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Collaborative study: Calibration of Replacement International Standard for Tetanus Toxoid for use in Flocculation Test

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 27 September 2019 and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: Dr Ivana Knezevic at email: knezevici@who.int.

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Summary

We report here the results of a study for the characterization of a preparation of tetanus toxoid (coded 16/302) and its calibration in flocculation (Lf) units. Calibration was performed using Ramon flocculation method, standardised using the 2nd International Standard (IS) for Tetanus Toxoid for use in Flocculation Test (04/150, 690 Lf/ampoule). 16/302 was found to have a unitage of 971 Lf/ampoule based on results from 17 laboratories in 10 different countries. There was good agreement in the results obtained (intra-laboratory GCV ranging from 0% to 7%, inter-laboratory GCV 11.7%).

Data from accelerated thermal degradation studies showed no temperature dependent loss in activity after storage for 9 months, indicating that the standard is likely to have good long-term stability. Further studies at later time points will be performed post establishment.

This study also provided an opportunity to assess the use of alternative methods for measuring antigen content. Participants were asked to determine the Lf value of 16/302 using an ELISA assay established at NIBSC, or other in-house methods for Lf determination. Nine laboratories participated by performing ELISA according to the NIBSC protocol, one laboratory performed flocculation using laser-light scattering according to an in-house protocol, and three laboratories performed other in-house ELISA methods. Results suggest that these methods may provide suitable alternatives to the Ramon flocculation test, subject to validation, and that the replacement WHO IS could act as a suitable reference preparation for these methods.

Based on the results of this study, and with the agreement of participants, it is proposed that 16/302 be established as the 3rd WHO International Standard for Tetanus Toxoid for use in Flocculation Test with an assigned potency of 970 Lf/ampoule.
Introduction

Tetanus is caused by a neurotoxin produced by the bacterium, *Clostridium tetani*. Active immunization against tetanus is based on the use of tetanus toxoid (TTxd), a chemically detoxified preparation of tetanus toxin, to induce protective antibody responses. Tetanus vaccines form an essential component of the primary immunization schedule of children and have been part of the WHO Expanded Programme on Immunization (together with diphtheria and pertussis components) since its inception in 1974. The bulk toxoid intermediates of tetanus vaccines can also be used as carrier proteins in polysaccharide conjugate vaccines against invasive bacterial infections caused by *N. meningitidis, H. influenzae* and *S. pneumoniae*.

TTxd is produced by growing the toxin-producing *C. tetani* in liquid media and converting the toxin to inactive toxoid by treatment with formaldehyde. Antigenic strength and purity of the bulk toxoid is evaluated by measurement of ‘limit of flocculation’ (Lf) units. TTxd for use in production of vaccines for human use must be shown to meet minimum requirements for purity (Lf units per milligram of protein nitrogen). The current WHO minimum requirement for antigenic purity of TTxd has been set as not less than 1000 Lf/mg of protein nitrogen [1]. Measurement of antigen content in Lf also serves as a good indicator of the consistency of production, and testing of the crude toxin prior to inactivation is recommended for monitoring purposes.

Flocculation is determined using an in vitro method based on the observation that antigen and antibody aggregate and form visible floccules when mixed in certain proportions in solution. The precipitate develops more rapidly when equivalent amounts of antigen and antitoxin are present than when an excess of either is available. In the original Ramon flocculation method, the antigen concentration is kept constant and different amounts of antitoxin are added to a series of tubes. The antitoxin content of the first tube to flocculate can be used to calculate the Lf value of the sample. The time in minutes for the first tube to flocculate is known as the Kf value and is a useful indicator of the quality of the antigen and antitoxin used.

The flocculation unit was originally a relative unit defined as the amount of toxin (or toxoid) equivalent to one International Unit (IU) of antitoxin in the flocculation test (Ramon version) [2]. In 1970 the WHO Expert Committee on Biological Standardization decided to examine the feasibility of defining the Lf unit internationally by means of a reference toxoid preparation calibrated in Lf, rather than by means of an antitoxin preparation [3]. Subsequently, the 1st International Reference Reagent of Tetanus Toxoid for Flocculation Test (TEFT) was established in 1988, calibrated against the second International Standard for Tetanus Antitoxin (TE), carrying the valid Lf definition at the time [4, 5]. TEFT was replaced with the 2nd International Standard (IS) for Tetanus Toxoid for use in Flocculation Test (04/150) in 2007 following depletion of stocks [6].

Due to its simplicity, speed and economy, flocculation remains the primary method used by vaccine manufacturers to evaluate toxin and toxoid concentrations in Lf. Most laboratories use the toxoid IS to calibrate a suitable antitoxin in Lf-equivalent (Lf-eq) units. This antitoxin can then be used routinely to determine Lf values of unknown toxin or toxoid samples. Various modifications of the original Ramon flocculation test exist, for example, keeping antitoxin...
concentration constant and adding different amounts of antigen (Dean-Webb method), or varying the concentrations of both components simultaneously (Levine-Wyman method). However, different versions of the method give different equivalence amounts, therefore it is important that the same version of the test is used for calibration of the local reference antitoxin and for routine use [5].

Stocks of the 2nd IS (04/150) are in limited supply and a project was initiated to calibrate and establish a replacement standard. Candidate material for the replacement standard was provided to NIBSC for formulation and filling prior to freeze-drying. A collaborative study (NIBSC code CS603) was initiated with the primary aim of calibrating this material in Lf units using Ramon flocculation test standardised against the 2nd IS. 19 laboratories in 12 countries (Argentina, Belgium, Canada, China, Denmark, France, Hungary, India, Indonesia, Japan, The Netherlands and the United Kingdom) participated in the collaborative study and 17 of these performed flocculation assays used for calibration of the candidate standard. A secondary aim of the collaborative study was to assess the suitability of alternative methods for measuring Lf of tetanus toxoid. 9 laboratories performed a capture ELISA assay developed at NIBSC [7]. In addition, 3 laboratories returned results from other in-house ELISAs and 1 laboratory performed an alternative flocculation method using laser light scattering to obtain a more objective detection of antigen-antibody complexes.

The participating laboratories are listed in the Appendix and are referred to throughout this report by a code number, allocated at random, and not related to the order of listing.

**Bulk material and processing**

Bulk purified tetanus toxoid (Lot 288S4015, 3480 Lf/ml, 1888 Lf/mg PN) was kindly provided by Serum Institute of India (SII, Pune, India). The antigen content was confirmed by in-house capture ELISA at NIBSC. Seven litres of tetanus toxoid 288S4015 was stabilized and diluted 1/3 by the addition of 0.1 M sodium chloride and 1% trehalose before freeze-drying. Filling (1 ml per ampoule) was performed within NIBSC’s Standard Processing Division on 18th November 2016 using a Bausch and Strobel Filling Machine (AFV5090). The material was stirred constantly during filling and the temperature was maintained between +4-8°C. The filled ampoules were freeze-dried using a Sorial CS100 freeze-dryer with a 4 day cycle initiated on the day of filling. Ampoules were sealed after completion of the freeze-drying cycle and stored in the dark at -20°C and the finished product was coded 16/302. A total of 21,022 ampoules of 16/302 were filled at NIBSC.

**Characterization of freeze-dried candidate standards**

After filling and freeze-drying, the candidate toxoid was examined for appearance, residual moisture content, oxygen head space and total antigen content (results are summarized in Table 1). The lyophilized product was of very good appearance, giving rise to robust and homogenous cakes. The precision of fill was determined by weighing ampoules after fill. Representative ampoules were weighed at one minute intervals throughout the production run. A total of 732 ampoules were weighed and the mean fill mass was 1.01 g with a coefficient of variation (CV) of 0.21%. Ampoules were sealed under boil-off gas from high purity liquid nitrogen (99.99%) and measurement of the mean oxygen head space after sealing served as a measure of ampoule
integrity. The mean oxygen head space was measured non-invasively by frequency modulated spectroscopy (FMS 760, Lighthouse Instruments, Charlottesville, USA). Residual moisture content was measured using the coulometric Karl Fischer method in a dry box environment (Mitsubishi CA100, A1 Envirosiences, Cramlington, UK) with total moisture expressed as a percentage of the mean dry weight of the ampoule contents. The results of measurements of residual moisture content and oxygen headspace are summarized in Table 1. The candidate preparation fulfils WHO requirements for reference preparations regarding precision of fill, residual moisture and oxygen head space.

The antigen content was determined by Ramon flocculation and in-house antigen ELISA against the 2nd WHO IS at NIBSC. For 16/302, the antigen content was measured as 1015 Lf/ampoule by ELISA (3 assays) and 891 Lf/ampoule by flocculation test (4 assays). Based on the antigen estimate obtained by ELISA for the bulk material, the recovery of antigen content after filling and freeze-drying was estimated to be approximately 88%.

Collaborative study design and methods

Study design
Since the original definition of the Lf unit was the amount of toxin or toxoid equivalent in the Ramon version of the flocculation test with one unit of antitoxin, the calibration of the candidate toxoid is based on the Ramon version. Participants were provided with method guidelines based on established World Health Organisation (WHO) and European Pharmacopoeia (Ph Eur) methods [8, 9]. Participants were asked to use their routine in-house antitoxin for flocculation test, and to pre-calibrate this in Lf-eq units using the 2nd IS to ensure traceability of the Lf unit and to standardise flocculation results. A summary of antitoxins used in the study is given in Table 2. Information for performing the pre-calibration step was included in the method guidelines provided to all participants.

Each laboratory was provided with sufficient ampoules of the 2nd IS and of the candidate tetanus toxoid (16/302) to perform one assay to pre-calibrate their antitoxin (Lf-eq) and four independent assays to calibrate the candidate material (Lf) using a new ampoule for every test. The materials were sent to participants with instructions for storage and use. Recommendations for suitable initial dilutions were provided to participants based on preliminary antigen estimates obtained at NIBSC.

Additional ampoules of the 2nd IS and of the candidate tetanus toxoid (16/302) were provided to some participants to perform an in-house ELISA developed at NIBSC for measuring relative antigen content, and/or other in-house methods for antigen quantification. For the NIBSC ELISA, participants were also provided with an experimental protocol and the critical antibody reagents used for capture and detection of the tetanus toxoid (NIBSC 10/134 and 10/132, respectively). Participants were asked to perform at least three independent assays for each alternative method using the same ampoule for all replicate assays. Estimates of tetanus toxoid antigen content in Lf were calculated relative to the 2nd IS.
Assay methods

**Ramon flocculation assay:**
For pre-calibration of the antitoxin, participants were recommended to prepare a series of seven tubes containing 39 IU to 51 IU of antitoxin and 50 Lf of the 2nd IS for tetanus toxoid for use in flocculation test (04/150) in a total volume of 2 ml. Based on the 1st tube to flocculate (which would contain 50 Lf-eq units of antitoxin) they were asked to calculate the Lf-eq value of their original antitoxin sample, taking any initial dilution factor into account, and to use this Lf-eq value for the subsequent assays for calibration of the candidate tetanus toxoid standard.

For calibration of the candidate standard it was recommended to initiate the assay with a series of seven tubes containing 30 Lf-eq units to 60 Lf-eq units of tetanus antitoxin, and tetanus toxoid to the assumed amount of 50 Lf, in a total volume of 2 ml. Participants were asked to record the first, second and third mixtures to flocculate as well as the time taken for the first flocculation to appear. The first tube in which flocculation appeared was used to calculate the Lf value of the toxoid sample. Participants could refine their dilution series after the first assay using a narrower range (e.g. 2 Lf increases from tube to tube) to obtain a more precise estimate of Lf.

If flocculation appeared first in the first or last tube of the series the test had to be repeated using either a different range of the antitoxin or different dilution of test toxoid.

**NIBSC Enzyme Linked Immunosorbent Assay (ELISA):**
A capture ELISA has been developed at NIBSC to monitor the tetanus antigen content and degree of adsorption in final vaccine products [7]. The method was performed as described previously [7], with the exception of using freeze-dried stocks of the capture and detection antibodies (reconstituted in 0.5 ml H2O, and diluted 1/200 for use in the assay). The reference toxoid (04/150) and the candidate tetanus toxoid standard were titrated in the range of approximately 0.069-0.0005 Lf/ml for the assay.

**Other methods:**
Flocculation assay by laser light-scattering [8, 10] was performed using a platelet aggregometer. The assay was performed with a series of five tubes containing 5.625 Lf-eq units to 15.625 Lf-eq units of tetanus antitoxin, and tetanus toxoid to the assumed amount of 10 Lf, in a total volume of 400 µl. The time taken to acquire particle counts of 50,000 was recorded and the data analysed using quadratic regression (log antitoxin concentration vs. log time). Lf values were calculated as the local minimum value of the regression curve.

Three other in-house antigen ELISAs were also performed:

1. Plates were coated with tetanus monoclonal antibody (SA13) diluted to 1.25 µg/ml. Following blocking with 2.5% skimmed milk powder in PBS + 0.05% Tween 20, 16/302 and reference toxoid (04/150) were titrated in the range of approximately 3-0.0004 Lf/ml. Bound toxoid was detected firstly with guinea pig polyclonal antibody followed by anti-guinea pig peroxidase conjugated antibody, and finally with substrate.

2. Plates were coated with polyclonal anti-tetanus toxoid sera (tetana®, Sanofi) diluted to 1 IU/ml. Following blocking with PBS + 3% BSA, 16/302 and reference toxoid (04/150) were titrated in the range of approximately 0.07-0.0005 Lf/ml. Bound toxoid was
detected firstly with mouse monoclonal antibody to tetanus toxoid followed by goat anti-mouse peroxidase conjugated IgG, and finally with substrate.

3. Plates were coated with anti-tetanus polyclonal equine serum (ST ATS 126-07, Biofarma) diluted to 1 IU/ml. Following blocking with PBS + 0.5% BSA, 16/302 and reference toxoid (04/150) were titrated in the range of approximately 0.5-0.004 Lf/ml. Bound toxoid was detected with HRP-conjugated horse anti-diphtheria serum, followed by substrate.

**Reporting of data and statistical analysis**

All raw data together with assay details were returned to NIBSC (using provided data sheets) to permit independent analysis. Any deviation from the method guidelines was reported to NIBSC.

Estimates from NIBSC ELISA for the candidate toxoid, 16/302 were calculated relative to IS 04/150 using CombiStats [11] by fitting a sigmoid model comparing assay response to log concentration using the full range of responses. Linearity was assessed visually and parallelism was assessed by calculation of the ratio of slopes for the test and reference samples. Samples were considered to be non-parallel when the slope ratio was outside of the range 0.80 to 1.25 and no potency estimates were then calculated.

Estimates from in-house ELISA were calculated in the same manner as for the NIBSC ELISA where the data permitted. For Lab 11, a parallel line model was used fitting log-response to log-concentration on a selected linear section of the dose-response curve. Linearity and parallelism were assessed as for the NIBSC ELISA.

Results from all valid assays were combined as unweighted geometric means (GM) for each laboratory and these laboratory means were used to calculate overall unweighted geometric means. Variability between assays and laboratories has been expressed using geometric coefficients of variation (GCV = \{(10^{s-1})\times100\%\} where s is the standard deviation of the log_{10} transformed estimates). Comparisons between flocculation and NIBSC ELISA assays have been made by unpaired t-test of log transformed results.

**Stability studies**

To determine the stability of the candidate toxoids an accelerated degradation study was initiated at NIBSC. For the accelerated degradation study, representative samples (ampoules) of 16/302 were stored at +4, +20, +37, +45 and +56°C in addition to the recommended storage temperature of -20°C. Data was obtained by flocculation test after 9 months of storage with further data to be collected at later time points (up to 4 years).

**Results**

**Results contributed to the study**

Flocculation assay results were returned by 17 laboratories and all performed four independent assays.

Five laboratories (lab codes 2, 8, 13, 15 and 16) chose to perform two tests with each reconstituted ampoule of the candidate tetanus toxoid standard - one ‘broad’ range according to
the dilution range suggested in the method guidelines, followed by one ‘narrow’ range using smaller increases from tube to tube to obtain a more precise estimate of Lf. Laboratory 5 performed a ‘broad’ range test followed by 4 ‘narrow’ range tests, and laboratory 14 performed a ‘broad’ range test followed by three ‘narrow’ range tests.

Lab 6 performed 3 tests to calibrate the antitoxin in Lf-eq units, with each test being performed by a different operator. Operator 1 performed two of the flocculation tests to calibrate the toxoid, and operators 2 and 3 each performed one test each. Lab 6 also performed the flocculation tests by making up each toxoid and antitoxin mixture in a total volume of 1 ml rather than 2 ml as suggested in the method guidelines. This should not have any adverse effect on the performance of the test.

NIBSC ELISA results were returned by 9 laboratories and all performed three assays except for Labs 7 and 16 where four assays were performed, and Lab 1 where 5 assays were performed, with all assays using one ELISA plate. Lab 19 performed two assays using three ELISA plates in each, followed by a third assay using a single plate.

Results from flocculation assay by laser light-scattering were returned by one laboratory. Five independent assays were performed, with assay 1 using one ampoule of 16/302, assay 2 using the same ampoule from assay one combined with a second ampoule, assay 3 using the second ampoule only, and assays 4 and 5 using a third different ampoule only.

Three laboratories returned results of three independent assays from their own in-house ELISA assays.

**Flocculation assay results**

Table 3 summarises the results (Lf/ampoule) obtained for 16/302 in the Ramon flocculation test. Where a laboratory performed both ‘broad’ and ‘narrow’ dilution ranges in the same test, the results obtained using the ‘narrow’ range have been used. An overall geometric mean of 971 Lf/ampoule (95% confidence limits: 917-1028; GCV 11.7%; n=17) was determined for 16/302. Within-laboratory GCV’s ranged from 0% (all assays giving the same result) to 7%.

The time taken for the first flocculation to occur, known as Kf, varied dramatically depending on the antitoxin preparation used, and was noticeably long for antitoxins 66/021 (NIBSC) and 10832-05-0004 (AJ Vaccines A/S). A summary of average Kf times observed for 16/302 with each antitoxin is shown in Table 2. The average Kf ranged from 3 minutes to 35 minutes. The Kf values observed for 16/302 are comparable to those obtained with the 2nd IS when determining the Lf-eq value of the antitoxin preparation (Table 2).

**NIBSC ELISA results**

Table 4 summarises the results (Lf/ampoule) obtained for 16/302 in NIBSC ELISA assays. Valid results were obtained for all laboratories. An overall geometric mean of 1050 Lf/ampoule (95% confidence limits: 988-1116; GCV 8.2%; n=9) was calculated. The result from Lab 1 was found to be an outlier (p<0.05 in Grubbs’ test on log laboratory means) and the result calculated excluding this lab was 1027 Lf/ampoule (95% confidence limits: 988-1067; GCV 4.7%; n=8). Within-laboratory GCV’s ranged from 1.2% to 12.3%. No significant difference was detected
between flocculation and antigen ELISA results in a paired 2-tailed t-test (p=0.349; n=9, Lab 1 excluded).

**In-House ELISA results**
Table 5 summarises the results (Lf/ampoule) obtained from in-house ELISAs. Valid results were obtained for all three laboratories. An overall geometric mean of 1466 Lf/ampoule (95% confidence limits: 350-6146; GCV 78.1%; n=3) was calculated. The result from Lab 3 is notably higher than other estimates, but there is insufficient data to reliably perform an outlier test.

**Other methods**
Lab 6 performed a flocculation test by laser light scattering, with a laboratory geometric mean value of 900 Lf/ampoule (95% confidence limits: 851-951). The within-laboratory GCV was 4.6% from 5 assays.

Laboratory geometric means for all assay types are shown in Figure 1.

**Stability**
Data from accelerated degradation studies at the 9 month time point showed no change in Lf value obtained for 16/302 over the range of temperatures used and therefore no prediction of stability could be made. This suggests that the preparation will be highly stable when stored at the recommended storage temperature of -20°C. Furthermore, results from the collaborative study to establish the 2nd WHO International Standards for diphtheria and tetanus toxoids for flocculation test suggest that, once freeze-dried, these materials are likely to be highly stable [12]. Further studies will be performed at later time points to try and obtain a prediction of the rate of loss of activity.

**Conclusions**
Ampoules coded 16/302 were tested and confirmed to fully comply with WHO recommendations for precision of fill, residual moisture content and integrity. Preliminary data from accelerated degradation studies indicate that the proposed standard will have adequate long-term stability. The results obtained in the Ramon Flocculation assay were comparable within and between laboratories and the results from this method are recommended for use in assigning a value to 16/302.

Comparable results to the original Ramon flocculation test were obtained when using a laser light-scattering platelet aggregometer as the detection system for flocculation. Similarly, Lf results returned by ELISA methods were not significantly different to flocculation. Together with results from other collaborative studies [12, 13], where ELISA has been included as a method for measuring antigen content, these findings suggest that ELISA might be an interesting alternative to flocculation testing and that this should be explored further, using tetanus toxoid from a range of vaccine manufacturers. Based on the performance of the candidate toxoid in this study, it is likely to be suitable for use as a reference preparation in ELISA methods for determining relative antigen content.
The results from this study confirm that the tetanus toxoid preparation coded 16/302 is suitable as a replacement WHO International Standard for Tetanus Toxoid for use in Flocculation Test. NIBSC will act as custodian of the standard which will be stored under assured temperature-controlled conditions within the Institute’s Centre for Biological Reference Materials, at the address listed in the introduction.

A total of 21,022 ampoules of 16/302 were filled at NIBSC. After collaborative study, in-house measurements and accelerated degradation studies, 20,602 ampoules remain available at NIBSC (-20°C) for use as the WHO IS. Based on current use, it can be predicted that this will be sufficient for approximately the next 40 years.

**Recommendation**

Based on the results of Ramon flocculation assay performed in the collaborative study, tetanus toxoid, 16/302 can be recommended as the 3rd **WHO IS for Tetanus Toxoid for use in Flocculation Test**. The recommended unitage assigned to 16/302 is proposed as 970 Lf/ampoule based on Ramon flocculation assay results returned by 17 laboratories.

**Comments from participants**

All 18 participants (not including NIBSC) were sent a draft report and asked to comment on the content and conclusions, and to confirm that their results had been reported correctly. Fifteen participants responded (83%) and all agreed with the findings of the report and the following specific comments were received:

1. **Comment**: Labs 1 and 4 asked for clarification on why ELISA results obtained from analysis of their raw data at NIBSC differed from the results they had calculated themselves.
   **Response**: Labs 1 and 4 had used parallel line analysis whilst NIBSC had used sigmoid curve analysis to calculate the results. The wrong analysis method was described in the report text which was subsequently corrected.

2. **Comment**: Lab 3 provided feedback explaining that their in-house ELISA was in development at the time of the study and that optimisation of the assay is still ongoing (in response to their result for this method being much higher compared to all other results in the study).

3. **Comment**: Labs 3 and 15 both identified the same typographical error where the wrong lab number was referred to in the text.
   **Response**: The error was corrected in the report.

4. **Comment**: Lab 5, although accepting the recommended units assigned 16/302, highlighted that the assigned unitage might cause approximately 15% bias in the flocculation test results for their laboratory and therefore they may need to apply a correction factor when 16/302 is implemented as a replacement standard.
   **Response**: Values assigned to WHO standards are typically based on a consensus value obtained in collaborative study. Even in this study where the between laboratory agreement is excellent, there is potential for an individual laboratory to experience a shift in their measured values for a given material following implementation of a replacement standard. While this is regrettable, it is unavoidable when replacing some biological reference materials and may be more noticeable for an assay like the flocculation test where the within
laboratory agreement is excellent (in this study the within laboratory variation for the floculation test was 0% in more than half of the participating laboratories).

5. **Comment**: Lab 5 requested changes to be made to the supplier of their flocculation antitoxin and to their honorific.
   **Response**: These changes were implemented in the report.

6. **Comment**: Lab 6 identified incorrect units used for a coating antibody in one of the in-house ELISA methods.
   **Response**: This error was corrected in the report.

7. **Comment**: Lab 7 identified that the results reported for all their flocculation tests were incorrect, and Lab 9 identified that the result reported for one of their flocculation tests was incorrect.
   **Response**: These erroneous results were corrected which altered the final geomean (but not the proposed units) of the candidate standard.

8. **Comment**: Lab 11 queried whether a global mean titre could be assigned including results from ELISA as they do not differ significantly from the floculation test results. They questioned the reporting of the overall geomean titre of the 3 in house ELISAs as one of the values is much higher than the others and they felt the mean value is not reliable and does not add any value. They also queried whether a specific ELISA unit could be assigned separately to that for use in flocculation test.
   **Response**: A response was provided explaining that the ELISA methods were included as a secondary objective to allow a comparison to be made with the results obtained by floculation testing. This gives preliminary information on whether ELISA might be suitable for measurement of absolute antigen content which could be assessed more thoroughly in a separate future study (which would have to include a much wider range of tetanus toxoids). Whilst this study does suggest that the candidate standard can be used in ELISA (i.e. it gives an acceptable dose response), the Lf values obtained using ELISA are provided for information only – they are not assigned values and will not be stated in the Instructions for Use that accompany the standard. With regards to excluding the high result (Lab 3) from the in-house ELISA geomean titre - there is insufficient data to determine whether this result is a statistical outlier and it is therefore included in the overall method geomean (which itself is shown for information only).

9. **Comment**: Labs 15 and 17 requested changes to be made to their laboratory addresses.
   **Response**: These changes were implemented in the report.

**Acknowledgments**

We especially thank Dr. Sunil Gairola (SII, India) for the donation of tetanus toxoid for establishment of an International Standard. We are extremely grateful to all participants who took part in the collaborative study. At NIBSC, Dr. Paul Matejtschuk, Chinwe Duru and Ernest Ezeajughi (Standardization Science) are acknowledged for their contribution to the formulation and lyophilisation studies, and staff of Standards Processing Division for the production and dispatch of the candidate materials. We are also grateful to Dr. Masaaki Iwaki from NIID for coordinating shipment of samples in Japan.
Abbreviations Used

References


Table 1. Summary of stabilised, freeze-dried candidate standard

<table>
<thead>
<tr>
<th>NIBSC Code</th>
<th>16/302</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxoid Manufacturer</td>
<td>Serum Institute of India</td>
</tr>
<tr>
<td>No. Ampoules filled</td>
<td>21,022</td>
</tr>
<tr>
<td>Appearance</td>
<td>Robust homogenous cake</td>
</tr>
<tr>
<td>Mean fill mass</td>
<td>1.01 g (CV 0.21%) ((n=732))</td>
</tr>
<tr>
<td>Mean dry weight</td>
<td>0.02 g (CV 0.82%) ((n=6))</td>
</tr>
<tr>
<td>Mean residual moisture</td>
<td>0.51% (CV 14.75%) ((n=12))</td>
</tr>
<tr>
<td>Mean oxygen head space</td>
<td>0.23% (CV 24.45%) ((n=12))</td>
</tr>
</tbody>
</table>

Table 2. Summary of antitoxins used by participants for flocculation test

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Batch</th>
<th>No. of labs</th>
<th>Lf-eq units/ml</th>
<th>Average Kf time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ Vaccines A/S</td>
<td>10832-05-0004</td>
<td>1</td>
<td>93(^a)</td>
<td>23(^a)</td>
</tr>
<tr>
<td>Bilthoven Biologicals B.V.</td>
<td>VSTET16/01</td>
<td>1</td>
<td>217</td>
<td>2</td>
</tr>
<tr>
<td>Central Drugs Laboratory</td>
<td>CDL/TATF-01/2018</td>
<td>1</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>WDT Serumwerk Memsen</td>
<td>WDT 628</td>
<td>1</td>
<td>2040</td>
<td>6</td>
</tr>
<tr>
<td>National Institute for Biological Standards and Control</td>
<td>66/021</td>
<td>4</td>
<td>1499 (1412–1585)</td>
<td>31 (19–42)</td>
</tr>
<tr>
<td>National Institute of Infectious Diseases</td>
<td>Lot 2</td>
<td>3</td>
<td>1215 (937-1492)</td>
<td>6.4(^b) (5.7-7.0)</td>
</tr>
<tr>
<td></td>
<td>Lot 3</td>
<td>3</td>
<td>1129 (915-1344)</td>
<td>3(^c) (2-4)</td>
</tr>
<tr>
<td>National Institutes for Food and Drug Control</td>
<td>0022</td>
<td>1</td>
<td>907</td>
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<tr>
<td>PT. Bio Farma</td>
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<td>6810</td>
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<tr>
<td>Serum Institute of India</td>
<td>ATS 1/2007</td>
<td>1</td>
<td>96</td>
<td>3</td>
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</tbody>
</table>

Where more than one participant used the same antitoxin, the average result taken across labs is shown with the range of lab means indicated in brackets. Individual Lf-eq units and Kf times with 04/150 are from 1 assay with three exceptions\(^a,b,c\). Individual Kf times for candidate tetanus toxoid, 16/302 are the mean of 4 assays with one exception\(^d\). Where four or more narrow range assays have been performed, only the Kf times for these assays have been included.

\(^a\) Mean of 4 assays; \(^b\) Mean of 4 assays for one of the 3 labs; \(^c\) Mean of 3 assays for one of the 3 labs; \(^d\) Mean of 7 assays.
## Table 3. Flocculation test results (Lf/ampoule) for 16/302

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
<th>GM</th>
<th>GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>1050</td>
<td>970</td>
<td>5.4%</td>
</tr>
<tr>
<td>2</td>
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<td>1029</td>
<td>1029</td>
<td>1029</td>
<td>1024</td>
<td>1.0%</td>
</tr>
<tr>
<td>3</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>0.0%</td>
</tr>
<tr>
<td>5</td>
<td>840</td>
<td>840</td>
<td>840</td>
<td>840</td>
<td>840</td>
<td>0.0%</td>
</tr>
<tr>
<td>6</td>
<td>835</td>
<td>835</td>
<td>835</td>
<td>835</td>
<td>835</td>
<td>0.0%</td>
</tr>
<tr>
<td>7</td>
<td>879</td>
<td>879</td>
<td>879</td>
<td>879</td>
<td>879</td>
<td>0.0%</td>
</tr>
<tr>
<td>8</td>
<td>1281</td>
<td>1260</td>
<td>1260</td>
<td>1302</td>
<td>1276</td>
<td>1.6%</td>
</tr>
<tr>
<td>9</td>
<td>1050</td>
<td>1050</td>
<td>1155</td>
<td>1050</td>
<td>1075</td>
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</tr>
<tr>
<td>10</td>
<td>900</td>
<td>900</td>
<td>900</td>
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<tr>
<td>12</td>
<td>1155</td>
<td>1155</td>
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</tr>
<tr>
<td>13</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>0.0%</td>
</tr>
<tr>
<td>14</td>
<td>945</td>
<td>882</td>
<td>945</td>
<td>945</td>
<td>929</td>
<td>3.5%</td>
</tr>
<tr>
<td>15</td>
<td>987</td>
<td>1029</td>
<td>1029</td>
<td>987</td>
<td>1008</td>
<td>2.4%</td>
</tr>
<tr>
<td>16</td>
<td>1050</td>
<td>1050</td>
<td>1050</td>
<td>1050</td>
<td>1050</td>
<td>0.0%</td>
</tr>
<tr>
<td>17</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>0.0%</td>
</tr>
<tr>
<td>18</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>945</td>
<td>0.0%</td>
</tr>
<tr>
<td>19</td>
<td>840</td>
<td>840</td>
<td>945</td>
<td>945</td>
<td>891</td>
<td>7.0%</td>
</tr>
</tbody>
</table>

Overall GM
95% Confidence Limits
GCV

971
917 – 1028
11.7%

## Table 4. NIBSC ELISA results (Lf/ampoule) for 16/302

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
<th>Assay 5</th>
<th>GM</th>
<th>GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1202</td>
<td>1413</td>
<td>1147</td>
<td>1350</td>
<td>1177</td>
<td>1254</td>
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</tr>
<tr>
<td>3</td>
<td>1106</td>
<td>973</td>
<td>928</td>
<td></td>
<td></td>
<td>999</td>
<td>9.5%</td>
</tr>
<tr>
<td>4</td>
<td>977</td>
<td>NL</td>
<td>1048</td>
<td></td>
<td></td>
<td>1012</td>
<td>5.1%</td>
</tr>
<tr>
<td>6</td>
<td>1256</td>
<td>1054</td>
<td>1009</td>
<td></td>
<td></td>
<td>1101</td>
<td>12.3%</td>
</tr>
<tr>
<td>7</td>
<td>961</td>
<td>1039</td>
<td>890</td>
<td>NL</td>
<td></td>
<td>961</td>
<td>8.0%</td>
</tr>
<tr>
<td>8</td>
<td>1114</td>
<td>1140</td>
<td>966</td>
<td></td>
<td></td>
<td>1071</td>
<td>9.4%</td>
</tr>
<tr>
<td>16</td>
<td>1019</td>
<td>1152</td>
<td>1041</td>
<td>1065</td>
<td></td>
<td>1068</td>
<td>5.5%</td>
</tr>
<tr>
<td>18</td>
<td>NL</td>
<td>1020</td>
<td>1003</td>
<td></td>
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<td>1012</td>
<td>1.2%</td>
</tr>
<tr>
<td>19</td>
<td>1027</td>
<td>1030</td>
<td>934</td>
<td></td>
<td></td>
<td>996</td>
<td>5.8%</td>
</tr>
</tbody>
</table>

## Table 5. In-House ELISA results (Lf/ampoule) for 16/302

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>GM</th>
<th>GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2588</td>
<td>3413</td>
<td>2645</td>
<td>2859</td>
<td>16.6%</td>
</tr>
<tr>
<td>11</td>
<td>1002</td>
<td>1176</td>
<td>990</td>
<td>1053</td>
<td>10.1%</td>
</tr>
<tr>
<td>12</td>
<td>842</td>
<td>1038</td>
<td>1317</td>
<td>1048</td>
<td>25.1%</td>
</tr>
</tbody>
</table>
Figure 1. Results (Lf/ampoule) for 16/302
List of participants (alphabetical order by country)

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Ontario K1A 0K9

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Mrs. Veronika Ondi  
Quality Control  
Biochemistry Laboratory  
GSK Biologicals Kft  
2100 Gödöllő  
Homoki Nagy István utca 1.
<table>
<thead>
<tr>
<th>Country</th>
<th>Name</th>
<th>Company/Institution</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>INDIA</td>
<td>Dr. Krishna Mohan</td>
<td>Bharat Biotech</td>
<td>Genome Valley Shameerpet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hyderabad – 500 078</td>
</tr>
<tr>
<td>INDIA</td>
<td>Dr. Arun Bhardwaj</td>
<td>Central Drug Laboratory</td>
<td>CRI Kasauli (HP)-173204</td>
</tr>
<tr>
<td>INDIA</td>
<td>Dr. Sunil Gairola</td>
<td>Serum Institute of India Pvt. Ltd.</td>
<td>212/2 Hadapsar</td>
</tr>
<tr>
<td></td>
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<td>Pune – 411 028</td>
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<tr>
<td>INDONESIA</td>
<td>Mr. Dori Ugiyadi</td>
<td>PT. Bio Farma</td>
<td>JL. Pasteur No 28</td>
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<td></td>
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<tr>
<td></td>
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<td>Bandung  40161</td>
</tr>
<tr>
<td>JAPAN</td>
<td>Dr. Masaaki Iwaki</td>
<td>Laboratory of Bacterial Toxins, Toxoids and Antitoxins</td>
<td>Department of Bacteriology II</td>
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<td></td>
<td>National Institute of Infectious Diseases</td>
</tr>
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<td></td>
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<td>4-7-1 Gakuen, Musashimurayama-shi</td>
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<td>Tokyo 208-0011</td>
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<tr>
<td>JAPAN</td>
<td>Mr. Hideki Nakagawa</td>
<td>The Research Foundation for Microbial Diseases of Osaka</td>
<td>University (BIKEN)</td>
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<td></td>
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<tr>
<td>JAPAN</td>
<td>Mr. Kazunori Morokuma, DVM</td>
<td>KM Biologics Co., Ltd.</td>
<td>1314-1 Kyokushi Kawabe</td>
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<td>Mr. Tetsuya Watanabe</td>
<td>Denka Seiken Co. Ltd.</td>
<td>1-2-2 Minami Honcho</td>
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<td>Niigata 959-1695</td>
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JAPAN
Ms. Rinko Ando
Kitasato Daiichi Sankyo Vaccine Co. Ltd.
6-111 Arai
Kitamoto-shi
Saitama 364-0026

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Mr. Sohei Murata
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Blanche Lane, South Mimms
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EN6 3QG
Appendix B.
Draft Instructions for Use for 16/302