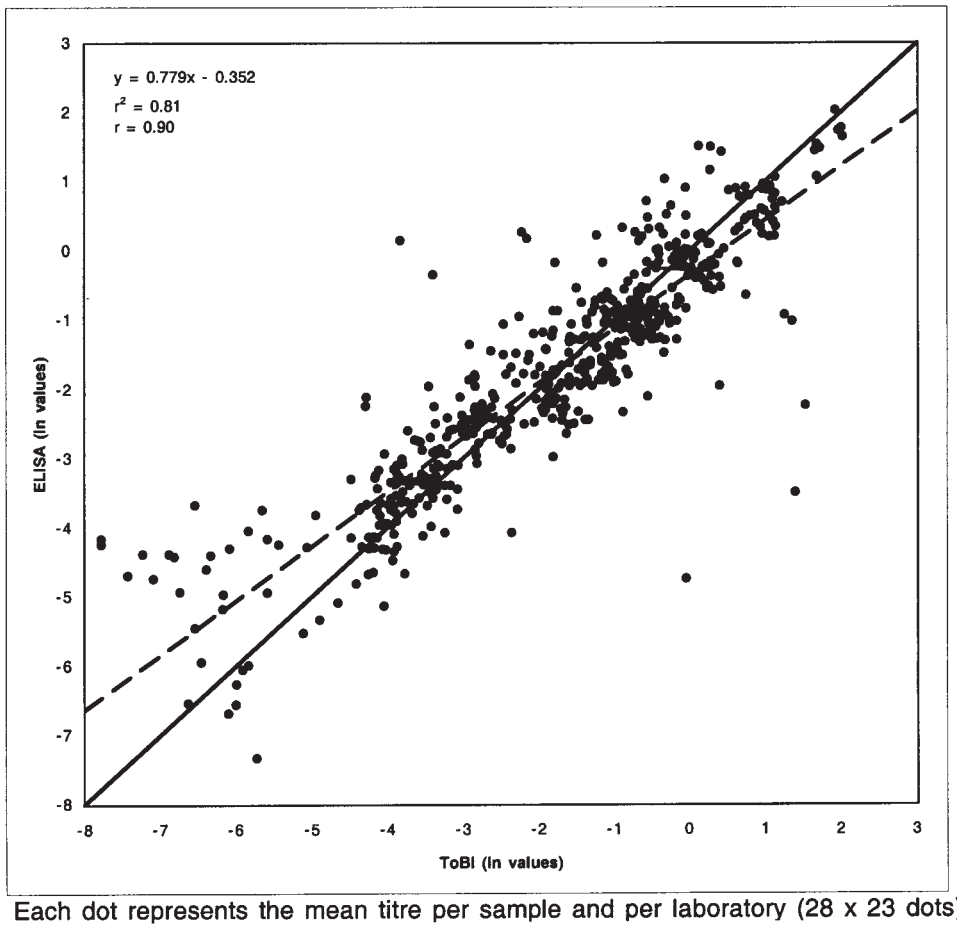
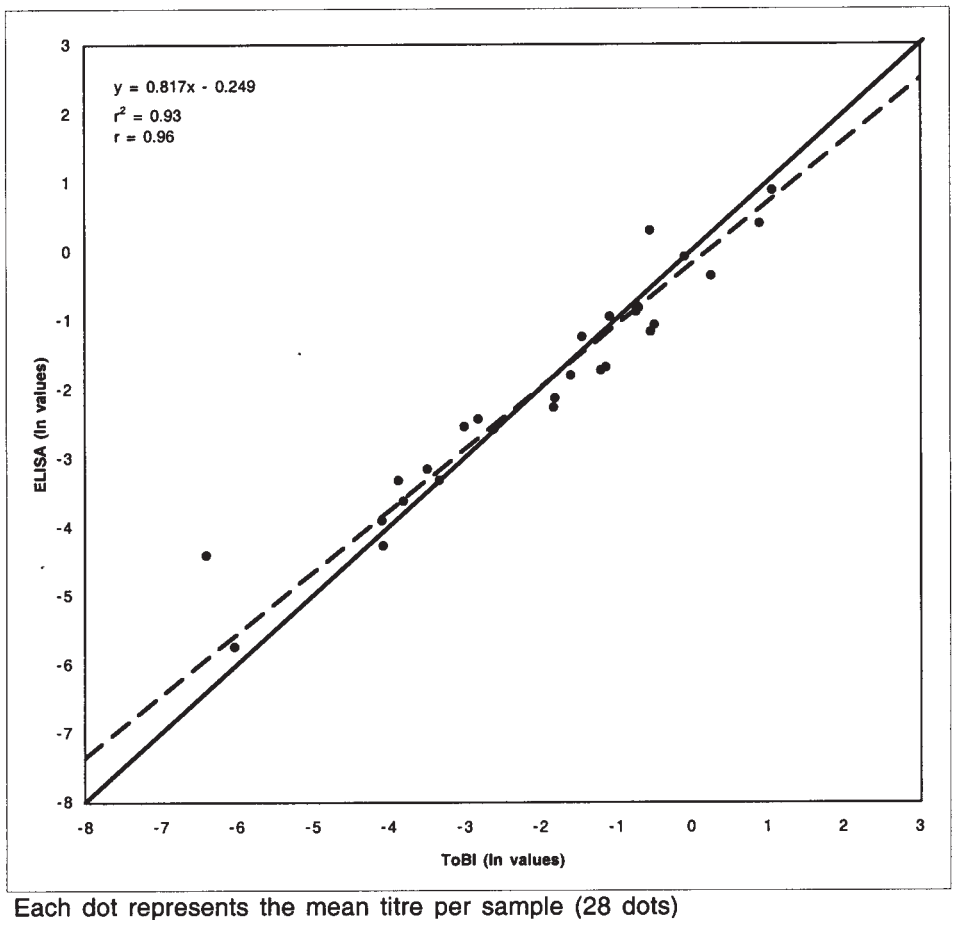


Figure 2.2 — Histograms of titres (ToBI)

This figure shows a 3-dimensional representation of the histograms for all sera in Figures 1.1 and 1.2 (ToBI).
The sera are ranked in increasing titres.



Each dot represents the mean titre per sample and per laboratory (28 x 23 dots)



Each dot represents the mean titre per sample (28 dots)

Figure 3. Correlation plots (ELISA vs. ToBI)

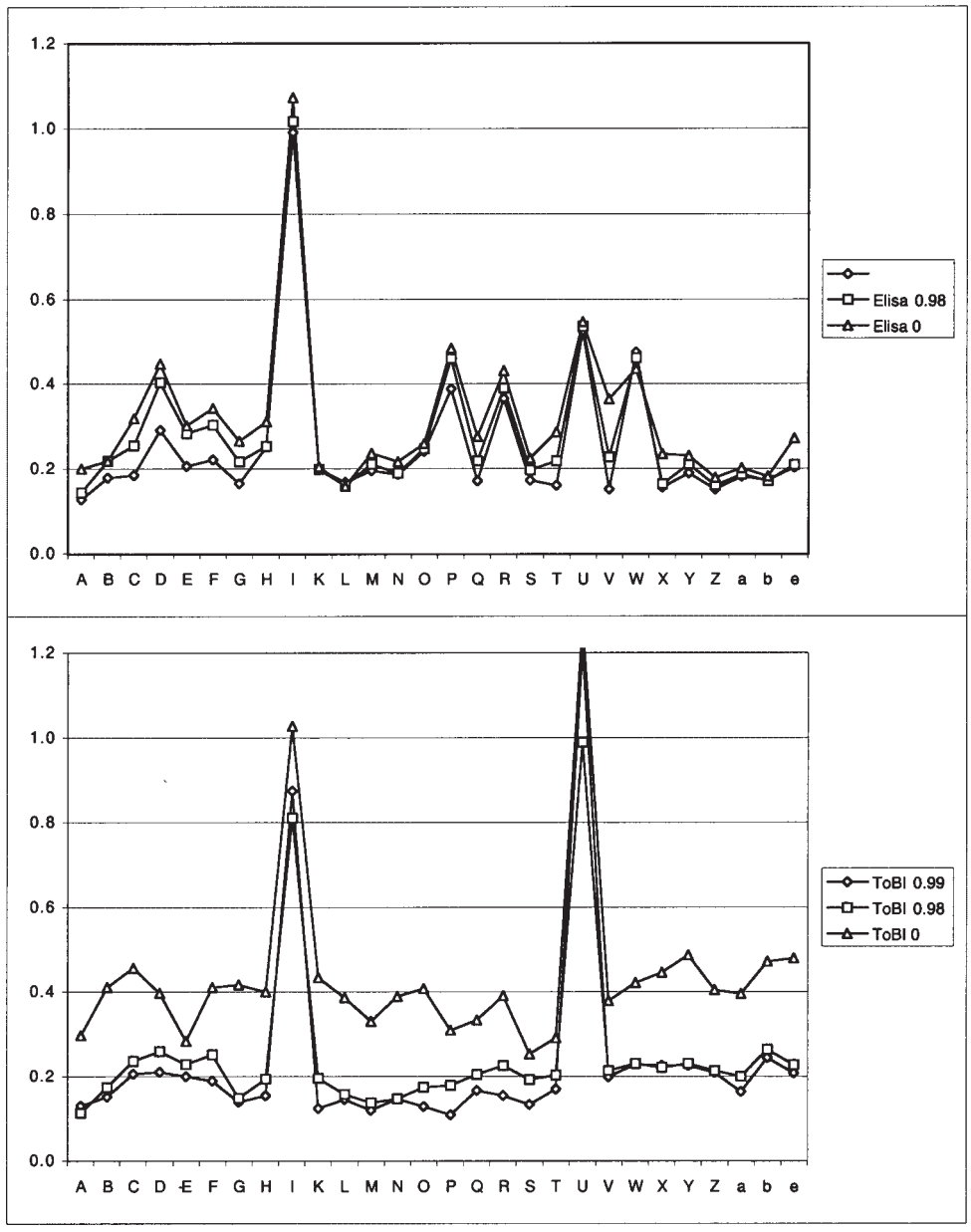


Figure 4.1. Reproducibility per method and per sample

The inter-laboratory standard deviation is shown on the vertical axis. The sera are shown on the horizontal axis.
0.99 means: Excluding assays with a correlation coefficient below 0.99.
0.98 means: Excluding assays with a correlation coefficient below 0.98.
0 means: Including all assays.
a, b and e correspond to serum samples α, β and ε.

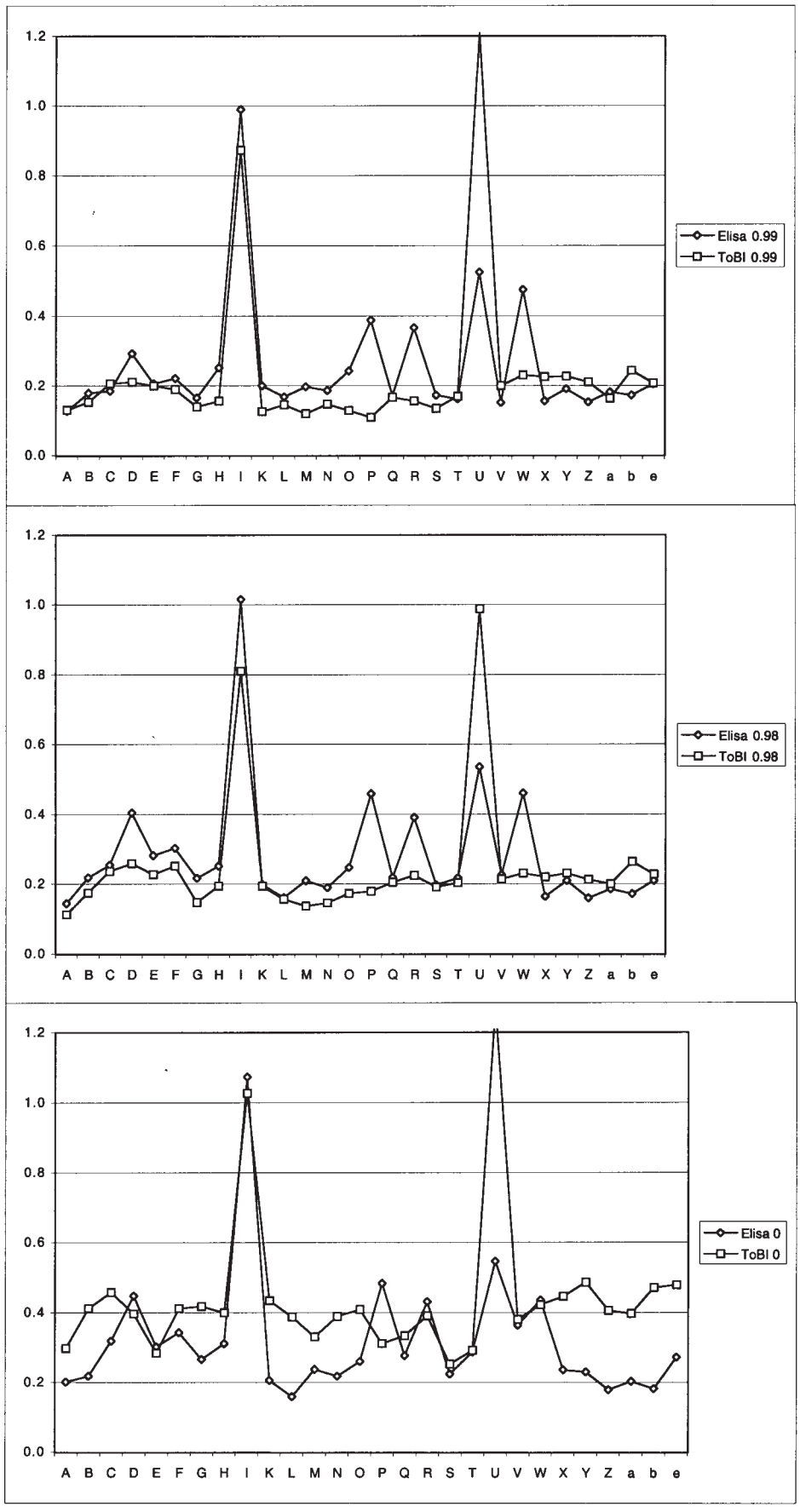


Figure 4.2. Reproducibility per method and per sample

The inter-laboratory standard deviation is shown on the vertical axis. The sera are shown on the horizontal axis.
0.99 means: Excluding assays with a correlation coefficient below 0.99.
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0 means: Including all assays.
a, b and e correspond to serum samples α, β and ε.

Collaborative Study for the Validation of Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use Summary of All Three Phases

Collaborative Study for the Validation of Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use - Summary of All Three Phases

Project leaders: Randi Winsnes[1], Coenraad Hendriksen[2]

1. INTRODUCTION

An international collaborative study on the evaluation of alternative methods for potency testing of tetanus toxoid vaccines for human use started in March 1996. This study was performed under the aegis of the Biological Standardisation Programme of the European Directorate for the Quality of Medicines (EDQM)[3] and supported by the Council of Europe, the European Commission and the European Centre for the Validation of Alternative Methods of the European Commission (ECVAM/IHPC/JRC). The study was divided into two projects (internal numbers BSP019 and BS035), and has been performed to validate two serological assays, Enzyme-Linked Immunosorbent Assay (ELISA) and Toxin Binding Inhibition test (ToBI) as alternatives to the direct challenge procedure for potency testing of tetanus toxoid vaccines for human use [Ph. Eur. monograph *Tetanus vaccine (adsorbed) (0452)*] for consistency testing of production (multiple-dilution serological assays) and for routine batch release testing (single-dilution serological assays).

The collaborative study was designed to demonstrate the relevance and reliability of the serological assays. Guinea pigs were immunised with tetanus toxoid vaccines from different manufacturers. The vaccines represented various types of combined products including one product of borderline quality. The procedure specified in the Ph. Eur. Chapter *2.7.8. Assay of tetanus vaccine (adsorbed)* was followed with two exceptions:

- The time interval between immunisation and challenge was extended from 4 to 6 weeks in order to achieve a good correlation between the various assays (based on data from the pre-validation study and from the literature).
- In order to allow comparison of the serological methods with the direct challenge method, a blood sample from each animal was taken 2-3 days before challenge for titration of specific antibodies.

Parameters that were analysed included:

a) correlation of vaccine potencies obtained by direct challenge test and by the serological assays,

b) prediction of survival based on antibody concentrations obtained in ELISA and ToBI, respectively, compared with actual survival/death.

c) correlation of antibody concentrations in ELISA, ToBI and Toxin Neutralisation Test in mice (TNT).

d) Assay repeatability and reproducibility study by titration of a panel of 28 serum samples in 23□laboratories.

2. DESIGN AND OBJECTIVES OF THE COLLABORATIVE STUDY

To allow interim evaluation of test results and to monitor study progress, the collaborative study was divided into four consecutive phases each with the following objectives:

- *Prevalidation*:

  To select the best time interval between immunisation and bleeding.

  To evaluate the use of tetanus toxoid as an alternative to tetanus toxin in ToBI.

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[2] Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven and Universiteit Utrecht, Utrecht (NL).

[3]Abbreviations: **BRP**: European Pharmacopoeia Biological Reference Preparation, **Cl.**: Clostridium, **ECVAM/IHPC/JRC**: European Centre for the Validation of Alternative Methods of the Institute for Health and Consumer Protection, Joint Research Centre, **EDQM**: European Directorate for the Quality of Medicines, **ELISA**: Enzyme-linked immunosorbent assay, **GMP**: Good manufacturing practice, **IS**: International standard; **IU**: International unit, **OD**: Optical density, **OMCL** : Official medicines control laboratory, **PC_{50}**: Dose protecting 50 % of the animals, **Ph. Eur.**: European Pharmacopoeia, **SOP**: Standard operating procedure, **ToBI**: Toxin binding inhibition test, **TNT**: Toxin neutralisation test in mice, **WHO**: World Health Organization.

- *Phase I (three laboratories):*

  To assess the correlation between potencies obtained in the direct challenge test and in the serological test for five tetanus toxoid vaccines of different composition.

  To assess the correlation between antitoxin titres of individual guinea pigs obtained by ELISA, ToBI and by TNT.

  To assess the correlation between protection after challenge and the antitoxin titres obtained by ELISA and ToBI.

  To analyse intra- and interlaboratory variation in ELISA and ToBI.

- *Phase II :*

  This phase was divided into two separate sub-studies, indicated as Phase IIa (three laboratories) and Phase IIb (two laboratories):

  Phase IIa: To confirm the results of the Phase I study in three additional laboratories. The study protocol was like the Phase I protocol except that the TNT was not performed.

  Phase IIb: Phase II was extended with an additional study for the following reasons. Firstly, part of the data of the Phase IIa study was invalid and could not be used. Secondly, half-way the Phase IIa study a tetanus toxoid of borderline quality became available. Furthermore, a combined tetanus vaccine, containing an acellular pertussis component, was included and, for comparison, one of the vaccines of Phase I and Phase IIa (vaccine F). The study design of Phase IIb was identical to that of Phase IIa.

- *Phase III:*

  To assess intra- and inter-laboratory variation in ELISA and ToBI test and to evaluate protocol transfer.

3. SUMMARY OF OUTCOME

After statistical analysis of the data of the collaborative study the following conclusions were drawn:

- Within each laboratory vaccine potencies estimated by the direct challenge test were in good agreement with potencies estimated by ELISA and ToBI test for all vaccines including the borderline product. The 95% confidence intervals of potencies obtained by ELISA and ToBI testing were only slightly smaller than those obtained by the direct challenge test. (This is most likely due to the fact that the immunising doses in this study were chosen in order to get optimal results from the challenge assay and were not always optimal for the serological assays. In general the 95% confidence intervals obtained by serological methods, using optimal doses for parallel-line assay calculation, are found to be smaller than for non-serological animal methods.)
- Potencies obtained sometimes differed substantially between the laboratories, both in the direct challenge assay and in the serological tests. (This might be related to the guinea-pig strain, the immunological status and health condition of the animals, differences in diet and environment.) Laboratories were in close agreement when rank orders of potencies of the test vaccines, estimated by challenge, ELISA and ToBI methods, were compared.
- For individual serum samples, a good correlation was observed between the predictive value of antitoxin concentration and survival after challenge; for ELISA: 90.5-94.6% and for ToBI assay: 91.8-97.0%. The range of PC_{50} serum antitoxin levels in the guinea pigs was comparable to the lowest antitoxin concentration which is, in general, considered to be protective in humans (0.01 IU/ml).
- For pooled serum samples, an overall excellent correlation was observed between antitoxin concentrations obtained by TNT and obtained by the *in vitro* serological tests; for ELISA : r = 0.93 – 0.99 and for ToBI assay : r = 0.97 – 0.99.
- Intra-laboratory variation for ELISA and ToBI test was acceptable (on average 0.14 and 0.20, respectively).
- Inter-laboratory variation for ELISA and ToBI test was acceptable. The difference between any two laboratories was generally less than 2-fold and only rarely more than 3-fold.

- The ratio between ELISA and ToBI test (correlation coefficient: 0.90) deviated from 1. The degree of deviation seems to depend on the particular serum sample. However, the ratio given here indicates the general trend.

Results of phase I-II of the collaborative study are published in Pharmeuropa (BIO 2000-1, August 2000, pp. 85-124 and Special Issue October 2000, pp. 29-61). The results of phase III are published in this issue (pp 3-44).

4. EVALUATION OF THE SINGLE-DILUTION TEST

Based on the data of the collaborative study (Phase I-II), the perspectives for a single-dose assay were explored (Pharmeuropa Special Issue, October 2000, pp.135-140). The single dilution test allows demonstration that the product under study meets the minimum requirement in IU/dose rather than assessment of the relative potency and 95% confidence intervals. It was shown that the number of animals and the number of replicate ELISA and ToBI assays might be dramatically reduced if the potency test is replaced by a limit test in routine situations. This may demand only one dilution per vaccine, and only one determination by ELISA or ToBI. Since the potencies of the vaccines are usually well above 40 IU/dose, a highly significant result may be achieved by using a limited number of animals. Another advantage of this method is that there is no absolute need to include a calibrated reference serum in the assay, because it is only used for cross-reference between plates.

All except one of the vaccines included in the study were of acceptable quality. It was therefore possible to show that an unacceptable or borderline vaccine would fail the test.

To study the suitability of the single-dose assay, a simulation study has also been performed using data from phase I of the collaborative study (Pharmeuropa Special Issue, October 2000, pp.141-144). Although data from the borderline vaccine was not included in this simulation study, the results seem promising for replacement of the direct challenge assay by a serological single-dilution assay.

The results of these studies confirm the conclusion of previous studies that replacing the multi-dilution test by a single dilution test is acceptable, the number of animals to be defined on a case by case basis.

5. PERFORMANCE OF SEROLOGICAL ASSAYS

Although Phase III study data are too limited for proper evaluation of the robustness of the in-house methods used for comparison, it is recommended from analyses of the data received that ELISA and ToBI test should be performed using the Standard Operating Procedures (SOP) used in the three phases of the collaborative study. These SOPs are published in this issue (pp. 79-92). For the purpose of in-house validation of the serological assays, the EDQM will provide Official Medicines Control Laboratories (OMCLs) and manufacturers with critical reagents for ELISA and ToBI test. For ELISA, tetanus toxoid and tetanus antitoxin (BRP) are the critical reagents necessary. For ToBI, tetanus toxin, equine anti-tetanus IgG, peroxidase-conjugated, equine anti-tetanus IgG and tetanus antitoxin (BRP), are the critical reagents.

6. IMPLEMENTATION OF SINGLE-DILUTION TEST BASED ON SEROLOGY

For proper in-house implementation of the multi-dilution tests, based on serology, results of at least 3 independent batches, from different bulks, will have to be analysed and submitted to licensing authorities by manufacturers. The choice of the design (dilutions used) of the multi-dilution test must be done so as to permit transition to the single-dilution test. Data of the multi-dilution tests can be used for computer simulation to evaluate the number of animals required.

The multi-/single-dilution tests will have to include an in-house reference vaccine having the same formulation as the test vaccine and being calibrated against the relevant WHO IS/Ph. Eur. BRP[4].

[4] Current standard : common 3rd IS/BRP Batch 2 for tetanus vaccine (adsorbed) Catalog Nr. T0400000

7. MONITORING OF THE SINGLE DOSE SEROLOGICAL ASSAY

Monitoring focuses on consistency in a) response of the animals and b) performance of the serological assays.

The following parameters are identified to monitor for test consistency:

- mean and standard deviation (SD) of antitoxin scores of the serum samples obtained after immunisation with a fixed dose of the in-house reference vaccine
- maximum optical densities (OD) and background ODs,
- antitoxin scores, or antitoxin titres of run controls (positive and negative serum samples).

Specific *Cl. tetani* guinea pig antiserum Ph. Eur. BRP Batch 1[5] can be used as the positive run control, and in-house positive serum controls may be calibrated against this BRP.

Parameters are monitored by the use of control charts.

8. CONCLUSIONS OF THE COLLABORATIVE STUDY

Considering a) the equivalence in relevance and reliability between the serological potency tests ELISA or ToBI and the challenge test, b) the suitability of the single dilution test and c) consistency in production, it is recommended to replace the quantitative direct challenge method by a single-dilution qualitative *in vitro* serological method for potency testing of tetanus vaccines for human use for routine batch release by manufacturers and OMCLs. The use of either ELISA or ToBI test should be a decision taken either by the quality control laboratory responsible for batch release, the manufacturer or OMCL.

The following exceptions are specified:

- in-house validation of the serological *in vitro* potency test,
- demonstration of consistency in production and
- calibration of in-house reference preparations.

In these cases the multi-dilution serological test should be performed.

9. ADVANTAGES OF THE SEROLOGICAL BASED POTENCY TEST

Compared to the multi-dilution direct challenge assay, the proposed *in vitro* serological procedures have a number of advantages:

a) reduction in the number of animals used (about 80% in a single-dilution test),

b) animal welfare (no challenge followed by severe distress to the animals),

c) improved safety for the staff in the animal laboratory (no toxin injection in the animals),

d) allowing for testing of more components of combined vaccines in one test (to be examined further for the diphtheria toxoid component),

e) storage of “biological results” (GMP: traceability),

f) possibility for exchange of serum samples for analyses and

g) improved monitoring for consistency in testing.

[5] Catalog Nr. C2424550

Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use: Protocols of Serological Assays Used in the Collaborative Study

Serological Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use: Protocols of Serological Assays Used in the Collaborative Study

INTRODUCTORY NOTE

This section describes in detail the protocols for the serological assays for potency testing of tetanus vaccines for human use that were performed in the international collaborative studies (BSP019, BSP035) organised by the European Directorate for the Quality of Medicines (EDQM)[1]. Any deviation from the protocol was requested to be reported.

From the results of the third phase of the collaborative study (BSP035) it could be concluded that the Enzyme-Linked Immunosorbent Assay (ELISA) and Toxin Binding Inhibition test (ToBI) protocols described herein enable titration of the tetanus antitoxin content of guinea pig sera with satisfactory repeatability and reproducibility.

In consequence the protocols provided here should be used as models to develop in-house Standard Operating Procedures (SOP) for serological potency assays of tetanus vaccines. In addition, SOPs designed for monitoring the production consistency (multiple-dilution serological assays) and for routine batch release (single-dilution serological assays) will have to include the use of appropriate reference materials to monitor the variability of the *in vivo* and *in vitro* part of the assays; furthermore the method will have to be validated using standardised reagents provided by the EDQM upon request.

For details on reference materials and reagents see Collaborative Study Report - Part 2 and Summary of all Three Phases, published in this issue.

[1] Abbreviations: **ABTS**: 2,2 Azino-di-ethylbenzthiazoline sulphonate, **AU**: Antibody unit, **BRP**: European Pharmacopoeia Biological Reference Preparation, **BSA**: Bovine serum albumin, **EDQM**: European Directorate for the Quality of Medicines, **ELISA**: Enzyme-linked immunosorbent assay, **ERTA:** Tetanus vaccine (adsorbed) Ph. Eur. BRP, **HRP:** Horseradish peroxidase, **i.m.**: Intra-muscularly, **i.p**.: Intra-peritoneal, **Lf:** Limes flocculation, **PBS**: Phosphate buffered saline, **OD:** Optical density, **PBST**: Phosphate buffered saline with Tween, **Ph. Eur.**: European Pharmacopoeia, **PS** : Polystyrene, **RIVM**: Rijksinstituut voor Volkgezondheit en Milieu, **s.c.:** subcutaneously**, SOP** : Standardised operating procedures, **ToBI**: Toxin binding inhibition test, **TMB**: Tetramethylbenzidine, **TNT**:Toxin neutralisation test in mice, **TT**: Tetanus toxin, **WHO**: World Health Organization.

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A. DESCRIPTION OF POTENCY TESTING IN GUINEA PIGS

48 guinea pigs, randomly subdivided into four groups of twelve animals, are used per vaccine.

Each subgroup is immunised with a dilution of Tetanus vaccine (adsorbed) Ph. Eur. BRP (ERTA) or of test vaccine.

Forty-two days after immunisation an individual blood sample is taken by an appropriate route. Blood sampling from *vena saphena* is preferred but if heart puncture is permitted and expertise in this technique is available, heart puncture may also be used. In the protocol based on heart puncture, all animals are immunised on the same day and consequently bled and challenged on the same day. Compared to heart puncture, blood sampling from the *vena saphena* is more laborious, thus requiring a modified immunisation schedule. When blood is drawn from the *vena saphena* the following procedure is recommended (NB: this schedule can also be applied for the heart puncture approach when blood sampling of all animals at the same day is not possible):

The day of immunisation (day 0) is the same for all of the animals of the same group. However, blood sampling is performed on three consecutive days (day 40, 41 and 42), each day on four new animals of each dilution group.

Vaccine potencies are calculated by probit analysis based on the individual antibody concentrations (ELISA and ToBI test). To allow probit analysis on ELISA and ToBI test data, individual antibody concentrations are transformed to dichotomised values.

Documentation

- Strain and breeder and breeders address of the guinea pigs;
- Sex and batch number of the guinea pigs;
- Data of microbiological control of the guinea pigs;
- Dates and specifications of sampling for microbiological control;
- Cage numbers and identification;
- Room number, temperature and humidity registration;
- Batch number of diet and bedding;
- Details of the material under test;
- Dates of weight of the guinea pigs at the beginning of the study;
- Date of immunisation;
- Date of drawing a blood sample of all the immunised guinea pigs and of the 8 challenge control guinea pigs;
- Data/results of microbiological control;
- Licensee.

1. Animals

1.1 Use 12 healthy guinea pigs of one sex, or an equal distribution of both, within the weight range 250-350 g, for each vaccine dilution.

1.2 Use 4 guinea pigs of the same group used for immunisation purposes. These animals will not be immunised and will be bled to produce a negative control serum sample.

1.3 Animals are weighed at the beginning of the experiment and weekly thereafter.

1.4 Animals are randomly distributed into the cages. All animals shall be identified individually to enable comparison of challenge result with antibody titre for each individual animal (cf. Work Sheet No. 1: Assay of tetanus vaccines).

2. Preparation of tests and reference vaccines

2.1 Prepare, in a safety cabinet, 4 two-fold dilutions for each of the test vaccines and the reference vaccine which shall be administered that day. Use 0.9% sterile sodium chloride saline (referred to as "saline") as the diluent. Prepare vaccine dilutions not more than one hour before immunisation.

NB: all the test vaccines and the Ph. Eur. BRP contain solid adjuvant to which most of the tetanus toxoid is adsorbed. It is therefore essential to mix properly **immediately before** performing dilutions! Avoid formation of air bubbles when mixing!

2.2 Recommended dilutions are as follows:

Reference vaccine (Tetanus Vaccine (adsorbed) Ph. Eur. BRP ref: T0400000), named ERTA

Reconstitute one freeze-dried ampoule of Ph. Eur. BRP for tetanus vaccine (adsorbed) with 2.00□ml of sterile distilled waterTransfer to a container filled with 30 ml of saline and rinse three times. Each ampoule contains 250 IU, therefore this gives a □7.81 IU/ml solution in a total volume of 32 ml:

1 amp. of ERTA	+	2.0 ml of sterile distilled water + 30 ml of saline	→	**7.81 IU/ml**	Solution□R1
15 ml Solution R1	+	15 ml saline	→	**3.91 IU/ml**	Solution R2
15 ml Solution R2	+	15 ml saline	→	**1.95 IU/ml**	Solution R3
15 ml Solution R3	+	15 ml saline	→	**0.98 IU/ml**	Solution R4

3. Immunisation of guinea pigs

Immunisation is performed using 4 dilutions of each of the vaccines. Use Work sheet No.1 - Assay of tetanus vaccines for the reporting of the immunisation details.

WORK SHEET NO. 1 - ASSAY OF TETANUS VACCINES

Vaccine: .....

Dilution No/Cage Group No.: .....

Cage No.	Animal No.	Identification	Date of	
			Immunisation	Bleeding
	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	11			
	12			
Attestation:				

Inject each immunisation group (n=12) of guinea pigs with one dilution of test or reference vaccine. The cages are numbered consecutively, and so are the animals.

Inject 0.5 ml subcutaneously (s.c.) in the skin fold of the axial region of each guinea pig, using a 2.5 ml syringe fitted with a 23 G × 1" needle. Tilt the syringe gently between the injections in order to maintain a homogeneous suspension.

4. Blood sampling

Blood sampling is performed on day 42 after immunisation by heart puncture or bleeding from *vena saphena*. If blood sampling in one day is not possible, then blood samples should be taken on three consecutive days (day 40, 41 and 42 after immunisation), at the rate of 4 animals of each vaccine dilution group per day.

4.1 Blood sampling by cardiac puncture

Animals are anaesthetised with a mixture of Ketamine/Xylazine/Atropine (KRA), approx. 0.05 ml/100 g body weight, intra-muscularly (i.m.). The ratio Ketamine : Xylazine : Atropine is 4 : 1.25 : 0.5. (N.B.: differences in sensitivity for KRA can be expected between the strains of guinea pigs. Before the blood sampling, information should be obtained on the sensitivity).

4.1.1 Blood sampling by cardiac puncture for volumes up to 2.5 ml

N.B.: because of the potential harmful sequelae to this procedure, cardiac puncture shall only be performed if expertise in this technique is available.

Anaesthetise the animal i.m. and wait until the animal is in a state of deep anaesthesia. Place the animal on its back on a table and stretch the front legs in a cranial direction. Use a 10 ml syringe with a 21G × 1.5 needle. The heart is reached by piercing the left ventricle through the chest wall at the sixth intercostal space, about one third of the ventral-dorsal distance. The puncture site can be confirmed manually, being the site at the chest with the strongest heart-beat. Puncture the skin and direct the needle in a cranio-dorsal direction. Draw the blood slowly in the syringe to a maximum of 2.5 ml. Remove the syringe carefully. Observe the animal for recovering from anaesthesia and for possible indications of cardiac tamponade (e.g. tachypnoea).

4.1.2 Cardiac puncture for terminal bleeding

Anaesthetise the animal with a mixture of KRA, approx. 0.05 ml/100 g body weight (see also 4.1.1), i.m. and wait until the animal is in a state of deep anaesthesia. Place the animal on its back on a table and stretch the front legs in a cranial direction. Use a 10 ml syringe with a 21G x 1.5 needle. The heart is reached by piercing the left ventricle through the chest wall at the sixth intercostal space, about one third of the ventral-dorsal distance. The puncture site can be confirmed manually, being the site at the chest with the strongest heart-beat. Puncture the skin and direct the needle in a cranio-dorsal direction. Draw the blood slowly into the syringe. Usually a total volume of 10-15 ml can be obtained. Remove the syringe. Check if the animal is dead, otherwise kill the animal by cervical dislocation or by intra-peritoneal (i.p.) injection of an overdose of pentobarbitone (100-150 mg/kg body weight).

4.2 Blood sampling from the vena saphena

Shave the thigh of the hind legs of the guinea pigs 1 to 3 days before the blood sampling. Shave thoroughly, particularly around the hollow of the knees where the *vena saphena* is most easily observed. Repeat the shaving on the morning of the blood sampling or the day before. In due time before the blood drawing, e.g. 15-20 min. before, the guinea pigs are given Hypnorm®, "Janssen" injection anaesthesia, 0.1 ml s.c. per 100 g body-weight, in a skin-fold at the top of the thigh, using a 1 ml syringe fitted with a 23 G × 1" needle.

For *vena saphena* puncture it is essential to hold the guinea pig properly, to push the knee joint to make the leg stretch out and to pinch or massage the musculature on the back of the thigh and around the knee, in order to let the *vena saphena* be filled with as much blood as possible. Grease the skin at the site of puncture with Dow Corning Valve Seal. Pierce the vein carefully with a 21 G × 1 1/2" needle. The blood then starts to drip and can be collected directly into centrifuge tubes.

The leg must be held tight all the time in order to maintain stasis. Massage during the blood taking may be advantageous. Preferably 2.5 ml of blood is collected from each guinea pig. A second puncture of *vena saphena* of the same hind leg thigh may be necessary. Alternatively, *vena saphena* puncture of the other hind leg thigh for blood sampling can be performed.

Use sterile tubes for blood sampling. Tubes containing a gel with a clot activator in order to make a rapid separation of the blood cells are appropriate.

5. Preparation of serum specimens

Procedure

5.1 When filled with blood, the vial is inverted six times.

5.2 The vial is left at 37 °C for 2 h followed by 2 h at + 4 °C.

5.3 Centrifuge for 20 min at 800 *g* at room temperature.

5.4 Transfer the serum into sterile tubes (not less than 40 % yield of serum is obtained by this procedure) and stored below - 20 °C.

B. GENERAL INFORMATION ON SEROLOGICAL ASSAYS

Serum samples obtained should be stored below - 20 °C. Before assaying they should preferably be inactivated by incubation at 56 °C for 30 min. Frequent freezing and thawing as well as microbiological contamination should be prevented. To ensure asepsis manipulations are best done in a laminar air flow cabinet.

Each individual serum sample should be titrated in triplicate in ELISA (chapter C) or ToBI (chapter D) against a guinea pig standard tetanus antitoxin, on three different days. Therefore the guinea pig standard should be included on every plate.

Apart from individual serum samples, from each vaccine dilution, serum pools are generated by mixing equal volumes of the respective individual serum samples. Each of the serum pool samples should be titrated in ELISA (chapter C) or ToBI (chapter D) against a guinea pig standard tetanus antitoxin, on three different days.

All tests should be performed according to the procedures given in the following annexes. For the two test systems, the reagents and materials that are used are divided into three categories:

First category: these items are essential for reasons of test standardisation and should therefore be used. They were supplied by the organising institutes in Phases I and II of BSP019.

Second category: these buffers and solutions should be of the same composition as described.

Third category: items listed are preferred. Reagents of other manufacturers but with the same specifications can equally be used.

For the washing step, each procedure that has demonstrated to wash effectively can be used. Three methods are commonly used: automatic plate washers, fountain washers and hand-washing. A description of the procedure should be given on the working protocol.

C. ELISA FOR THE ESTIMATION OF TETANUS ANTIBODIES IN GUINEA PIG SERUM SAMPLES

Principle

This protocol describes the ELISA test for the estimation of tetanus antibodies in guinea-pig sera obtained in phase I. It is based on the NIBSC SOP entitled “ELISA for Anti-Tetanus Antibody in Guinea-pig Sera” May 1996 version. Sera should be titrated on three different days. A guinea pig standard tetanus antitoxin (standard GPTA-6) must be included on each plate.

On an ELISA plate, coated with tetanus toxoid, twofold dilution series of standard- and test sera are made. After addition of a peroxidase conjugated rabbit-anti-guinea pig IgG, the amount of antibodies bound to the coat can be visualised by the addition of a substrate. The antibody titre can be estimated by comparing the dose response curves, based on optical densities (OD), of test and standard serum.

1. Materials

Materials and reagents for the ELISA can be divided into three categories.

First category (1.1 to 1.4):

1.1 ELISA plates, NUNC-immunoplate, Maxisorp, Cat. No. 442404.

1.2 Standard guinea-pig tetanus anti-serum GPTA-6, 0.08 IU/ml (obtained by TNT).

1.3 Rabbit-anti-guinea pig horseradish peroxidase (HRP) conjugate (Sigma A5545).

1.4 Tetanus toxoid, lot MWC S208/A/F-6, 2567 Lf/ml, NIBSC.

Second category (1.5 to 1.12):

1.5 Carbonate coating buffer pH 9.6

Requisites:

1. Na_2CO_3, anhydrous	1.59 g	(0.015 M)
2. $NaHCO_3$	2.93 g	(0.035 M)
3. Distilled water	1 L	

Preparation: Stir until the solids have dissolved. Dispense into 150 ml glass bottles and sterilise by autoclaving at 121 °C for 15 min.

1.6 Phosphate Buffered Saline pH 7.4 (PBS)

Requisites:

1. NaCl	80.0 g	(1.37 M)
2. KH_2PO_4	2.0 g	(0.015 M)
3. $Na_2HPO_4.2H_2O$	14.3 g	(0.08 M)
4. KCl	2.0 g	(0.027 M)
5. Distilled water	1 L	

Preparation: Stir the mixture until the solids have dissolved. This is a 10-times concentrated buffer which needs to be diluted 1/10 before use. Store at room temperature to prevent crystallisation.

1.7 Citrate buffer

Requisites:

1. $C_6H_8O_7.^1H_2O$	10.51 g	(0.05 M)
2. Water (Milli Q), or distilled water	1 L	

Preparation: Dissolve citric acid and adjust to pH 4.0 with 10 M NaOH.

1.8 *Washing buffer PBST*: PBS containing 0.05 % Tween 20 (1.13).

1.9 *Diluent*: PBS containing 0.05 % Tween 20 (1.13) and 2.5 % dried skimmed milk (1.14).

1.10 *Block-buffer: same as diluent*; PBS containing 0.05 % Tween 20 (1.13) and 2.5 % dried skimmed milk (1.14).

1.11 *Negative control buffer*: carbonate coating buffer pH 9.6 (1.5) containing 2.5 % dried skimmed milk (1.14).

1.12 *Substrate*: 2,2 Azino-di-ethylbenzthiazoline sulphonate (ABTS) (1.15) in 10 mg tablets. Dissolve one tablet of ABTS (10 mg) in 20 ml citrate buffer. Immediately before use add 5 µl of a 30 % hydrogen peroxide solution (1.16).

[1] Citric acid

Third category (1.13 to 1.17):

1.13 Tween 20.

1.14 Skimmed milk (Marvel).

1.15 ABTS (Sigma A 9941).

1.16 Hydrogen peroxide 30 % H_2O_2 (Merck 10128).

1.17 Distilled water.

1.18 Negative control serum, being a pooled serum sample, obtained from non immunised guinea pigs (e.g. 4) of the same batch of guinea pigs used for immunisation purposes.

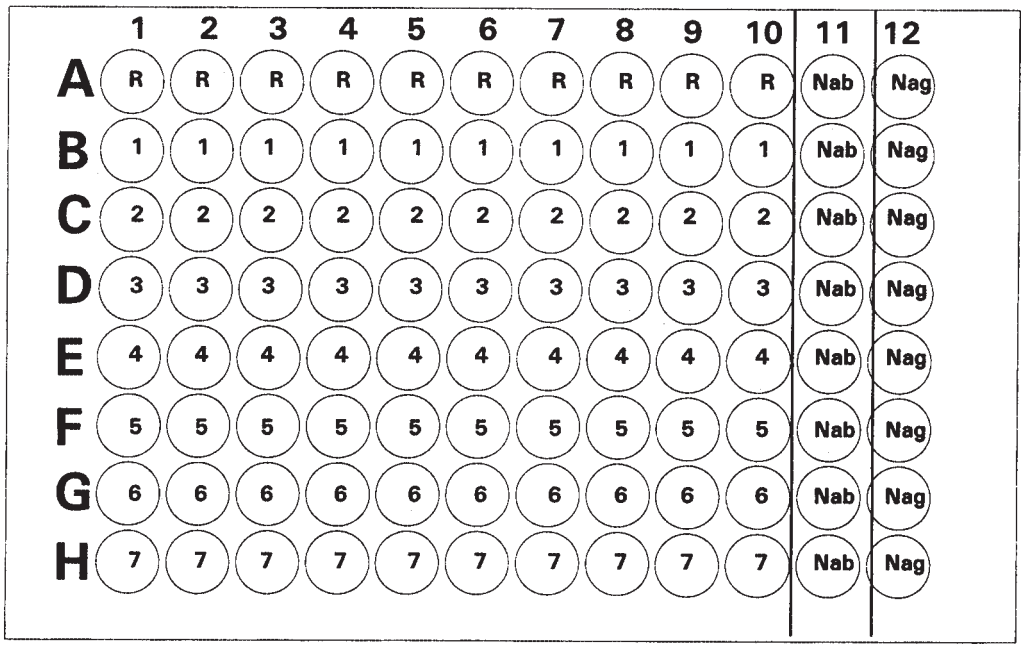
2. Performance

In this study, plates should be coated immediately before use (night before).

2.1 Prepare a solution of 0.5 Lf/ml of tetanus toxoid (1.4) in carbonate coating buffer (1.5).

2.2 Coat all wells of the ELISA plates (1.1) with 100 µl volumes of tetanus toxoid solution (2.1).

2.3 Incubate the plates overnight at 4 ºC in a humid container. Due to the temperature gradient don't stack more than four plates on top of each other.



R = standard GPTA-6
Nag = negative control serum sample (antigen with negative control serum)
Nab = negative antibody control (antigen but no primary antibody)
1-7 = test sera

Next day

2.4 Wash the ELISA plates thoroughly(1) with washing buffer (1.8).

2.5 To minimise non-specific interactions block the plates by addition of 100 µl of block-buffer (1.10) to all the wells.

2.6. Incubate the plates for 1 hour at 37 ºC in a humid container.

ELISA:

2.7 Wash the ELISA plates thoroughly(1) with washing buffer (1.8).

2.8 **Except the wells of columns 1 and 12** fill all wells of the plate with 100 µl of diluent (1.9).

2.9 Dilute standard serum GPTA-6 by 1/10 in a tube (Eppendorf 1.5 ml). Ideally 1.4 ml Micronic tubes are used. An independent dilution of the standard should be made for each plate. Potency of GPTA-6 is 0.08 IU/ml.

2.10 Dilute each test sample by 1/10 in a tube (Eppendorf 1.5 ml). Ideally 1.4 ml Micronic tubes are used.

2.11 **On each plate** add 100 µl of diluted GPTA-6 to well A1 and A2.

2.12 Introduce 100 µl of diluted test samples to wells 1B-H and 2B-H as appropriate.

2.13 Introduce 100 µl of the 1/10 diluted negative control serum pool (1.18.) to all wells of column 12.

2.14 Where Micronic tubes have been used introduction of diluted standard and test samples can be done by using an 8-channel multipipette. In this way the immediate binding of high titre sample is avoided.

2.15 Use a multichannel micropipette. Make twofold dilution series across the plate by mixing intensively the wells of column 2 (five times up and down) and transfer 100 µl of each mixture to the adjacent well in column 3 and mix intensively.

Make a similar dilution and transferring process from the wells in column 3 up to and including the wells of column 10. Avoid air bubbles in the tips! Discard 100 µl from the last column of wells (column 10). Every well on the plate should now contain 100 µl.

2.16 Incubate for 2 hours in a humid atmosphere at 37 °C.

2.17 Wash the ELISA plates thoroughly with washing solution (1.8).

2.18 Make a dilution of the conjugate rabbit anti-guinea pig HRP (1.3) of 1/2000 in diluent (1.9). Add 100 µl of the dilution to all wells.

2.19 Incubate for 1 hour in a humid atmosphere at 37 °C.

2.20 Wash the ELISA plates thoroughly with washing solution (1.8)

2.21 Prepare substrate solution shortly before use:

Substrate: Dissolve one tablet of 10 mg ABTS (1.15) in 20 ml citrate buffer (1.7). Immediately before use add 5 µl of 30 % hydrogen peroxide solution (1.16).

2.22 Add 100 µl of substrate to each well.

2.23 Leave for 30 min at room temperature, protected from light.

2.24 Read the plates at 405 nm in the same plate-order as the substrate has been added.

2.25. Record the absorbance.

D. TOBI TEST FOR THE ESTIMATION OF TETANUS ANTIBODIES IN GUINEA PIG SERUM SAMPLES

Principle

This protocol describes the ToBI test for the estimation of tetanus antibodies in guinea-pig sera obtained in phase I and II studies. It is based on the RIVM SOP. Sera should be titrated on three different occasions. A guinea pig standard tetanus anti-toxin (standard GPTA-6) must be included on each plate.

On a polystyrene micro-titration plate, twofold dilution series of standard- and test serum are made in phosphate buffered saline (PBS). After addition of the test dose of tetanus toxin, the serum/antigen mixtures are incubated overnight. The following day “non-neutralised” toxin is determined on a tetanus antitoxin coated ELISA-plate. The antibody titre is estimated by comparing the dose response curves, based on optical densities, of test and standard serum.

1. Materials

Materials and reagents for the ToBI test can be divided into three categories.

First category (1.1 to 1.7):

1.1 Polystyrene (PS) round-bottomed micro-titration plates, rigid (Greiner 650101).

1.2 Immunoassay (ELISA) micro plates, flat bottomed (Greiner 655092).

1.3 Tetanus toxin, lot T417, 300 Lf/ml (RIVM).

1.4 Standard guinea-pig tetanus anti-serum GPTA-6, potency 0.08 IU/ml (calibrated by TNT).

1.5 Equine anti-tetanus IgG, lot GTL34, 200 AU/ml (RIVM).

1.6 Equine anti-tetanus IgG, peroxidase conjugated, (HATPO, lot 32-33) (RIVM).

Second category (1.7 to 1.13):

1.7 Carbonate buffer, pH 9.6

Requisites:

1. Na_2CO_3, anhydrous	1.5	g
2. $NaHCO_3$	2.39	g
3. NaN_3	0.2	g
4. Distilled water	1	L

Preparation: Dissolve 1, 2 and 3 in 4. N.B.! adjust to pH 9.6. Autoclave for 20 min at 120 °C.

1.8 Sodium acetate buffer, pH 5.5

Requisites:

1. CH_3CO_2Na, anhydrous	90.2	g
2. Saturated $C_6H_8O_7$[1]. H_2O solution	x	ml
3. Distilled water	1	L

Preparation: Dissolve 1 in most of 3. Adjust to pH 5.5 using 2 and fill up to 1 litre with 3.

1.9 Phosphate Buffered Saline (PBS), pH 7.2

Requisites:

1. NaCl	135.0	g
2. Na_2HPO_4. $2H_2O$	20.55	g
3. NaH_2PO_4. H_2O	4.80	g
4. Distilled water	up to 15	L

Preparation: Dissolve 1, 2 and 3 in a part of 4 and fill up to 15 litres. Autoclave for 60 min at 100 °C.

1.10 *Diluent*: PBS containing 0.5 % bovine serum albumin (BSA) (1.15) and 0.05 % Tween 80 (1.14).

1.11 *Block-buffer*: PBS containing 0.5% BSA (1.15).

1.12 Tetramethylbenzidine (TMB) (1.16) solution in ethanol (1.18) (6 mg/ml, soluble within 30-40 min at room temperature).

1.13 *Substrate*: 90 ml of distilled water
10 ml of 0.1M sodium acetate buffer (1.8)
1.67 ml of TMB solution in ethanol (1.12)
and 20 µl of a 30 % solution of H_2O_2 (1.17)

[1] Citric acid

Third category (1.14 to 1.21):

1.14 Tween 80 (Merck 822187)

1.15 Bovine serum albumin (BSA, Boseral Organon Teknika)

1.16 Tetramethylbenzidine (TMB, Sigma T2885)

1.17 Perhydrol 30 % H_2O_2 (Merck art. 8597)

1.18 Ethanol 96 %

1.19 Distilled water

1.20 2 M H_2SO_4

1.21 Washing solution: tap water containing 0.05 % Tween 80 (1.14)

2. Performance

2.1 Block the round-bottomed polystyrene (PS) micro-titration plates (1.1) for pre-incubation of serum dilutions and antigen mixtures, by filling each well with 150 µl block-buffer (1.11). Cover the plates with a lid or sealer.

2.2 Incubate for 1 hour at 37 °C in a humid atmosphere.

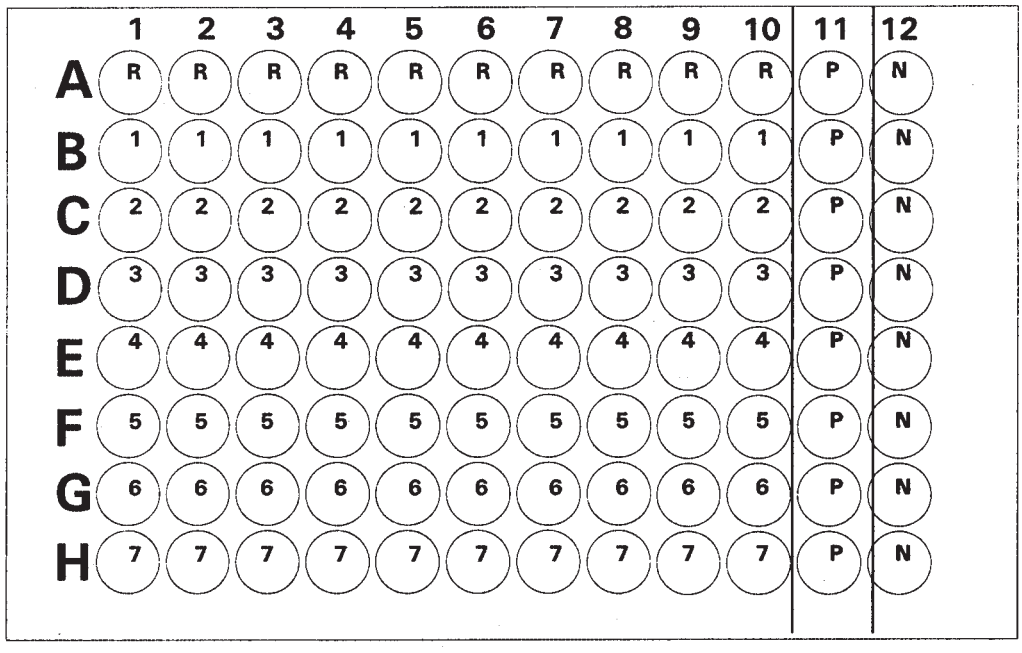
2.3 Wash the plates thoroughly with washing solution (1.21)

2.4 Fill all wells of the PS micro-titration plate with 100 µl PBS (1.9)

2.5 On each plate add 100 µl of GPTA-6 standard serum (undiluted) to well A1 (see template).

2.6 Add 100 µl of the undiluted sera under test to the wells B1 to H1 (see template).

2.7 Use a multi-channel micropipette. Make twofold dilution series by mixing intensively (five times up and down) and transfer 100 µl of each mixture to the adjacent well in column 2 and mix intensively.



R = standard GPTA-6
P = positive control
N = negative control
1-7 = test sera

Make a similar dilution and transferring process from the wells in column 2 up to and including the wells of column 10. **Avoid air bubbles in the tips!** Discard 100 µl from the last column of wells.

2.8 Dilute the tetanus toxin to a concentration of 0.1 Lf/ml in PBS.

2.9 Add 40 µl quantities of tetanus toxin (0.1 Lf/ml) to all wells except those of column no. 12. The wells of row 11 are used as a positive control.

2.10 Add 40 µl quantities of PBS (1.9) to the wells of column 12 which functions as a negative control.

2.11 Shake the plates gently and cover them with lids.

2.12 Coat the ELISA plates. Immediately before use make a dilution of the equine-anti-tetanus IgG (1.5) to a concentration of 1.0 AU/ml in carbonate buffer (1.7). Add 100 µl to all wells and cover the plates with lids.

2.13 Incubate the plates of point 11 and 12 overnight at 37 °C in a **humid** atmosphere. Due to temperature gradient don't stack more than four plates on top of each other.

Next day

2.14 Wash the ELISA plates from point 12 thoroughly with washing solution (1.21)

2.15 Block the ELISA plates by filling each well with 125 µl of block-buffer (1.11).

2.16 Incubate for **1 hour** in a humid atmosphere at 37 °C.

2.17 Wash the ELISA plates thoroughly with washing solution (1.21)

2.18 Transfer 100 µl of the pre-incubation mixture from the PS plates to the corresponding wells of the ELISA plates. **Start with column 12 followed by 1 to 11.** Cover the plates with a lid.

2.19 Incubate for **2 hours** in a humid atmosphere at 37 °C.

2.20 Wash the ELISA plates thoroughly with washing solution (1.21)

2.21 Make a dilution of the conjugate HATPO (1.6) of 1/4000 in diluent (1.10). Add 100 µl of the dilution to all wells and cover the plates with a lid.

2.22 Incubate for **1.5 hour** in a humid atmosphere at 37 °C.

2.23 Wash the ELISA plates thoroughly with washing solution (1.21)

2.24 Prepare the TMB ethanol substrate (1.13)

Add to each well 100 µl of the substrate. A blue colour will develop.

The substrate consists of:
- 90 ml of distilled water
- 10 ml of 0.1M sodium acetate buffer (1.8)
- 1.67 ml of TMB solution in ethanol (1.12)
- 20 µl of a 30 % solution of H_2O_2(1.17)

2.25 Incubate the plates at room temperature (20 - 25 °C).

2.26 Stop the reaction within 10 minutes after incubation by the addition of 100 µl of 2 M H_2SO_4 (1.20) to each well in the same plate-order as the substrate has been added. The colour will change from blue to yellow.

2.27 Measure the absorbance at 450 nm using an automatic plate reader preferably immediately after the addition of 2 M H_2SO_4. If not, the plates have to stay in darkness until read. Maximum OD in the wells of row 11 are preferably in between 0.500 and 1.300.

2.28 Record the absorbance data.