



Research Article

A New Lymphocyte Proliferation Assay for Potency Determination of Bovine Tuberculin PPDs

Christina Spohr¹, Eva Kaufmann¹, Sibylle Battenfeld¹, Karin Duchow¹, Klaus Cussler², Elisabeth Balks¹ and Max Bastian¹

¹Division of Veterinary Medicine; ²Division Safety of Medicinal Products and Medical Devices, Paul-Ehrlich-Institut, Langen, Germany

Summary

The tuberculin skin test is the method of choice for tuberculosis surveillance in livestock ruminants. The exact definition of the biological activity of bovine tuberculin purified protein derivatives (bovine tuberculin PPDs) is essential for the reliability of a test system. PPDs consist of heterogeneous mixtures of mycobacterial antigens, making it difficult to determine their potency *in vitro*. The commonly used batch potency test is therefore based on the evaluation of skin reactions in mycobacteria-sensitized guinea pigs. The aim of the present study was to test an alternative *in vitro* method that reliably quantifies tuberculin PPD potency. This novel approach may prevent animal distress in the future.

To this end a flow cytometry-based lymphocyte proliferation assay using peripheral blood mononuclear cells (PBMCs) from sensitized guinea pigs was established. Potency estimates for individual PPD preparations were calculated in comparison to an international standard. The comparison with results obtained from the guinea pig skin test revealed that the lymphocyte proliferation assay is more precise but results in systematically higher potency estimates. However, with a manufacturer specific correction factor a correlation of over 85% was achieved, highlighting the potential of this *in vitro* method to replace the current guinea pig skin test.

Keywords: alternative potency assay, bovine tuberculosis, *Mycobacterium bovis*, guinea pig, lymphocyte proliferation assay

1 Introduction

Bovine tuberculosis (Tb) is a zoonotic disease that causes significant economic losses and a high animal and public health burden. The causative agent is *Mycobacterium bovis*. In the middle of the twentieth century concerted efforts succeeded in virtually eradicating *M. bovis* in Central Europe through test and slaughter programs (Caffrey, 1994). Consequently, several countries abandoned the annual testing of cattle herds by intradermal tuberculin tests. However, a recent outbreak of bovine Tb in the southern parts of Bavaria illustrates that – even in officially Tb-free countries – ongoing screening programs are required (Moser et al., 2014). It is now thought that an abattoir surveillance strategy must be complemented by regular herd testing. This is reflected in the revised German bovine Tb regulation, which

reintroduces regular skin testing of cattle older than 24 months (Federal Ministry of Food and Agriculture, 2014).

In veterinary medicine the tuberculin skin test remains the standard method to detect Tb-infections (OIE, 2011). It is based on the intradermal injection of a defined volume of PPD. If an animal is infected with mycobacteria or a closely related species, a swelling develops at the site of injection that is measured 72 ± 4 hours later (Monaghan et al., 1994). The swelling represents a delayed-type hypersensitivity reaction and is indicative for an adaptive immune response against mycobacteria (Huebner et al., 1993).

PPDs are manufactured from mycobacterial cultures. Mycobacteria are grown in synthetic, liquid media, inactivated and filtered. The water-soluble antigens are concentrated, washed and redissolved (European Pharmacopeia Monograph

Abbreviations

CFSE, carboxy-fluorescein-succinimidyl-ester; FSC, forward-scatter; SSC, side-scatter; PPD, purified protein derivative

Received February 10, 2015;
Accepted April 13, 2015;
Epub May 3, 2015;
<http://dx.doi.org/10.14573/altex.1502101>



This is an Open Access article distributed under the terms of the Creative Commons Attribution 4.0 International license (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is appropriately cited.



0536, 2008). Due to the manufacturing process, PPDs, though protein-enriched, represent complex and heterogeneous mixtures of mycobacterial components. Since the skin test relies on the identification of heterogeneous anti-mycobacterial immune responses, it is very sensitive but displays a low specificity. To differentiate between animals infected with *M. bovis* and those responding to bovine PPD as a result of exposure to other cross-reactive (myco)bacteria, a comparative tuberculin test with *M. bovis* and *M. avium* derived PPDs injected simultaneously at different sites may become necessary (OIE, 2011). In order to reliably discriminate infected from non-infected animals and to detect differences in reactivity in the comparative test, a consistent composition of the PPDs is of pivotal importance.

The complex composition of PPD prohibits an all-encompassing *in vitro* quantification of active ingredients. In several publications the composition of PPDs has been studied by mass spectrometry and an array of immunogenic proteins has been identified (Borsuk et al., 2009; Prasad et al., 2013). However, since the quantitative contributions of these proteins and possibly undefined components to skin reactivity remain to be determined, PPD potency is still measured by an *in vivo* assay. The current test procedures performed according to the European Pharmacopoeia (European Pharmacopoeia Monograph 0536, 2008) rely on the sensitization of guinea pigs either with live or inactivated mycobacteria. After at least four weeks the animals' flanks are shaved and different dilutions of a reference preparation and the PPD under evaluation are injected intradermally. Doses are chosen that produce lesions ranging from 8 to 25 mm. Diameters are measured and the potency of the test batch is calculated by comparing the reactions induced by the test and the corresponding reference preparation. This test suffers from several drawbacks including the subjective read-out, the limited number of replicates and/or dilutions due to the limited area that is available on the flanks of the guinea pig, the poor reproducibility and the significant distress for the laboratory animals caused by painful, sometimes necrotizing lesions (Weisser and Hechler, 1997). Based on early, pioneering work by Haslöv and colleagues (1984) we aimed to establish an alternative *in vitro* assay using lymphocytes from mycobacteria sensitized guinea pigs. This approach is a refinement and a reduction approach in accordance with the 3R principle of Russel and Burch (1959). The guinea pig sensitization test is significantly refined as the animals are still sensitized but do not undergo PPD challenge; also the approach has the potential to reduce animal numbers as more than one batch can be tested per animal and replicate testing improves test reliability.

2 Animals

Female Dunkin-Hartley guinea pigs were obtained from Charles River Laboratories, Sulzfeld, Germany. A total of 165 guinea pigs were used in this study. The animals were kept under conventional conditions in accordance with Appendix A of the European Convention for the Protection of Vertebrate

Animals Used for Experimental and Other Scientific Purposes as adopted by the German Animal Welfare Legislation (Federal Ministry of Food and Agriculture, 2013). The animals were housed in groups of maximum five animals in stainless steel-cages on dust-free wooden bedding. The animals had free access to dry pellets and water. Twice a week they were offered vegetables. The cages were enriched with two plastic refuges and hay for chewing and concealment. The facility is air-conditioned and has an average temperature of 20° C. It is open to daylight and allows for a natural day and night cycle. Before each experiment a veterinarian clinically inspected the animals. All treatments and manipulations were performed in a separate room within the animal facility. The experiments were performed during the morning. After completion of the study the animals were anesthetized as described below and euthanized by carbon dioxide inhalation in an in-house built carbon-dioxide device. When guinea pigs had to be anesthetized in non-terminal experiments, during the wake up period the animals were carefully put back into the cages, covered with hay to prevent cooling and their eyes were treated with Thilo-Tears® Gel (Alcon Pharma, Freiburg) to prevent drying. All information concerning the individual experimental groups is detailed in Table S1 in the supplementary data file at <http://dx.doi.org/10.14573/altex.1502101s>.

According to German Animal Welfare legislation the study was announced to and approved by the competent authority (Veterinary Department, Regierungspräsidium Darmstadt, Germany; ref.: V54-19c20/15-F107/110).

3 Material and methods

Bacteria and Tuberculin PPDs

Live attenuated Bacille Calmette Guerin (BCG), Pasteur strain 1173, was kindly provided by Dr W. Matheis, Paul-Ehrlich-Institute (Langen, Germany). Bacteria were grown at 37°C in 7H9 Middlebrook Medium (Becton Dickinson) supplemented with OADC enrichment (Becton Dickinson) and 0.05% Tween80 (Sigma). The numbers of colony forming units were determined by plating on 7H11 Middlebrook-agar. The bacteria were washed, resuspended in Middlebrook-medium containing 10% glycerol, aliquoted and stored at -70°C until use. Inactivated wet mass of *M. bovis* strain AN5 was kindly provided by Dr Schaufuß, WDT Serumwerk Memmen (Memmen, Germany) and stored at 4°C.

International standards for Purified Protein Derivatives of *M. bovis* (PPDBOV; (NIBSC, 2010a)) and *M. tuberculosis* (PPDT; (NIBSC, 2010b)) were obtained from the National Institute for Biological Standards and Control (Potters Bar, UK) and stored lyophilized at -20°C until use. One vial of PPDBOV contained 58,500 international units (IU) corresponding to a net weight of 1.8 mg. One vial of PPDT contained 5,000 IU. This corresponded to a net weight of 0.14 mg. Test batches of bovine PPD were either purchased or kindly provided for research purposes by three different European manufacturers. The products have stated potencies of 50,000 IU, 25,000 IU

and 30,000 IU per ml. The products were obtained refrigerated and stored until use at 4°C.

Sensitization of guinea pigs

The sensitization method was adopted from the respective monograph (European Pharmacopeia Monograph 0536, 2008). Briefly, mycobacteria-naïve guinea pigs (8–15 per group; weighing more than 400 g) were anesthetized by subcutaneous injection of 10 mg ketamine and 0.5 mg xylazine (Bayer, Leverkusen) per 100 g body weight and sensitized either by subcutaneous injection behind the left forelimb with live BCG (2×10^5 - 3×10^6 bacteria in 0.5 ml phosphate buffered saline (PBS, in-house) or by intramuscular injection of 2 mg heat-inactivated wet mass of *M. bovis* strain AN5 resuspended in 0.5 ml light mineral oil (Merck, Darmstadt) distributed into both hind limbs.

Tuberculin Skin Test

Thirty days after sensitization, guinea pigs were anesthetized as described above. Both flanks were shaved and three doses (509 IU, 169 IU and 56 IU) of the standard tuberculin (PPD_{BOV}) dissolved in 100 μ l PBS containing 0.05% Tween80 (Sigma) were injected intradermally. Test PPDs were diluted according to the stated potency and the same nominal doses were applied. Each animal received three dilutions of the reference and the respective test PPD in duplicate. Injection sites were randomized according to the Latin square. Twenty-four hours later the diameters of the resulting rashes were determined with a transparent ruler. Each lesion was measured twice in two directions. Usually, the reddening disappeared after two to three days. 20% of the animals developed lesions with an inner anemic area surrounded by a hyperanemic inflammatory ring. Whenever this was observed it was usually at the injection site of the highest concentration of the WHO standard. In about 2% of the animals these lesions ulcerated, see Table S1 in the supplementary data file at <http://dx.doi.org/10.14573/altex.1502101s>. Those animals were treated with 20 μ g per 100 g body weight of the non-steroid antiphlogistic Metacam (Boehringer, Ingelheim).

Lymphocyte stimulation assay

Directly before the intradermal skin injection whole blood was obtained from the anesthetized animals by cardiac puncture. According to the approved trial protocol we obtained less than 20% of the minimum estimated blood volume, e.g., from an animal weighing 400 g a maximum of 5 ml. To this end, the needle was inserted between the *Processus xyphoideus* and the left costal arch in cranio-dorsal direction. Under slight aspiration the needle was pushed gently forward into the Apex cordis. Although the procedure was usually well tolerated as a rare complication we sometimes observed the development of a hemopericardium. Overall the loss rate was below 2%, see Table S1 in the supplementary data file at <http://dx.doi.org/10.14573/altex.1502101s>.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (1.074 g/ml density; GE Healthcare) gradient centrifugation as described elsewhere (Bastian et al., 2011).

Cells were resuspended in PBS and stained with the green fluorescent dye carboxy-fluorescein-succinimidyl-ester (CFSE; Alexis). Cells were washed and resuspended in Iscoves Modified Dulbeccos Medium (IMDM, in-house) containing 5% autologous serum. 1×10^5 cells were seeded into 96 well round bottom plates (NUNC). The reference tuberculin PPD (PPD_{BOV}) was added to 30 IU/ml and serially diluted 1:3 to 0.01 IU/ml. Test PPDs were diluted according to the stated potency and added to the same nominal concentration of international units (IU). Phytohemagglutinin (PHA; Oxoid Germany) served as positive control; wells without stimulation (medium only) served as negative control. Every dilution was tested in duplicate. After 5 days of incubation at 37°C and 5% CO₂ cells were harvested and analyzed by flow cytometry using a C6 Accuri flow cytometer (Becton Dickinson). CFlow Plus Analysis software (Becton Dickinson) was used to identify proliferated lymphocytes according to loss of CFSE staining. The percentage of “CFSE low” cells was determined and used for further analysis.

Statistical analysis

Potency estimates for individual PPD preparations in comparison to the international standard tuberculin *M. bovis* PPD (PPD_{BOV}) were calculated according to a sigmoid curve assay (proliferation assay) or by means of a parallel line assay (skin test). An individual potency estimate was calculated for each animal of an experimental group. In addition, the geometric mean and the 95% confidence interval were determined for the entire group. The calculations were performed according to Ph. Eur. (European Pharmacopeia Monograph 50300, 2008) using the CombiStats software (EDQM). Two-tailed parametric Student's t test was used to calculate the significance of differences. The geometric coefficient of variation was calculated as $CV = \sqrt{e^{\sigma_{\ln}^2}} - 1$, where σ_{\ln}^2 is the squared standard deviation of the data after natural log transformation. Spearman non-parametric correlation test was used to calculate correlation coefficients assuming a non-Gaussian distribution of potency estimates.

4 Results

4.1 PPD induces specific T cell proliferation in guinea pig lymphocytes

To measure proliferation of lymphocytes from guinea pigs sensitized against mycobacteria, we adapted a flow cytometric assay developed to study human T cell responses to mycobacterial lipid antigens (Bastian et al., 2008). Before and 30 days after sensitization, blood was obtained from the same animals, PBMCs were isolated, stained with CFSE and stimulated with PPD. After five days cells were analyzed by flow cytometry. Fig. 1A shows representative dot plots of the flow cytometric analysis: Living cells were identified according to their forward- and side-scatter (FSC and SSC) characteristics (Fig. 1A, left dot plot) and then analyzed for CFSE staining. Each condition was tested in duplicate. Before sensitization, the CFSE-low population made up less than 20% of the cells (Fig. 1A, second



dot plot), and sensitized but unstimulated cells also displayed less than 20% CFSE-low cells (Fig. 1A, third dot plot). In contrast, stimulation 30 days after sensitization caused massive proliferation – represented by a prominent CFSE-low cell population (Fig. 1A, right dot plot). CD4-positive T cells were the dominant responding population (data not shown). Figure 1B and 1C show results for the assay done with lymphocytes from ten tested animals. Prior to sensitization the CFSE-low population made up less than 20% of the cells upon stimulation, but four weeks later PPD stimulation caused a robust lymphocyte proliferation in cells from all tested animals as shown by the high ratio of cells in the CFSE-low population (Fig. 1B). In the absence of PPD stimulation the CFSE-low population was less

than 20% (Fig. 1C). Together this indicates that the lymphocyte proliferation detected by loss of CFSE staining in the flow cytometric assay is antigen-specific.

4.2 The sensitization method influences the course of the T cell response

For the current potency assay three different methods to sensitize guinea pigs are described in Ph. Eur. Depending on the PPD being tested, animals either receive defined amounts of live or heat-inactivated mycobacteria, resuspended either in sodium chloride or paraffin oil. The skin test is performed at minimum four weeks later. For bovine PPD the sensitization with live *M. bovis* of the virulent strain AN5 is advised (Eu-

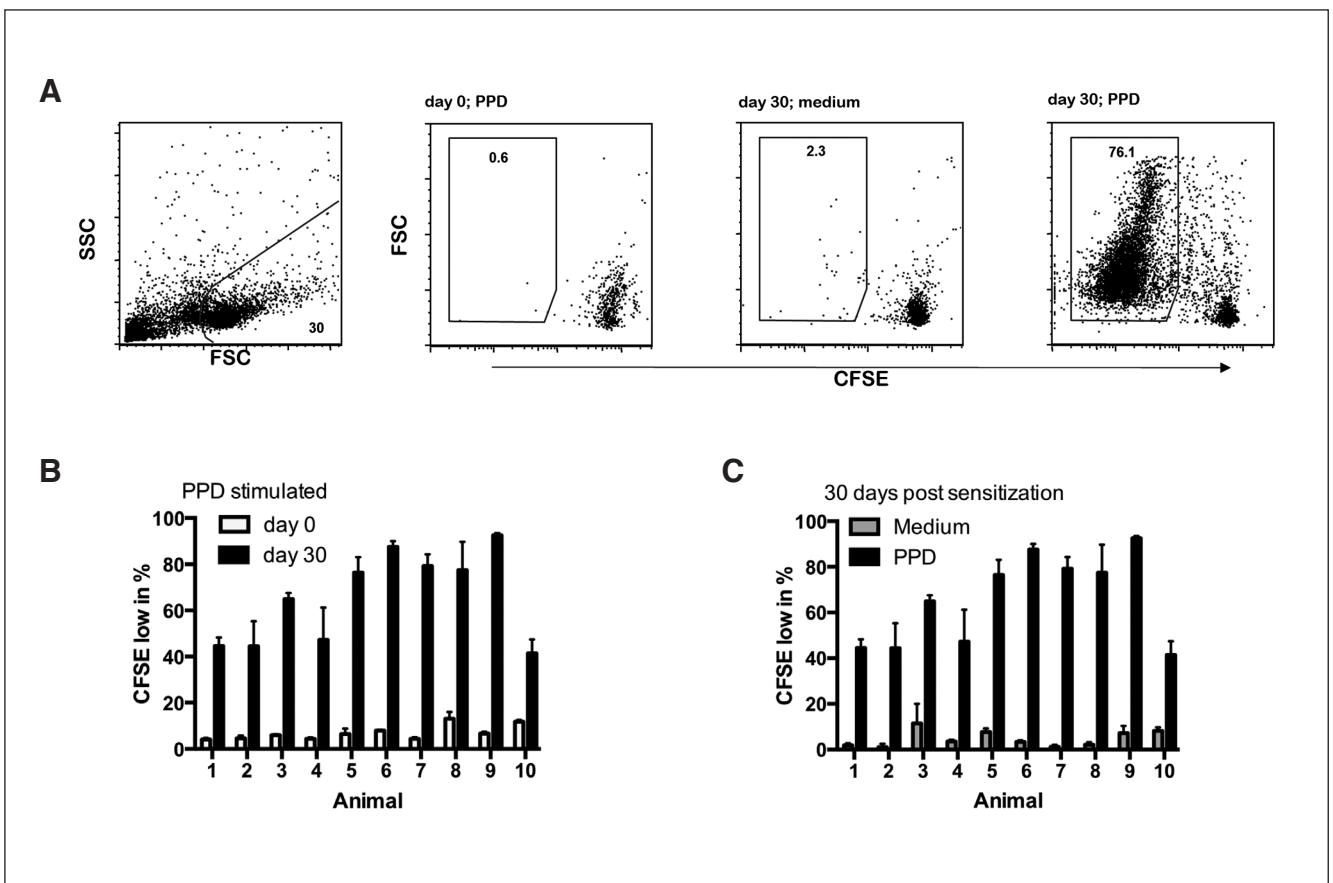


Fig. 1: Bovine tuberculin PPDs induce specific lymphocyte proliferation in mycobacteria sensitized guinea pigs

PBMCs were isolated from guinea pigs prior to or 30 days after sensitization with inactivated wet mass of *M. bovis*, stained with CFSE and stimulated with 30 IU/ml of the international standard for PPD of *M. bovis* (PPD_{BOV}). After 5 days of incubation lymphocyte proliferation was assessed by flow cytometry. (A) The left dot plot shows the typical pattern of a lymphocyte population. The black lined area represents the live cell gate. Numbers indicate the percentage of living cells. The other dot plots show CFSE versus FSC staining within the population of living cells. From left to right the first graph shows a population of PPD-stimulated lymphocytes prior to sensitization (day 0), 30 days after sensitization in the absence of PPD and, in the presence of PPD. Proliferated cells are identified as CFSE-low events within the black-lined area. Numbers indicate the percentage of CFSE CFSE-low. (B) Lymphocytes were isolated from ten animals prior to and 30 days after sensitization and stimulated with PPD. Open bars represent the percentage of CFSE-low cells at day 0, black bars at day 30. (C) From the same ten animals lymphocytes were isolated at day 30 after sensitization and cultivated in the absence (grey bars) or presence (black bars) of PPD. Error bars indicate the standard deviation of duplicates.

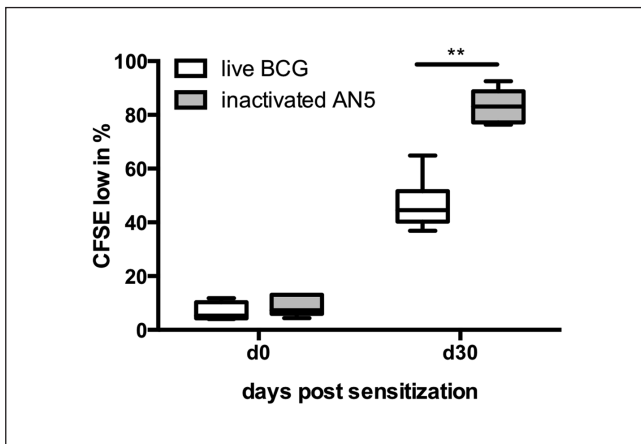


Fig. 2: The sensitization method influences the course of the T cell response

Two groups of six guinea pigs were sensitized either with inactivated mycobacterial wet mass of AN5 or with live attenuated BCG. PBMCs were isolated and stimulated with PPD prior to or 30 days after sensitization. Frames of the boxes indicate the 25 and the 75 percentile, the bar in the middle the respective median and whiskers the maximum or minimum percentage of CFSE low cells. Asterisks indicate the significance of difference ($p = 0.0022$) as determined by the two-tailed Mann-Whitney test.

ropean Pharmacopeia Monograph 0536, 2008). For biosafety and animal welfare reasons we did not use live-virulent mycobacteria. Instead, we tested whether it is feasible to sensitize the animals either with live-attenuated BCG, or – in analogy with the Ph. Eur. monograph for PPD for human use (European Pharmacopeia Monograph 0151, 2008) – with inactivated bacterial wet mass of the virulent strain AN5. To this end we immunized two groups of six guinea pigs either with BCG or with inactivated strain AN5. Blood was obtained prior to and 30 days after sensitization, and lymphocyte proliferation was analyzed. Lymphocytes from all animals responded 30 days after sensitization, although live BCG immunization caused a significantly weaker lymphocyte proliferation response (Fig. 2). This indicates that a robust response can be induced by either of the two sensitization methods. However, since the inactivated strain AN5 wet mass resulted in more consistent and pronounced responses, we chose this method to sensitize guinea pigs for the following experiments.

4.3 T cell proliferation is dose-dependent and discriminates between PPDs

To analyze the dose dependency of the PPD induced lymphocyte proliferation, we tested different concentrations of the international standard for PPD of *M. bovis* (PPDBOV). PBMCs were isolated from a group of nine animals 30 days

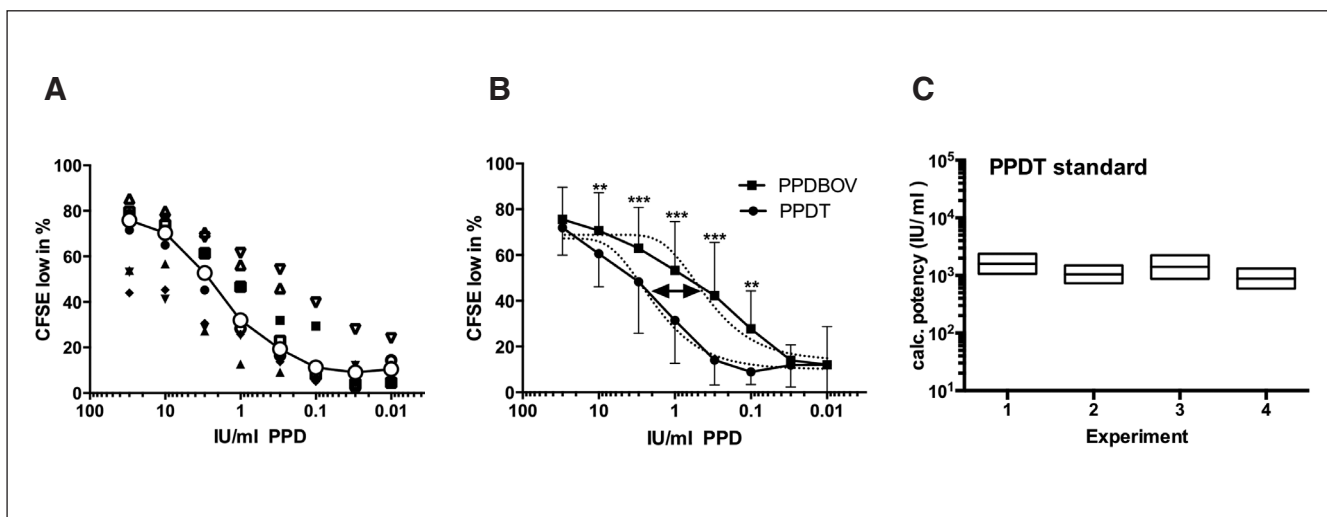


Fig. 3: Lymphocyte proliferation is dose dependent and allows for potency estimation of tuberculin PPDs

(A) PBMCs were isolated from a group of nine guinea pigs 30 days after sensitization with inactivated wet mass of AN5 and stimulated with serial dilutions of the international standard for PPD of *M. bovis* (PPDBOV). Small symbols represent the mean percentage of CFSE-low cells for individual animals. Connected open circles represent the mean over the entire group for the respective PPD concentration. (B) In a different experiment using ten animals PPDBOV was compared to the international standard for PPD of *M. tuberculosis* (PPDT). The arrow illustrates the left shift of the dose-response curve for PPDT. Error bars indicate the standard deviation. For clarity reasons only upper or lower error bars are shown. Two asterisks indicate a level of significance for the indicated dose of $p < 0.01$; three asterisks represent a level of $p < 0.001$ as determined by two-tailed, paired Student's *t* test. The dotted lines represent the non-linear fitted curves as calculated by Boltzmann equation. (C) Lymphocyte proliferation to PPDBOV and PPDT was compared in four independent experiments using groups of ten to fourteen guinea pigs. The potency of PPDT was calculated in relation to PPDBOV by the sigmoid curve assay. Individual boxes represent one independent experiment. The inner bar depicts the respective potency estimate, the frame the upper and lower limit of the 95% confidence interval of the potency estimates.



after sensitization with inactivated *M. bovis* AN5 wet mass. Although the percentage of proliferated cells varied between the animals, the overall response curves ran in parallel, allowing us to calculate group means for each dose (Fig. 3A). To assess if it is possible to detect differences between PPD batches we tested serial dilutions of the PPDBOV standard in parallel to the international standard for PPD of *M. tuberculosis* (PPDT). The difference in the stimulatory capacity between the two PPDs is reflected by the left shift of the dose response curve of the human PPDT in comparison to PPDBOV (Fig. 3B). We applied the sigmoid curve assay as described above to calculate the potency of the PPDT standard relative to the PPDBOV standard for each individual. Subsequently, the geometric mean and the 95% confidence interval were calculated for the entire group. To test the inter-assay precision we determined the potency of PPDT in relation to PPDBOV in four

independent experiments using eight to fourteen animals per group. The potency estimates varied between 882 and 1,590 IU/ml with overlapping confidence intervals (Fig. 3C). This shows that the proliferation assay is able to reproducibly detect differences between different PPDs.

4.4 Potency estimates correlate between the skin test and proliferation assay

Next, we tested to what extent potency estimates correlate between skin test and proliferation assay. Thirty days after sensitization, we drew blood samples and performed the skin test with the same animals. All conditions were tested in duplicate. After 24 h the size of the skin lesions was measured. For individual animals individual potencies, and consequently the geometric group mean and the 95% confidence interval were calculated on the basis of international units (IU). After

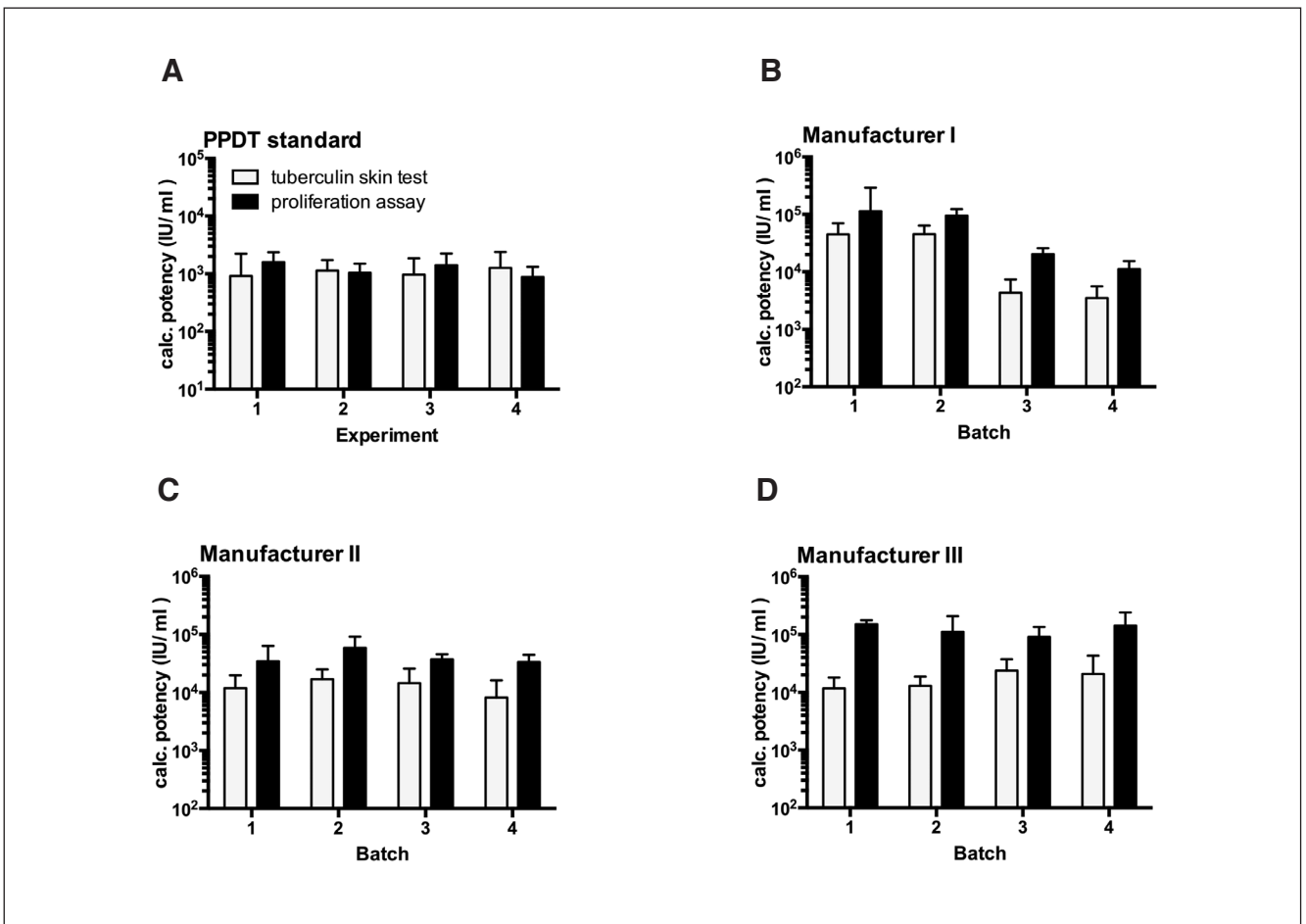


Fig. 4: Proliferation assay and skin test yield comparable potency estimates

(A) The potency of the PPDT standard in relation to the PPDBOV standard was determined in parallel by the proliferation assay and the skin test. Within the independent experiments the same animals were used for both assays. Dose response curves were used to calculate the relative potency estimate for PPDT on the basis of international units. (B-D) Subsequently, four different batches of bovine PPDs from three different European manufacturers were tested against PPDBOV standard. For each test a minimum of eight animals was used. Empty bars represent the calculated potency as determined by skin test, black bars as determined by proliferation assay. Error bars indicate the upper limit of the 95% confidence interval.

5 days T cell proliferation was analyzed by flow cytometry and potency estimates were determined accordingly. In four independent experiments, similar results were obtained with both methods for the PPDT standard: While potency estimates varied from 882 to 1.590 IU/ml for the proliferation assay, a range of 917 to 1.261 IU/ml was calculated for the skin test (Tab. 1 and Fig. 4A). To assess whether the alternative approach is suitable for batch release testing of bovine

PPDs, four batches from three different manufacturers were tested in comparison to the PPDBOV standard. Again, both methods yielded comparable results (Fig. 4B-D). To compare the precision of the two methods we calculated the coefficient of variation based on individual potency estimates. Biological potencies are usually log-normally distributed (Kenakin, 1997; Ratkowsky and Reedy, 1986), therefore the geometric coefficient of variation (CV) was used. Taking all results

Tab. 1: Test repeats with the international standard for PPD of *M. tuberculosis* (PPDT)

Experiment	Proliferation Assay			Skin Test		
	Potency estimate ¹ (IU/ml)	Confidence limits (P = 0.95)	CV ²	Potency estimate ¹ (IU/ml)	Confidence limits (P = 0.05)	CV ²
I	1590.2	67 - 149%	55%	917.6	41 - 243% ³	192%
II	1046.7	70 - 143%	57%	1132.9	66 - 153%	75%
III	1404.3	63 - 160%	86%	965.8	52 - 192%	149%
IV	882.5	67 - 150%	58%	1261.1	53 - 190%	100%

¹ From individual potency estimates, the overall potencies and the respective confidence intervals were calculated for each experimental group in relation to PPDBOV, the international bovine tuberculin standard. Confidence limits are given as percentage of the calculated potencies.

² The geometric coefficients of variation were calculated from the individual potency estimates after natural log-transformation. They are representative for the inter-animal variation.

³ This skin test would have to be repeated, as it does not meet the validity criteria as defined in Ph. Eur. of confidence limits (P = 0.95), i.e., not less than 50 and not more than 200 percent of the estimated potency (European Pharmacopeia Monograph 0536, 2008c).

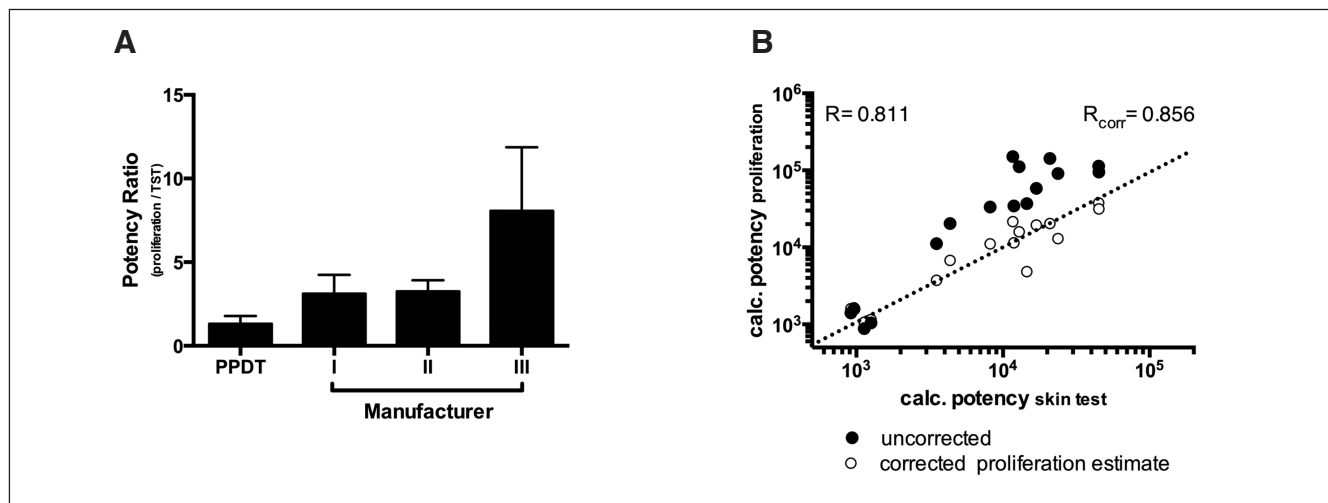


Fig. 5: The proliferation assay overestimates tuberculin PPD potency manufacturer-dependently

(A) The ratio was formed between corresponding potency estimates obtained with the proliferation assay and the skin test. Black bars represent the mean ratio over the four experiments using the PPDT standard or over the four batches tested from each of three manufacturers. Error bars depict the respective standard deviation. (B) All calculated potencies shown in Figure 4 are plotted in a single two dimensional graph (The x-axis shows the results obtained with the skin test, the y-axis shows proliferation assay-derived estimates). The dotted, bisecting line indicates full concordance of test results. Black circles indicate uncorrected test results; open circles depict results for which the proliferation assay derived potency estimates were adjusted with the manufacturer-specific correction factor. Spearman correlation coefficient was calculated for uncorrected and for corrected potency estimates.



together, the inter-individual variation reached on average 56% on the basis of the proliferation assay and 75% on the basis of the skin test (see Tab. 1).

When comparing proliferation assay and skin test based potency estimates, the proliferation assay systematically yielded higher potency estimates. This effect was manufacturer-dependent and was visualized by forming the ratio between proliferation assay and skin test derived potency estimates: no discrepancy was noted for the human PPD; for the different bovine PPDs proliferation assay based estimates were three- (Manufacturer I and II) to sevenfold (Manufacturer III) higher compared to skin test results (Fig. 5A). Therefore, we tested whether the correspondence between the two methods can be improved by a manufacturer specific correction factor. The effect is depicted in Figure 5B: all test results shown in Figure 4 are plotted in a two-dimensional dot plot. Uncorrected values invariably scatter above the bisecting line. When the proliferation-based estimates are divided by three for manufacturer I and II or by seven for manufacturer III, the corrected values lie on or in close proximity to the line. The improved correlation is also reflected by the increase of the correlation coefficient from 0.811 to 0.856. Together this indicates that it is feasible to calculate batch potency estimates for individual PPD batches using the proliferation assay, and that the discrepancy between proliferation assay and skin test can be corrected as it is specific for each manufacturer.

5 Discussion

In the current study we present an alternative method for the potency determination of bovine PPDs. We adapted an approach that was proposed by Hasløv and colleagues in 1986, who developed a method to measure PPD potency by thymidine incorporation (Hasløv et al., 1986). The authors succeeded in calculating relative potencies from lymphocyte proliferation assays. However, unspecific mitogenic effects of PPD on naïve lymphocytes at doses of 1 µg/ml PPD and more were observed. At similar concentrations, we did not observe lymphocyte proliferation prior to mycobacterial sensitization. We therefore conclude that the observed lymphocyte responses are antigen-specific.

The Eur. Ph. monograph for bovine PPD prescribes to sensitize guinea pigs with live *M. bovis* AN5 (European Pharmacopeia Monograph 0536, 2008), but for biosafety and animal welfare reasons we did not use virulent mycobacteria. We therefore tested two alternative methods using live attenuated BCG instead of virulent *M. bovis* AN5 and heat-inactivated *M. bovis* AN5 instead of live *M. bovis* AN5. The latter approach was adopted by analogy to the monographs on human and avian PPD (European Pharmacopeia Monograph 0151, 2008 and European Pharmacopeia Monograph 0535, 2008). We observed pronounced PPD-specific responses. For comparison of the two read-out systems, proliferation assay and skin test, the use of inactivated AN5 can therefore be regarded

a feasible approach, although it is not exactly the same sensitization method as prescribed for bovine PPD in the Eur. Ph. This is particularly true because there was a very good correlation between stated and calculated potencies with a Spearman correlation coefficient of $R = 0.927$ for the proliferation assay and $R = 0.916$ for the skin test (data not shown).

It is of note that sensitization with inactivated AN5 yielded higher proliferation rates and also more prominent skin test reactions (data not shown) as compared to BCG vaccination. The reason is that the heat-inactivated AN5 wet mass used for guinea pig sensitization represents process material derived from a PPD manufacturing procedure. The composition of the sensitizing antigens therein closely matches the repertoire of antigens in the PPD. In contrast, the BCG used for guinea pig vaccination was grown at our institute. Most likely there are substantial differences in the antigen composition compared to AN5 derived PPD. In line, it is known that BCG strains lack important immunogens, such as CFP-10 and ESAT-6 (Harboe et al., 1996). These antigens are present in the *M. bovis* AN5 derived PPDs (Borsuk et al., 2009) and account for a substantial part of the adaptive, anti-mycobacterial immune response (Pollock and Andersen, 1997). Similarly, differences between *M. bovis* and *M. tuberculosis* are likely to explain the responses to the two standard PPDs: both preparations were adjusted to the same concentration according to the stated potency. Since the animals were sensitized with inactivated *M. bovis* AN5, they responded significantly more strongly to the bovine PPD compared to the human PPD produced from *M. tuberculosis*. For our study the reduced reactivity to the heterologous PPD was deliberately utilized to show that the test is able to detect differences between PPDs.

Potency estimates obtained with both methods correlated well, which is in accordance with the notion that the skin reactivity is due to a T cell induced delayed type hypersensitivity reaction (Huebner et al., 1993). Nevertheless, we observed some discrepancy between the two methods. This may indicate that the induction of the inflammatory response in the skin requires additional signals compared to the *in vitro* stimulation of lymphocyte proliferation. Along that line, Hasløv and colleagues showed with *M. tuberculosis* infected guinea pigs that culture filtrate proteins smaller than 10 kDa preferentially induced *in vitro* T cell proliferation, while the major contribution to skin reactivity was conferred by antigens of about 20 to 30 kDa size (Hasløv et al., 1995). In our study, the differences between skin and *in vitro* reactivity were manufacturer-dependent. Probably this can be explained by differences in the antigenic repertoire of the AN5 production strains and/or divergent manufacturing processes. Since these parameters are controlled, as manufactures use seed lot systems and standardized production processes, it is justified to address the systematic discrepancies between the two methods by introducing manufacturer specific correction factors. Ongoing efforts are aiming to further substantiate this approach.

Due to the complexity of the underlying immunological processes, the variability of the test results – in particular the

differences between individual animals of one group – was relatively high with both methods. This is an observation that is frequently made with biological potency assays (Weisser and Hechler, 1997). However, in a direct comparison the proliferation assay was clearly more precise than the skin test, which indicates that the new assay yields more reliable test results. This reduces animal numbers, because it avoids test repeats. The alternative method further reduces animal numbers, because the assay allows for simultaneous testing of several batches: To obtain statistically robust potency estimates test results from at least six guinea pigs are required. This is also the case for the *in vitro* test. However, due to the limited area on the side of the animals, for the *in vivo* skin test only one test preparation can be tested per guinea pig. With the *in vitro* method one blood withdrawal from one animal yields sufficient PBMCs to test up to three batches in parallel. Accordingly, the number of animals can be reduced by three. Since the blood withdrawal is well tolerated (see above), it is theoretically also possible to use the animals several times. However, in this study we did not assess the effect of repetitive bleeding on the accuracy of the potency estimate. Another 3R aspect reducing distress for the animals is that the *in vitro* method replaces the intradermal injections and the development of skin lesions with blood withdrawal under anesthesia. With this, the alternative approach is fully in accordance with the 3R concept proposed by Russel and Burch (1959).

Based on these considerations and on the data presented in the current manuscript, we conclude that the proliferation assay holds promise to replace the *in vivo* potency assay for bovine PPDs. As a next step we are now going to validate the new method and we will perform both tests in parallel to collect further experience. The final goal is to include the method into the European Pharmacopeia monograph and eventually to abandon the current *in vivo* testing.

References

- Bastian, M., Braun, T., Bruns, H. et al. (2008). Mycobacterial lipopeptides elicit CD4⁺ CTLs in Mycobacterium tuberculosis-infected humans. *J Immunol* 180, 3436-3446. <http://dx.doi.org/10.4049/jimmunol.180.5.3436>
- Bastian, M., Holsteg, M., Hanke-Robinson, H. et al. (2011). Bovine neonatal pancytopenia: Is this alloimmune syndrome caused by vaccine-induced alloreactive antibodies? *Vaccine* 29, 5267-5275. <http://dx.doi.org/10.1016/j.vaccine.2011.05.012>
- Borsuk, S., Newcombe, J., Mendum, T. A. et al. (2009). Identification of proteins from tuberculin purified protein derivative (PPD) by LC-MS/MS. *Tuberculosis* 89, 423-430. <http://dx.doi.org/10.1016/j.tube.2009.07.003>
- Caffrey, J. P. (1994). Status of bovine tuberculosis eradication programmes in Europe. *Vet Microbiol* 40, 1-4. [http://dx.doi.org/10.1016/0378-1135\(94\)90041-8](http://dx.doi.org/10.1016/0378-1135(94)90041-8)
- European Pharmacopeia 0151 (2008). *Tuberculin Purified Protein Derivative for Human Use*. Monograph 0151. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe.
- European Pharmacopeia 0535 (2008). *Tuberculin Purified Protein Derivative, Avian*. Monograph 0535. Ph. Eur. 6th edition, Strasbourg, France: Council of Europe.
- European Pharmacopeia 0536 (2008). *Tuberculin Purified Protein Derivative, Bovine*. Monograph 0536. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe.
- European Pharmacopeia 50300 (2008). *Statistical Analysis of Results of Biological Assays and Tests*. Monograph 50300. Ph. Eur. 6th edition. Strasbourg, France: Council of Europe.
- Federal Ministry of Food and Agriculture (2013). Verordnung zum Schutz von zu Versuchszwecken oder zu anderen wissenschaftlichen Zwecken verwendeten Tieren (Tierschutz-Versuchstierverordnung)
- Federal Ministry of Food and Agriculture (2014). Verordnung zum Schutz gegen die Tuberkulose des Rindes (Tuberkulose-Verordnung).
- Harboe, M., Oettinger, T., Wiker, H. G. et al. (1996). Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. *Infect Immun* 64, 16-22.
- Hasløv, K., Closs, O., Moller, S. et al. (1984). Studies on the development of tuberculin sensitivity in immunized guinea pigs with demonstration of a close relationship between results of skin tests and the lymphocyte transformation technique. *Int Arch Allergy Appl Immunol* 73, 114-122. <http://dx.doi.org/10.1159/000233450>
- Hasløv, K., Bentzon, M. W. and Moller, S. (1986). Comparison in sensitized and unsensitized guinea-pigs of tuberculin PPDs RT 23 and PPD-M by skin tests and lymphocyte stimulation tests. Effect of immunization time. *J Biol Stand* 14, 143-152. [http://dx.doi.org/10.1016/0092-1157\(86\)90033-8](http://dx.doi.org/10.1016/0092-1157(86)90033-8)
- Hasløv, K., Andersen, A., Nagai, S. et al. (1995). Guinea pig cellular immune responses to proteins secreted by Mycobacterium tuberculosis. *Infect Immun* 63, 804-810.
- Huebner, R. E., Schein, M. F. and Bass Jr, J. B. (1993). The tuberculin skin test. *Clin Infect Dis* 17, 968-975. <http://dx.doi.org/10.1093/clinids/17.6.968>
- Kenakin, T. P. (1997). *Pharmacologic analysis of drug-receptor interaction*. Philadelphia: Lippincott Williams and Wilkins.
- Monaghan, M. L., Doherty, M. L., Collins, J. D. et al. (1994). The tuberculin test. *Vet Microbiol* 40, 111-124. [http://dx.doi.org/10.1016/0378-1135\(94\)90050-7](http://dx.doi.org/10.1016/0378-1135(94)90050-7)
- Moser, I., Kohler, H. and Menge, C. (2014). Tuberculosis in cattle – surprisingly re-emerging or continuously present? *Tierarztl Prax Ausg G Grosstiere Nutztiere* 42, 240-249.
- NIBSC (2010a). Instructions for use: WHO international standard purified protein derivative (PPD) of Mycobacterium bovis. <http://www.nibsc.org/documents/ifu/PPDBOV.pdf>
- NIBSC (2010b). Instructions for use: WHO International Standard. The 1st international standard for purified protein



- derivative (PPD) of *Mycobacterium tuberculosis*. <http://www.nibsc.org/documents/ifu/PPDT.pdf>
- OIE – World Organisation for Animal Health (2011). Chapter 2.4.7, Bovine Tuberculosis. In OIE (ed.), *OIE Terrestrial Manual 2011*.
- Pollock, J. M. and Andersen, P. (1997). The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. *J Infect Dis* 175, 1251-1254. <http://dx.doi.org/10.1086/593686>
- Prasad, T. S., Verma, R., Kumar, S. et al. (2013). Proteomic analysis of purified protein derivative of *Mycobacterium tuberculosis*. *Clin Proteomics* 10, 8. <http://dx.doi.org/10.1186/1559-0275-10-8>
- Ratkowsky, D. A. and Reedy, T. J. (1986). Choosing near-linear parameters in the four-parameter logistic model for radioligand and related assays. *Biometrics* 42, 575-582. <http://dx.doi.org/10.2307/2531207>
- Russell, W. M. S. and Burch, R. L. (1959). *The principles of humane experimental technique*. London, UK: Methuen & Co, Ltd.
- Weisser, K. and Hechler, U. (1997). *Animal welfare aspects in the quality control of immunobiologicals: A critical evaluation of the animal tests in pharmacopoeial monographs*. Nottingham, UK: FRAME for ECVAM and PEI. ISBN: 0-9501700-4-6

Disclosure

The authors have no financial conflict of interest.

Acknowledgements

This work was supported by the German Ministry for Research and Education (BMBF; grant number: 0316009A). We are grateful for the technical support of F. Mohs and A. Geißler and we acknowledge the contribution of colleagues of the Paul-Ehrlich Institut, namely J. Kirberg, G. van Zandbergen and W. Matheis for provision of reagents and antibodies; K.-M. Hanschmann for help with the biostatistical analysis and V. von Messling for inspiring discussions. We thank H. Schäfer of the Robert-Koch Institut for the generous gift of guinea pig-specific test material.

Correspondence to

Max Bastian, DVM
Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51
63225 Langen, Germany
Phone: +49 6103 77 7464
Fax: +49 6103 77 1254
e-mail: basma@pei.de