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BINACLE Assay for tetanus neurotoxin: outcomes of project BSP136

12 November 2024

Webinar Summary

The following is a summary of the live webinar event hosted by the European Directorate for the Quality of Medicines & HealthCare (EDQM) on 12 November 2024. Slides for the respective presentations and a recording of the full proceedings can be accessed here.

Introductory welcome from the Head of the Department for Biological Standardisation, OMCL Network and HealthCare, Laurent Mallet, EDQM

Laurent Mallet welcomed the webinar participants and gave a brief overview of the mission of the EDQM and the Biological Standardisation Programme (BSP) before introducing the BINACLE assay, its evaluation in BSP136 and the main webinar topics. In the speech, the role of the project leaders from the Paul-Ehrlich-Institut (PEI) before and during the BSP136 study was highlighted, and the contributions of all parties involved in the study and the webinar were gratefully acknowledged.

EDQM & the Biological Standardisation Programme (BSP), Catherine Milne, EDQM

The EDQM BSP was established in 1991 and has been cosponsored by the Council of Europe and the EU Commission since 1994. Its goal is to promote standardisation of quality control testing for biologicals through the establishment of common European Pharmacopoeia (Ph. Eur.) reference standards and methods, in particular, those with a focus on replacement, refinement or reduction of animal use. The programme organises large-scale collaborative studies to achieve its goal. The reference standards established (Biological Reference Preparations (BRPs), Chemical Reference Substances (CRS) and Biological Reference Reagents (BRRs)) are linked to Ph. Eur. methods and are adopted by the Ph. Eur. Commission (EPC). Successful methods are also referenced in the Ph. Eur. to promote implementation. A key focus of the BSP is also to promote global harmonisation and the EDQM team actively interacts with the World Health Organization (WHO) and other regulatory authorities with this aim. The presentation provided an overview of the programme, highlighting steps in the process and key achievements.

Entry poll, Marie-Emmanuelle Behr-Gross & Christina Göngrich

A short poll at the start of the webinar showed that most participants who responded to the questions were based in Europe and worked in licensing departments of regulatory authorities or in public or vaccine manufacturers' quality control laboratories**.** Approximately one third of respondents had no prior knowledge of BSP136. The expectations for the webinar were evenly distributed between learning more about the method's scientific background, the study results and the regulatory implications of the project.

The BINACLE (binding and cleavage) assay for *in vitro* activity determination of tetanus neurotoxin, Heike Behrensdorf-Nicol, PEI, Germany

Tetanus neurotoxin (TeNT) is a potent toxin inducing severe muscle spasms. Chemically inactivated TeNT serves as tetanus vaccine. During vaccine production, inactivated toxoid bulks must be tested in guinea-pigs for the absence of active toxin. If the animals do not develop tetanus symptoms, the toxoid is considered safe. This test has never been validated in line with current guidelines. Replacement by a suitable *in vitro* method would be preferable.

TeNT consists of two subunits linked by a disulfide bond: the heavy chain mediates the binding to neuronal receptors and the translocation of the light chain into the cytoplasm, whereas the light chain cleaves synaptobrevin and thereby blocks neurotransmitter release. Based on this mechanism of action, PEI developed the binding and cleavage (BINACLE) assay for *in vitro* detection of active TeNT: the toxin first binds to a receptor-coated microplate, then the light chains are released by chemical reduction and transferred to a plate containing immobilised synaptobrevin. Active light chains cleave the synaptobrevin, and the cleavage fragment is detected using an antibody.

PEI initially demonstrated that this assay can detect spiked TeNT in toxoids with a detection limit similar to the estimated *in vivo* detection limit. However, the applicability of BINACLE testing varies between toxoids from different sources. The transferability of the BINACLE assay to other laboratories was also shown.

Based on these data, the BSP Steering Committee initiated the collaborative study BSP136 to examine the applicability of the BINACLE assay as an alternative to the animal test for the toxicity testing of tetanus toxoids.

Review and harmonisation of toxicity testing requirements in Ph. Eur. tetanus vaccine monographs: 3Rs achievements, Gwenaël Ciréfice & Catherine Lebrun, EDQM

In parallel to the BSP136 project, the work of the Ph. Eur. groups of experts in charge of human and veterinary vaccines and sera (Groups 15 and 15V) was presented. The toxicity testing requirements of both Ph. Eur. monographs for tetanus vaccines for human and veterinary use have been revised to be rationalised and harmonised. As a result of the exercise, both monographs now include similar toxicity testing, i.e. a test for absence of tetanus toxin where at least 500 Lf of bulk purified toxoid is to be injected into 5 guinea-pigs weighing 250-350 g. During this reassessment of the toxicity testing requirements for tetanus vaccines and based on the review of collected data (published and in-house data, data from a survey with vaccine manufacturers), two animal tests have been deleted: the test for irreversibility of toxoid as well as the test for residual toxicity/test for specific toxicity, carried out in guinea-pigs. The different steps of this exercise and the work on the Ph. Eur. monographs was explained. BSP136 was aimed at replacing the only remaining animal safety test in tetanus vaccine monographs: the test for absence of tetanus toxin in guinea-pigs.

BSP136 collaborative study – outcomes part 1, Marie-Emmanuelle Behr-Gross, EDQM

An international collaborative study aimed at verifying the suitability of the BINACLE assay as a potential alternative to the guinea-pig toxicity test for tetanus toxoids was organised by the EDQM under the aegis of the BSP. Within the framework of this study, coded BSP136, a feasibility phase was run to select and qualify critical study reagents and samples and to assess the performance of the BINACLE test Standard Operating Procedure developed by the project leaders. Then the international collaborative study aimed at evaluating the BINACLE was started. A total of 19 international laboratories (comprising vaccine manufacturers as well as national control laboratories) were supplied with a detailed assay protocol, critical reagents required for the assay, 3 samples consisting of 3 different bulk tetanus toxoids donated by major European vaccine manufacturers and one international standard toxoid, and were asked to purchase a centrally reserved commercial tetanus toxin from the same batch. Each of the participants had to perform 3 independent BINACLE assays following the provided protocol. The statistical analysis of the results showed that most of the participating laboratories were able to perform the BINACLE assay according to the provided protocol. However, the results obtained by the participants varied widely, and not all the laboratories were able to achieve a sensitive detection of active tetanus toxin. Factors that may have contributed to the elevated variability of the BSP136 study results were analysed and strategies were developed to help increase the standardisation of the BINACLE assay and obtain more consistent results in a follow-up validation study, BSP136 Part 2.

Challenges identified in BSP136 part 1, Heike Behrensdorf-Nicol, PEI, Germany

From the results of BSP136 part 1, it was concluded that the BINACLE assay has the potential to detect TeNT with a sensitivity similar to the *in vivo* test. However, with regard to the high variability of the results, it was agreed that an optimisation and enhanced standardisation of the method would be required. Several factors that may have contributed to the increased variability were identified and used as starting points for optimising the BINACLE protocol and the study design. The improved protocol was then tested in BSP136 part 2.

The following improvements were introduced in this context:

- More prequalified reagents were distributed to the participants to make sure that all materials used were of appropriate quality.
- In study part 1, each participant had to prepare the toxin solutions in a multi-step and potentially error-prone procedure. For part 2, ready-to-use TeNT solutions were provided to all participants.
- Some protocol steps that had turned out to be critical were improved to facilitate handling and enhance standardisation. For example, measures were introduced to prevent an inappropriate drying of the microplate wells.
- More extensive information about the BINACLE method was provided to the participants, and the importance of strict compliance with the protocol instructions was communicated clearly.
- The plate layout was optimised to make the handling less error-prone and allow a better estimation of the detection limit.

BSP136 collaborative study – outcomes part 2, Christina Göngrich, EDQM

BSP136 part 2 was carried out using an optimised and more standardised study protocol compared to BSP136 part 1, with the goal of testing the applicability of the BINACLE method as alternative to the compendial *in vivo* test "Absence of tetanus toxin". Applicability was assessed based on the assay precision and the limit of detection of the method under the improved study conditions. As an important standardisation point, almost all reagents were supplied as prequalified and, in most cases, ready-to-use solutions to avoid introduction of variability due to the reagents used. Four manufacturers of tetanus vaccines for human use, four manufacturers of tetanus vaccines for veterinary use and the laboratory of the project leader participated in this collaborative study and provided data for a total of 38 assays.

The presentation focused on the study design and results, which demonstrated good assay precision both with respect to repeatability and reproducibility. Importantly, the limit of detection estimated in this study part indicated that the BINACLE assay can detect TeNT with similar sensitivity to *in vivo* toxicity tests.

The study outcomes suggested that the method is a promising alternative to the current test, and it was therefore presented to the Ph. Eur. Groups 15 and 15V for their consideration as an alternative method to the compendial *in vivo* test.

Considerations for method validation and implementation, Heike Behrensdorf-Nicol, PEI, Germany

Various topics related to the implementation and validation of the BINACLE assay were addressed:

- In BSP136, toxoids were tested in diluted form based on the specifications of the former test for "irreversibility of toxoid". However, the currently prescribed test for "absence of toxin" is performed using concentrated toxoids. In-house experiments confirmed that toxoids that are suitable for BINACLE testing can also be applied to the BINACLE assay in concentrated form. For toxoids inducing elevated background signals, however, testing at high concentrations may not be possible.
- A prerequisite for the substitution of animal-based safety tests is that the alternative method is at least as sensitive as the animal test. The results of BSP136 indicated that the detection limit of the BINACLE assay for TeNT in toxoids is approximately equivalent to the estimated *in vivo* detection limit.
- For several BINACLE reagents, information about key characteristics and availability was provided. As internationally accepted reference toxins or toxoids for the BINACLE assay are not yet available, in-house reference preparations should be established.
- The users of the BINACLE assay need to validate the method for their specific toxoid product. Essential components of this validation are a profound characterisation of the toxoid in the BINACLE assay and a comparison of the detection limits of the *in vitro* and *in vivo* tests. It must be demonstrated that the BINACLE assay reliably detects TeNT-containing toxoids.
- Recommendations for routine use of the BINACLE assay were made in terms of microplate layout, relevant controls, and validity and acceptance criteria.

Towards the introduction of BINACLE in Ph. Eur. tetanus vaccine monographs, Gwenaël Ciréfice & Catherine Lebrun, EDQM

Further to the successful completion of the BSP study in 2024, the EPC has added the revision of both Ph. Eur. monographs for tetanus vaccines for human and veterinary use to its work programme to introduce a reference to BINACLE as an *in vitro* alternative to the test for absence of tetanus toxin in guinea-pigs. Ph. Eur. Groups 15 and 15V worked together to discuss how the outcome of BSP136 could be translated in tetanus vaccine monographs. The revised monographs will be published in Pharmeuropa 37.2 (commenting period from 1 April to the end of June 2025). The participants were invited to take part in the public enquiry.

BINACLE assay for tetanus neurotoxin: optimisation perspectives for quality control use, Romain Pizzato, Sanofi, Marcy-l'Étoile

In light of Sanofi's commitment to the 3Rs and its ambition to fully replace *in vivo* analytical testing, the company has been involved in the BSP136 collaborative study since its early phases in order to implement an *in vitro* alternative to the current guinea-pig model for the detection of active tetanus toxin. In parallel, with the aim of introducing a method fully applicable to an industrial QC environment and fully in line with the company's 3Rs policy, the analytical development laboratory has been performing assay optimisations, notably regarding the use of alternatives to critical animal-derived reagents and chemical reagents with occupational safety concern as well as assay cycle time. The presentation therefore described the optimisations already performed or ongoing along with comparative results obtained on different matrices. Lastly, the presentation highlighted remaining challenges for next steps to further develop and implement this assay in collaboration with the EDQM and other stakeholders.

BINACLE Requirement for industrialisation of the assay, Shahjahan Shaid, GSK, Marburg, Germany

GSK's animal studies are conducted with high standards of humane care and treatment. GSK's moral and scientific responsibility led to a company-wide strategy and programme to set up state-of-the-art non-animal technologies for QC testing regarding the safety and efficacy of vaccines.

Active engagement in collaborative studies to identify alternative methods, as well as transparent communication to regulators on the equivalence or even superiority of these methods, enables collective agreement to be reached. This is a cornerstone of GSK's strategy to reduce the use of laboratory animals in vaccine release by 75% by 2025 when compared to 2015. To date the company has achieved a 66% reduction.

In this talk GSK presented their efforts to industrialise the BINACLE assay evaluated in the BSP136 study and described critical elements to move the assay into cGMP release testing as the sole safety assay to test the detoxification of TeNT in tetanus vaccines. Elements that were discussed are 1) availability of critical reagents such as antibodies, antigens and stabilisers; 2) the advantage of standardising toxins and toxoids and 3) potential requirements of regulators outside of Europe.

While the work toward non-animal-based research and development continues, GSK is committed to a culture of care, acting ethically and practising good animal welfare where animal use is still inevitable.

Exit poll – outcomes

In a second poll, respondents identified the availability of a reference tetanus toxin, the commercial availability of the antibody to cleaved synaptobrevin and the international harmonisation of testing requirements as factors that would most support the implementation of the BINACLE method in the QC strategy for tetanus vaccines.

On the subject of future activities, 52% of respondents indicated that their organisation would be willing to participate in future BSP studies, and 38% responded that their organisation could support the reduction of animal use by submitting to the EDQM relevant information on alternative methods that were developed in-house for the QC testing of biologicals. In a final word cloud on products, assays and tests to be considered as priorities for 3Rs activities, potency assays in general were frequently mentioned.

Webinar highlights and closing of the meeting, Catherine Milne, EDQM

Catherine Milne closed the webinar with a summary of the main points that were addressed, highlighting the webinar's goal of facilitating the implementation of the BINACLE method, which is a promising opportunity to end the need for using animals in toxicity testing for a number of tetanus vaccines in the human and veterinary field. It was considered of importance for the global community that two major vaccine manufacturers openly shared their results and experiences with the method implementation in this webinar in the spirit of promoting 3Rs. Thanks were expressed to all webinar speakers, organisers and project contributors, first and foremost to the project leaders and the PEI as a whole.