General Public

Annual newsletter 3

EUROBIOTOX UPDATES

2020

EuroBioTox project made significant progress !

It is a great joy for the EuroBioTox partners to publish the third annual newsletter, aiming at describing the progression of the project, the different meetings, the trainings courses and to shed light on one of the toxin involved in the scope of the project: the Botulinum neurotoxin.

Thanks to the involvement of the EuroBioTox partners significant progress was made during the first half of this project.

Three of the candidate reference materials are now produced and filled and their characterization is far in progress. So far the materials look very good: They passed first purity, homogeneity and stability tests successfully.

We finished the production of tools and reagents (e.g. antibodies, toxic supernatants, lateral flow assays) for detection of the molecules in the scope of EuroBioTox. An important effort was made to perform a parallel validation of immunological, MS based and functional detection methods for ricin, abrin, SEB and STX.

The first tools are now available since early 2020 for authorised lab in the EuroBioTox network.

All basic training courses were given and four large proficiency tests (PT) were organised.

Guidelines and practical protocols for first responders on sampling, detection and decontamination of biological toxins in case of an incident were presented at the 3 day workshop for first responders in March 2020 at CEA Saclay FR.

Face to face meetings

The second small project meeting was organised among all beneficiaries in Umeå, SE, in May 2019. During this meeting, discussions focused on the progress reached in all work packages in detail, especially progress in WP1 (reference materials), WP2 (repository/validation), preparation of WP3 (proficiency test scheme), status of WP4 (quality assurance), WP5 (training), WP6 (*in-situ* detection/ forensic procedures) and WP7 (animal replacement methods). This served to efficiently implement the different WPs and to streamline the scientific and administrative decision processes.

Participants of the 2nd EuroBioTox face-to-face meeting among all beneficiaries in Umeå, Sweden.

WP6 meeting

Apart from the second small project meeting, another face-to-face meeting joining selected partners working together on WP6 tasks was organised. This meeting was held on December 4, 2019 with participants from FOI, CEA and the Swedish company SAAB Technologies. The meeting focused on finalising the agenda and deciding on speakers and companies to invite to the workshop for first responders in March 2020. The procedure for the demonstration was also discussed and adjusted according to feedback from the CEA first responder team. The Swedish company SAAB, producer of the backpack used at the workshop, was invited to decide on the most suitable materials for sampling and detection of biological toxins (see pictures below).

Participants of the face-to-face meeting on the workshop for first responders under WP6 at CEA, France.

Anti-bioterrorist sampling kit for toxins from SAAB.

First EuroBioTox publication !

During the second reporting period, the EuroBioTox partners were able to publish the first publication on EuroBioTox contents highlighting the intricacies involved in the generation and characterisation of toxin reference materials. If you are interested, the paper is available at:

https://edoc.rki.de/handle/176904/6290

Zeleny R, Rummel A, Jansson D, Dorner BG (2019). Challenges in the Development of Reference Materials for Protein Toxins. In "Applications in Forensic Proteomics: Protein Identification and Profiling", Merkley E, Ed.; ACS Symposium Series #1339; American Chemical Society; DOI: 10.1021/bk-2019-1339.ch012

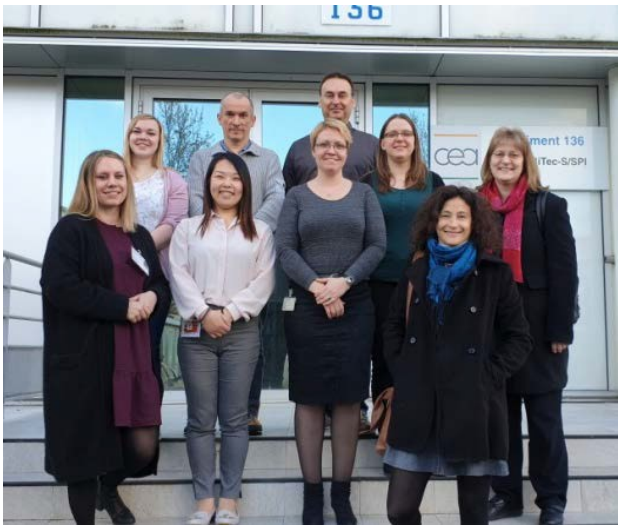
Training courses

BoNT/A detection

Three basic training courses for the detection of BoNT by immunological methods were organised by CEA and RKI at Saclay in March 2019. There were 10 scientific institutions represented at the training courses: from Cyprus, Finland, Germany, Italy, Norway, Slovakia, Sweden, The Netherlands and United Kingdom. The aim was to train participants to analyse BoNT/A containing samples based on different immunological methods and to implement this approach in their own laboratory as part of an overall analysis strategy.

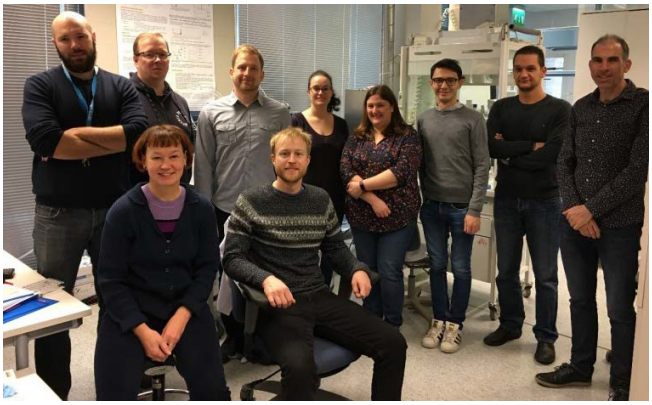
This training involved theoretical lectures on BoNT, Antibody/ELISA and safety aspects as well as an overview of existing immunological methods for BoNT/A detection. The focus was laid on how to set up sandwich ELISA starting from monoclonal antibodies available from the EuroBioTox repository. Two BoNT ELISA protocols (CEA and RKI) and one LFA were trained. Feedbacks from the trainees was very positive!

A proficiency test for the detection of BoNT was organised was organised by RKI in February 2020 and the training participants were given the opportunity to participate with tools and protocols during this course.



Trainees and instructors of EuroBioTox training course 14 at CEA, Saclay, in March 2019

Saxitoxin detection



Trainees and instructors of EuroBioTox advanced training course 5 at VERIFIN in October 2019.

Two advanced training courses on immunological, chromatographic and LC-MS/MS methods for STX analysis were also organised. Five persons signed up for the HPLC-FLD course organised at VERIFIN in October 2019 and eight trainees from both outer and inner partner laboratories took part in the LC-MS/MS methods course.

HPLC-FLD course participants received introduction into the official regulatory PSP monitoring method and practical training on adapting it for analysis. Teaching focused on how to prepare analytes from complex mixtures using different solid phase extraction methods and how to apply prechromatographic oxidation HPLC–FLD with reference controls for identification and quantitation of STX and its analogs.

During the LC-MS/MS course, trainees prepared samples from complex matrices by two clean-up methods to be tested, analysed all samples using LC-MS/MS analysis method. Results were calculated and discussed. After the course, the participants were able to analyse PSP containing samples based on the trained method and to implement the technique in their own laboratory as part of an overall analysis strategy.

Trained laboratories are expected to participate and to apply the trained methods in the forthcoming STX PT in 2020.

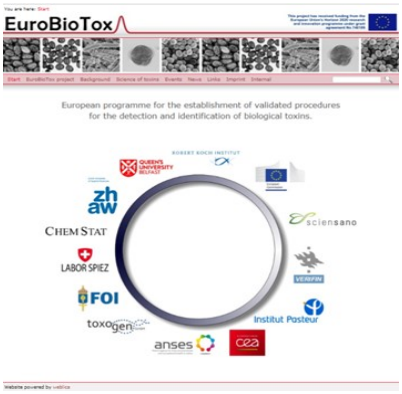
EuroBioTox website

The EuroBioTox website- launched on 31 January 2018 was updated to improve visibility of 'News' and a newsletter subscription tool was incorporated, by a new link allowing direct registration to receive the news from EuroBioTox including the annual newsletter.

Non-confidential training material of all basic training courses was also deposited on the EuroBioTox website and is accessible by login to all trained institutions to multiply and to spread information.

An animated movie explaining the objectives of EuroBioTox for general public was produced and will be added soon to the website.

You can visit the website using this link: https://eurobiotox.eu



Interim report

All network activities, results obtained and materials generated in the different workpackages were described in an interim report and evaluated by the European Commission call Horizon 2020 Framework Programme. A second review meeting took place in Bruxelles in February 2020. During this meeting the consortium presented all the activities carried out so far including the deliverables submitted, the project management and use of resources, ethics issues as well as dissemination and communication activities.
Our laboratories have been working hard to actively contribute to the project and experts from the commission were very satisfied by the project activities. Feedback from EC on the scientific report states "Project has delivered exceptional results with significant immediate or potential impact". Moreover, no changes were requested to be addressed.

Proficiency tests

Four large proficiency tests (PT) were organised so far. These PTs covered qualitative and quantitative detection of STX, SEB, ricin/abrin and BoNT. STX and SEB PTs are finished. Individual summary reports for the ricin/abrin PT were sent to the participants in early January 2020. The PT samples on the qualitative and quantitative detection of BoNT were dispatched in February 2020. This BoNT PT was foreseen to be organised by Pasteur Institute. However, due to technical and administrative problems at Pasteur that could not be resolved in time, it was decided that the organisation of the two BoNT PTs was switched: RKI took over the organisation of the first BoNT PT, while Pasteur will organise the second BoNT PT. Results obtained by the participating laboratories were already sent to the organiser and are currently under evaluation.
Six more proficiency tests will be organised within the next years. We will inform all EuroBioTox partners on the timeline.

New EuroBioTox outer network partners

Based on the work performed in the first half of the project, three additional outer network partner were recruited to join EuroBioTox : The first one is Netherlands Organisation for Applied Scientific Research (TNO) from the Netherlands.

In early 2020 three more institutions also joined the outer network partners : the "Institut de Recherche Biomédicale des Armées" (IRBA) from France, "Unidade Militar Laboratorial de Defesa Biológica e Química" from Portugal and Ministry of Interior - Czech Fire Rescue Service DG, Population Protection Institute, from Czech Republic.

The fact that additional outer network partners could be recruited to EuroBioTox indicates that the project is seen from the outside as an important and successful European initiative.

EuroBioTox and coronavirus crisis

Due to the actual coronavirus crisis situation and after internal discussions with all core members of the project a significant number of tasks had to be postponed.

The next large meeting which was initially planned for the end of May 2020 at ANSES is postponed to 6-7 October 2020 and will be executed as Web meeting.
All training courses in March, April, May and June were shifted to next year starting from January/ February 2021. Training course organisers have made a suggestion for a new timeline that you can find in the table below.

The *In-situ* PT and the second STX PT were also postponed. We will keep you informed about the timeline.

Original timeline	Task	Postponed to
16-18 March 2020	Training: ELISA validation / SEB (Anses)	01-05 February 2021
March 2020	Training: ELISA validation / SEB II (Anses)	08-12 February 2021
20-23 April 2020	Training: Proteomics I on SEB (FOI)	18-20 May 2021
April 2020	*In-situ* PT (FOI)	21 October 2021
11-14 May 2020	Training: Proteomics II on SEB (CEA)	12-15 April 2021
May 2020	STX PT2 (Verifin)	Early September 2020
25-29 May 2020	2nd large Meeting in Paris (Anses)	05-09 October 2020
09-11 June 2020	Training: ELISA validation / ricin quantification by ELISA I (CEA, RKI)	02-04 March 2021
17-19 June 2020	Training: ELISA validation / ricin quantification by ELISA II (CEA, RKI)	08-10 March 2021
Sep/Oct 2020	SEB PT2 (Sciensano)	Nov/Dec 2021
28-30 Sep 2020	Training : Ricin detection by functional MS (CEA)	03-05 Nov 2021
07-09 and 14-16 Dec 2020	Training : BoNT detection by functional MS (CEA)	01-03 and 08-10 Dec 2021
January 2021	3rd small Meeting in Geel (JRC)	Mar/Apr 2021

Workshop for first responders

A three-day workshop for first responders focusing on *in-situ* sampling, detection and decontamination was organised at CEA (Saclay, France) between March 3 – 5, 2020.

The workshop gathered 17 external participants from seven different EU countries including police, emergency units and fire brigades.

The first day focused on theoretical lectures on toxins, bioterrorism and the guidelines. The second day focused on discussion of the sampling and detection guidelines after a scenario-based demonstration with a team of CEA first responders. The last day was dedicated to presentations and practical demonstrations of existing and emerging detection equipment/kits from the invited commercial partners.

Participants of the workshop

The participants had the opportunity to visit a mobile lab from the Population Protection Institute, Czech Republic. The laboratory and all its equipment was presented to the attendees.

(See photos on the left)

Day 2 started with a scenario-based demonstration in a "clandestine lab" where the CEA first responder team showed the procedures from the guidelines live. The demonstration covered the step-by-step procedure including reconnaissance, documentation, sampling, packaging and in-situ LFA detection of three sample types (liquid, solid, wipe).

Botulinum Neurotoxins

2020

PART II : FOCUS ON...

General overview

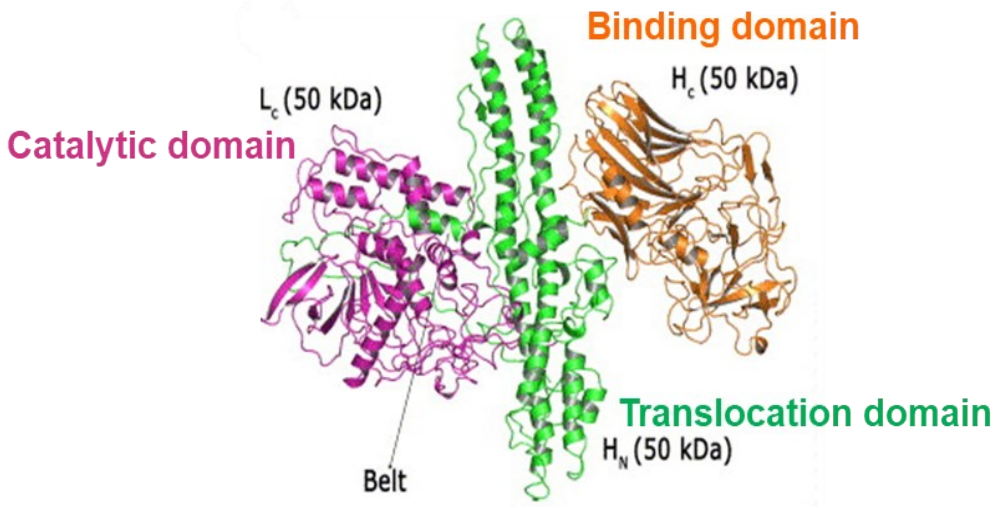
Botulinum neurotoxins (BoNTs) are the most potent toxins among all bacteria, animal and plant toxins. BoNTs are responsible for botulism in humans and other vertebrates. This disease is characterized by flaccid paralysis and inhibition of secretions leading to respiratory distress and death in the most severe cases. The extremely high *in vivo* toxicity of BoNTs results from a unique cascade of sequential steps resulting in cumulative effects. The latter include trafficking from the intestinal tract to neuronal cells, recognition of specific neuronal cell receptors, receptor-mediated endocytosis followed by translocation into the cytosol of their catalytic domain (light L chain) that is a protease, and cleavage of SNARE proteins inducing decreased neurotransmitter exocytosis at the nerve endings of the peripheral nervous system. Therefore, cleavage of a small proportion of SNARE proteins is sufficient to silence synaptic neurotransmission, and partial blockade of acetylcholine release at neuromuscular junctions is sufficient to weaken respiratory and pharyngeal muscles so that respiratory insufficiency and asphyxia ensue.

Given their extreme potency, BoNTs are considered among the most potent agents at risk of bioterrorism and are classified as a Tier 1 select agent by the United States Centers of Disease Control and Prevention.

BoNTs are classically divided into 7 types (A, B, C, D, E, F, and G) according to the inhibition of their biological activity by specific neutralizing antibodies. Each toxinotype is neutralized by its corresponding antiserum and not by the sera against the other toxinotypes. BoNT genes have been sequenced in numerous *Clostridium botulinum* strains, and amino acid sequence variations have been observed between BoNTs of each toxinotype. Thereby, based on amino sequence variations, BoNTs of each toxinotype are subdivided into subtypes. Up to now, more than 40 subtypes have been identified from the 7 toxinotypes. Recently, additional BoNT types have been characterized including BoNT/H (or F/A, or H/A) and BoNT/X.

BoNTs are produced by *C. botulinum* which is a heterogeneous bacterial species subdivided into 4 groups (I, II, III, and IV or *C. argentinense*), and by atypical strains from other Clostridium species such as *C. baratii*, *C. butyricum*, and *Paraclostridium bifermentans*. In addition, related BoNT sequences have been identified in non-clostridium species such as BoNT/Wo and BoNT/Cp1 in the Gram-negative *Weissella oryzae* and *Chryseobacterium piperi*, respectively, as well as BoNT/En (or eBoNT/J or BoNT/J) in an *Enterococcus faecalis* strain.

Structure of BoNT



Strategies for BoNT analysis

The complexity and diversity of BoNT types and subtypes as well as their biological activities are seriously hampering the development of sensitive *in vitro* tests for detection and identification. Up to now, based on the high *in vivo* lethal potency of BoNTs, the mouse bioassay is the most sensitive and it is considered as the gold standard test for BoNT identification and titration. An additional and relevant advantage of the mouse bioassay is that mice are sensitive to all BoNT types and subtypes. It is noteworthy that the therapeutic units of BoNT are defined as mouse lethal doses 50 (LD50), but with relative precision. Due to the more and more restricted use of laboratory animals and the heavy ethical concerns linked with the mouse bioassaysensitive *in vitro* tests are required to perform BoNT detection and identification by routine laboratories and for accurate determination of BoNT therapeutic doses.

In EuroBioTox, specific work packages are devoted to BoNTs including preparation of materials representative of each BoNT type and subtype as well as evaluation of currently available *in vitro methods as* replacement of the mouse bioassay.

Alternatives to the mouse bioassay: Where do we stand, where do we go?

The mouse bioassay (MBA) has been the gold standard for the detection of botulinum neurotoxins (BoNTs) for the last 100 years [1] for several reasons: The MBA is very sensitive; depending on the BoNT serotype it can detect between 5–50 pg/mL of the toxin mostly within 8–24 hrs. For an experienced operator, the MBA is very easy to use and requires minimal handling time. Most importantly, it is sensitive to so far all known sero- and subtypes and covers all three biological steps in BoNT-mediated toxicity (receptor binding to nerve terminals, translocation of the light chain into the cytosol and enzymatic degradation of SNARE proteins). However, it is associated with several drawbacks: It is ethically highly questionable due to the suffering of the animals. Furthermore, it requires the operator to have continuous access to an animal facility. Regarding the specificity of the MBA, matrices with a high load of microbiota (e.g. stool or certain foods) as well as other toxic components, drugs, or auto-antibodies have been reported to induce false positive results. Therefore, blocking of the toxic action in vivo by serotype-specific antibodies is necessary to proof presence of BoNTs [2, 3].

There are two major fields intended to be addressed by animal replacement methods: on the one hand testing of pharmacological grade BoNT for which the sero-/subtype as well as the matrix is known and well-defined; in this case the assay duration is not of relevance; and on the other hand diagnostic/surveillance purposes, which have to cover all known and unknown sero- and subtypes as well as many different matrices; here the assay time is often paramount. Numerous alternatives have been proposed to replace the MBA, many of them overcoming the one or other drawback of the MBA, but none was able to fulfill all the requirements for a bona fide replacement so far [4, 5]. For diagnostic/surveillance applications in particular, the major obstacles are the high degree of variability between the sero- and subtypes being reflected by the involvement of different receptors of which not all have been elucidated yet and, furthermore, by the proteolytic action at various positions on the three synaptic proteins targeted by BoNTs, the proteins VAMP, syntaxin, and SNAP-25 [6, 7].

What have been the key strategies to replace the MBA? Since half a century, immunoassays (e.g. RIA, ELISA) have been developed to detect the toxin itself but fell short to assess its activity. As all assays involving antibodies, immunoassays need to be tested against all existing sero- or subtypes to make sure that there no diagnostic gaps. In addition, they can result in false negative and false positive results depending on the quality of antibodies applied and on the matrix components present in the samples [8]. Cell culture based assays depict all steps required for BoNT toxicity, but sensitivity, assay time, and applicability to complex matrices are most often insufficient for diagnostic/surveillance purposes. Yet they became valuable replacements for the MBA in testing of pharmaceutical BoNT preparations, able to save hundreds of thousands of animal lives [2]. The third important detection strategy focuses on the detection of the enzymatic activity of BoNT's light chain to cleave at least one of the three synaptic proteins. Each serotype cleaves its target at a unique position, which narrows the more than 40 subtypes down to 8 cleavage sites [4]. Proteolysis of the synaptic proteins can either be assessed by cleavage assays utilising different read-outs like Förster resonance energy transfer [9, 10], detection of cleavage products by mass spectrometry (Endopep-MS assay, [11]), or alternatively, by neoepitope-specific antibodies recognising the newly generated neo-epitope after substrate cleavage (e.g. Endopep-ELISA [12-14], SPR [15]). Usually endopeptidase assays show good sensitivities, sometimes even exceeding the sensitivity of the MBA. Nevertheless, to be compatible with the problems arising from complex clinical, food, or environmental matrices (e.g. endogenous proteases, inhibitors), an upstream purification step utilising either antibodies or receptor molecules is necessary [12, 16, 17]. Antibodies for all serotypes are available in various

qualities; here, the recognition of all subtypes of one specific serotype is paramount [18-20]. Unfortunately, comprehensive characterisation of the antibody quality and subtype specificity is difficult to conduct as the access to many subtypes is restricted and published data on subtype-recognition are sparse. The use of native receptor molecules might overcome this issue due to its intrinsic binding capability of multiple BoNT subtypes, but well defined receptor molecules are only available for individual serotypes. Additionally, it must be considered that subtypes might bind to those receptors with different affinities [21-23]. Thus, the major obstacles for any *in vitro* replacement assay for the detection of BoNT in the framework of diagnostics is to assure broad subtype recognition and assay compatibility with complex matrices. To address this EuroBioTox has set-up a repository of culture supernatants covering a number of available BoNT subtypes to be used for assay validation within the network. In addition, the EuroBioTox repository contains a selection of pre-evaluated antibodies which can be used by all network partners to establish and to validate own detection methods. Finally, EuroBioTox develops (certified) reference materials for toxins to facilitate a better comparison of data across laboratories. In the future, the reference materials as well as the BoNT containing culture supernatants will be used to evaluate a set of five animal replacement methods next to each other.

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