Final Report club 5 joint research project, covering 2011-2012
Title: In vitro multiplex botulism bioassays using neurotoxin capture systems.
Participating partners:
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3. Øystein Angen; National Veterinary Institute (DTU), Copenhagen, D
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Summary project plans (no more than 100 words) :
Alternative assays for the 7 serotypes of botulism neurotoxins are desired to replace standard mouse bioassays, able to detect toxin levels down to 10-20 pg/ml. Recently either with synaptosomes or antibodies, toxin capture experiments (serotypes A, B, E and F) have been shown to detect serotype specific endoprotease activity at sufficient sensitivity (Evans 2009; Kalb 2010). Developing toxin capture techniques (synaptosomes, antibodies) and subsequent toxin type detection assays (ELISA, mass spectrometry) with peptide substrates for toxin-specific proteolysis, this project will find proof that at sufficient sensitivity BoNT/C and D toxin presence can be revealed. These two toxins are the most frequently encountered serotypes in veterinary labs.

State if there were any changes according to the original research plan:
The plans were reviewed and approved in a kick-off meeting at Weybridge of which the minutes are attached to this report (date: 9 Feb 2011). One addition to the original proposed project was that MS final samples should be inactivated to prevent any chance for intoxication of personnel, yet the process should also leave the test material (peptides) intact. This process was not yet available. The MS analyses on the first synaptosomal toxin substrate samples had been planned too optimistically and were only possible from early 2012 onwards. The approach using antibody capture - to be performed parallel outside this CoVetLab environment - has been stopped halfway 2012 (intended by AHVLA) due to changes in research priority.

Formal Date started:	1 Dec 2010
Length of project	2 years (with a go decision in Oct 2011)
Formal End of project:	30 Nov 2012

Project Team		
Names	Institute	Role in project
Jan Langeveld	CVI	coordinator, synaptosome capture approach
Maurice Sauer	VLA	analysis by Mass Spec; antibody capture approach (outside this project)
Mikael Hedeland/Viveca Baverud	SVA	analysis by Mass Spec; supply field sample(s)
Ostein Angen/Heegard	DTU	analysis by Mass Spec; supply sera & field samples

Milestone #	Milestone title	Month	Achieved
1	Synaptosomal capture performed	0-1	yes
2	Candidate products being tested in MS (VLA, SVA)	1-3	yes
2	Endopeptidase activity tested by MS (VLA, DTU, SVA)	1-3	yes 2012
3	Synaptosome-toxin capture protocols optimized (CVI)	2-4	yes
4	Toxin inactivation studies	new	yes
6	Protocols for synaptosme-toxin capture and preparations of synaptosome ready to be transferred to DTU	2-4	yes
7	Start immunisation rabbits (CVI)	6-12	yes, funded from other CVI funding
8	Spiking experiments	11-12	no
9	2nd year testing developments and opportunities for further funding.	11-12	accomplished at CVI

Deliverable #	Deliverable title	Month	Achieved
1	Method for synaptosome preparation	1	yes
2	Method for synaptosomal toxin BoNT/C and D capture	4	yes
3	MS data on substrate cleavage products (DTU, VLA and SVA) from exprimental non-captured and captured toxin(s)	3 & 8	yes
4	Antisera specific for cleavage products	7	yes
5	Spiked field samples tested	12	no
6	Final report of project	24	yes
7	New plans for year 2 CoVetLab	12	yes
8	Plans for acquiring for external funding	10-12	ongoing

Results / progress:

Capture conditions: Synaptosomal preparations were prepared under currently standardized conditions for the past period. BoNT/D and BoNT/C samples had been both found to be captured properly using an in-house available toxin C and D specific monoclonal antibody (Figure 1). Freezing of brain tissue did not affect the synaptosomal work-up procedure. In the mean time proteolytic activity of the synaptosomes has been given attention, which is present and can be largely reduced to acceptably low back ground levels. Other factors that have been investigated for the capture were salt, carrier protein additives, centrifugation circumstances, temperature, and pH.

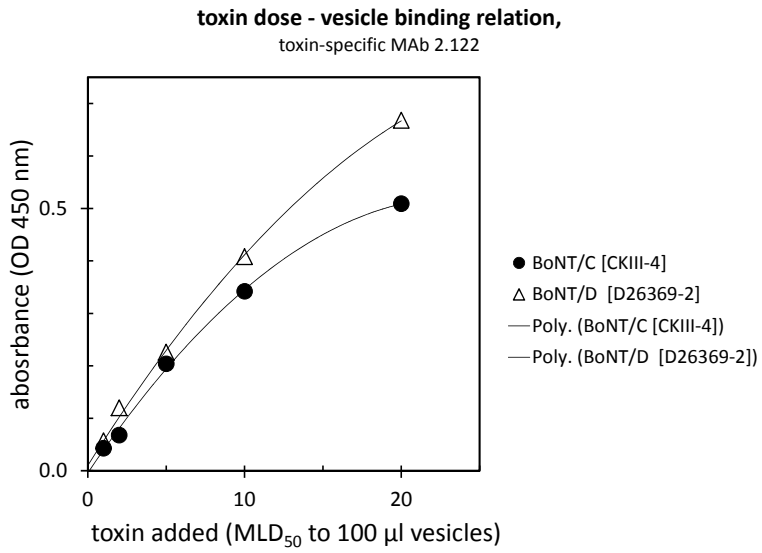

Figure 1: BoNT/C&D dose-response relation for capture by synaptosomes.

Control: while toxin binding was studied with BoNT/C&D specific mAb 2.122, a further specificity control was performed using a control mAb: antibody 3C9 that has strong affinity for VP2 of canine parvovirus (CPV) was completely negative which excluded the possibility of an a-specific binding of antibodies to synaptosomes. Therefore, toxin (or toxin-complex) binding to synaptosomes is a specific process. This showed that this kind of brain vesicle preparations were ready to be used for our studies of toxin capture and BoNT-type specific substrate proteolysis.

Detection of toxin activity: Substrate cleavage testing had to be developed and detection by ELISA appeared possible after raising rabbit antisera to the substrate cleavage products of BoNT/C and BoNT/D using polystyrene coated peptide-substrates (Jones, 2009). Since the BoNT/C reference sample (in Cardella culture medium) was available as a homogenous product in contrast to BoNT/D preparations, BoNT/C was used for further improvements in a C-type specific detection assay. For example we could investigate the conditions for heat-inactivation of toxin sample. Inactivation of toxin was performed and required a 95-99°C treatment for assuring a full inactivation (Fig. 2). Stability of the substrate peptides and cleavage products during this inactivation step has been confirmed (80-100% recovery of intact peptide, tested by CVI+Pepscan BV using HPLC-ESI/MS).

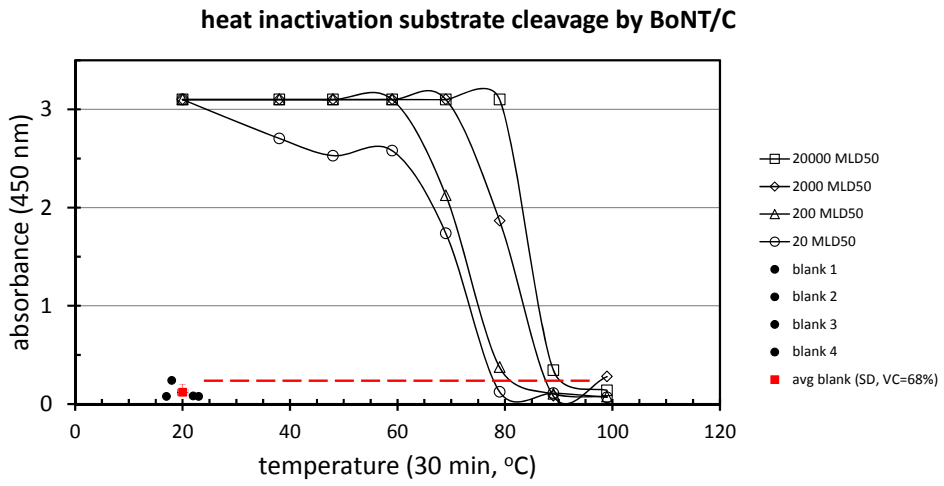

Figure 2: Heat inactivation analysis of BoNT/C preparation from culture CKIII-4 (stock concentration: $4x10^6$ MLD/ml).

Proof of principle of type specific cleavage by toxins after capture with synaptosomal preparations was obtained by both mass spectrometry and immunochemical (ELISA) assays.

A, MS analyses: AHVLA (spring 2012) showed that both the N- and C-terminal cleavage products were detectable in HPLC-ESI/MS (electron spray ionisation/MS), though only at the highest toxin concentration tested (33 µg substrate peptide, 1500 MLD_{50} BoNT/C per mL digest), and only in presence of non-captured toxin. Afterwards, SVA (July 2012, October 2012) performed twice analyses. The first time was unsuccessful. CVI developed an alternative digestion immuno-assay with substrate in liquid phase instead of solid phase for both BoNT/C and BoNT/D and realized that the digestion conditions had to be adapted omitting culture medium which could explain the low sensitivity found at AHVLA and SVA. At the 2nd time, MS-analyses (both MALDI- and ESI- MS system) successfully showed the formation of N-terminal cleavage product. And while there was quite a high activity of substrate degradation, the detected cleavage product without or with capture was shown to be toxin dose related and yielded at highest toxin concentration up to 50% of N-terminal cleavage product based on the concentration of the in-put substrate (Table I). Thus, this high yield of a specific cleavage product confirms BoNT/C specificity.

		S=substrate, N=N-fragment, C=C-fragment	0-300-3000 MLD addition	0-150-1500 MLD addition
		MS	ESI quantification (N-fragment)	
toxin	substrate (1 µM)	maldi	capture	direct
BoNT/C	SCJ15-SNAP25 (CVI)	S, N	0.02-0.1-0.5 µM	0-0.4-0.5 (µM)
	CHEM1969-SNAP25 (AHVLA)	S, N	nd	nd
BoNT/D	SCJ15-SNAP25 (CVI)	S, N	nd	0-0.05-0.4 (µM)
	SCJ048-VAMP2 (CVI)	S	nd	nd

Table I: Summary of analyses by SVA using two mass spectrometry techniques: MALDI/MS and ESI/MS. The BoNT/C and BoNT/D preparations used were from CVI cultures. The substrate and N-terminal cleavage products that were detected by MALDI/MS were further quantified by ESI/MS analyses. The results show that there is a dose-response relation between toxin added and resulting N-terminal cleavage product. The recovery of the cleavage product was very high at the higest toxin doses added (0.5 µM i.e. ±50% of added substrate). It was impossible to detect the specific BoNT/D cleavage products, though this toxin type did show C-type cleavage specificity (also observed in ELISA), pointing towards a possible mosaic status or mixed composition, which was already suspected by immunoassay before submission to SVA. n.d. = no substrate or cleavage product detected.

B, ELISA assays: To obtain proof-of-principle for the CVI ELISA based detection system three aspects were investigated: **i,** Firstly, reference toxin samples from Metabiologics Inc. USA were tested in parallel with the CVI home BoNT/C product (in cardella culture medium). All samples appeared to be active in our ELISA assay for substrate cleavage after synaptosomal capture. All toxin preparations could be captured and contained proper cleavage specificity. **ii,** Secondly: three different substrate peptides were used to confirm the cleavage in ELISA: a 75mer, a biotinylated 75-mer and a biotinylated 22-mer peptide, each covering the cleavage site. All three substrates were cleaved. **iii,** Thirdly, the specificity of the antiserum R44470 directed against the C-terminus of the N-terminal cleavage product was investigated. A set of 18 synthetic peptides with mostly SNAP-25-derived sequences around arginines and lysines were tested with our cleavage specific antiserum R44470. The antiserum did only bind a QR C-terminus and it could also bind the terminus if amidated. So it is highly BoNT/C cleavage site specific (Fig 3).

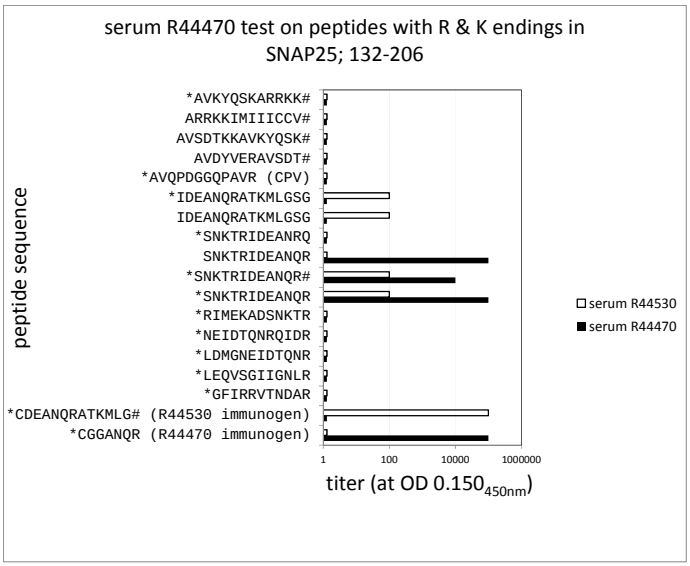

Figure 3: The R44470 serum is specific for the QR sequence of the BoNT/C specific cleavage product in SNAP-25 as tested by ELISA with coated peptides. The C-terminal R can be present with a free carboxygroup as well as with an amidated terminus. However, the R should not be preceded by any other amino acid than Q, nor is there any cross-reactivity with the other basic amino acid lysine (K) at the terminus. Control serum R44530 was elicited to the intact QRAT sequence; it exhibits specificity for this sequence and does not bind the cleaved product that is recognized by R44470. The top 4 peptides are based on the sequence of syntaxin, which is in addition to SNAP-25 one of the two proteins that can be cleaved by BoNT/C. CPV indicates a synthetic peptide with a C-terminal R derived from VP2-protein of canine parvovirus. * = acetylated N-terminus; # = amidated C-terminus. Sequence of the SNAP-25 region used for above peptides design is: GFIRRVTND ARENEMDENL EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT KMLGSG.

Dose-relation and sensitivity of the synaptosomal capture based BoNT/C assay: The sensitivity of the BoNT/C capture test reached a 10 MLD_{50} level of sensitivity. There was a good dose-response correlation between toxin dose added and substrate specific proteolytic activity activity measured at constant synaptosomal concentration (Fig. 4). The sensitivity of the whole assay should still be 10-fold improved to a 1 MLD_{50} level.

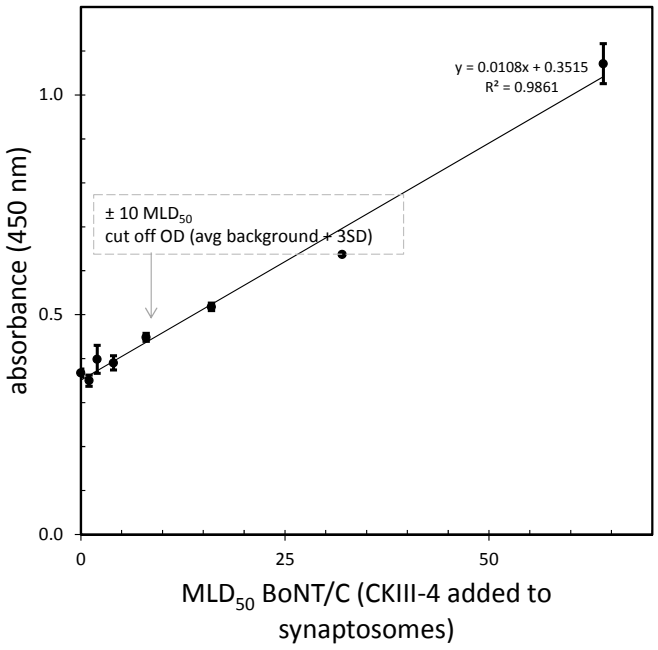

Figure 4: Dose-response relation between added BoNT/C and ELISA signal for captured BoNT/C toxin. Variable amounts of toxin (2-4-8-16-32-64 MLD_{50}) were added to constant amount of synaptosomal protein. The linearity in the toxin range tested is surprisingly consistent between experiments. Standard deviations are based on duplicate samples.

References:
Evans et al., J Appl Microbiol 2009, 107:1384–1391.
Jones et al., 2009. J. Immunol. Methods 343 (2009) 21–27
Kalb et al., 2010. PLoS ONE 5(8): e12237. doi:10.1371/journal.pone.0012237

Problems experienced:
At CVI, testing synaptosomal binding of toxins is a laborious activity that has absorbed much time, while the final procedure appears practically feasible for a research assistant. Due to these efforts, a crucial stage has been reached. The progress remained promising because the proof of principle has been obtained, both by immunochemical means (ELISA) and MS. In addition, a check was carried out to assure the stability of the cleavage products for incubation conditions and toxin-heat inactivation (99^{o}C, 30 min) to enable safe working for the partners receiving the materials. This is new knowledge.
AHVLA has stopped her botulin-test development research project in 2nd half of 2012. SVA and DTU were willing to analyse CVI products in their MS facilities, which had started at AHVLA, and were ultimately finished at SVA. Due to the labour-time involved to improve the capture and assay conditions, spiking experiments could not be not performed in real practice samples.

Value of cooperation for club 5 institutes
The issue whether an *in vitro* toxin test would be feasible using synaptosomal capture also for BoNT/C and BoNT/D has been of interest for each of the four partners involved, since this is new knowledge. Especially SVA and CVI have profited from the experimental experience (substrate choice, possibility to perform different kinds of immuno-chemical toxin activity tests, use of synaptosomal preparations from brain, heat inactivation conditions for safe working).

Dissemination activities
Within the EU network ANIBIOTHREAT the partners have realized how little coherence was existing with respect to botulin testing and developments. The experiences have been communicated in:

a paper:

Skarin H, Tevell-Aberg A, Woudstra C, Hansen T, Löfström C, Koene M, Bano L, Hedeland H, Anniballi F, De Medici D, and Olsson Engvall E. 2013. The Workshop on Animal Botulism in Europe. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science. 11, Supplement1, S183-S190, DOI: 10.1089/bsp.2012.0076.

and posters:

17-18 April 2012. Papendal. Springmeeting 2012 of the NVvM and NVMM. In vitro botulism test development with C toxin type specificity. Erkens JHF, Roest HIJ, Koene MGJ, Langeveld JPM.
7-8 Juni 2012. AniBioThreat workshop EU project meting on botulism in Europe. Uppsala, Sweden. In vitro botulism test development with C toxin type specificity. Erkens JHF, Roest HIJ, Koene MGJ, Langeveld JPM.

and presentations:

9 June 2011, Sophia Antipolis FR, CoVetLab meeting. Clost. botulinum toxin testing. JPM Langeveld.
24 mei 2012, AHVLA Weybridge, Addlestone, CoVetLab meeting. In vitro botulism assays using neurotoxin capture systems. JPM Langeveld
7-8 Juni 2012. AniBioThreat workshop EU project meting on botulism in Europe. Uppsala. Sweden. In vitro botulin assays: using a BoNT capture system & immunochemical analysis. Langeveld JPM, Erkens JHF, Koene MGJ, Roest HIJ.

Suggestions for future: Improve sensitivity, apply field studies including spiking experiments in parallel to mouse assays, develop the testing also for BoNT/D, simplify and shorten procedures.

Summary:

Aim & Background: The project aimed at design of an *in vitro* method suitable to replace the mouse bioassay as alternative for botulin testing. Capture methods followed by the toxin specific endoprotease activity testing appear as the best candidates to specifically detect the toxins from its interfering environment. Collaboration was agreed: CVI invested man-power and materials in developing capture conditions using brain vesicle derived materials (synaptosomes) and immunochemical test methods, while AHVLA, DTU and SVA would show that MS analysis was capable to proof the endo-protease specificity. (A separate initially planned approach at AHVLA to use capture by antibodies on beads did not reach maturity, and in 2012 AHVLA withdraw from the collaboration). From the 7 known toxin types BoNT/C & /D were chosen as target for capture, the most involved botulins in veterinary testing. Partners supported the approach of CVI to biologically capture toxin using brain vesicles, and subsequently try to obtain proof-of-principle. The only pre-existing basis for this was a paper of Evans et al., 2009.

Results: BoNT/C capture conditions have been confirmed after developing ELISA methods that can sensitively detect the cleavage of synthetic peptide substrates with sequences based on the substrate proteins from the synaptosomal SNARE-complex. The ELISA itself *i.e.* without capture now can detect BoNT/C levels below 0.5 MLD_{50}/ml, while BoNT/D activity probably also is detectable. BoNT/C substrates from both AHVLA and CVI were immunochemically shown to be cleaved. For additional proof-of-principle, *in casu* by mass spectrometry (MS), CVI generated new information about efficient inactivation of BoNT/C which required near boiling temperatures; peptides remained sufficiently intact. In two phases MS analysis yielded proof-of-principle: AHVLA initially could proof the existence of the three expected products of proteolysis (substrate and two cleavage products), and after a further phase of improvement in conditions of endo-protease digestion at CVI, SVA did show the presence of cleavage product and a dose-response relation between toxin and substrate cleavage in two situations: either with or without capture. This is the first time that MS does confirm the suitability of this type of in vitro biological capture approach.

We have obtained continuous progress towards obtaining an in vitro BoNT/C test, Nevertheless, due to the complex aspects in the botulin test development, we did not yet perform tests using real practice materials (feed samples, organ tissue, serum, spiking experiments). Further experiments to do still are indeed confirmation of test performance under these reality conditions and improvements towards a ten-fold better sensitivity.

CoVetLab total budget: 39 kEuro for the first year; Go/No-go after year 1 not formally confirmed.

Indicative contribution per partner:

CVI: 36 k€ (labour, shipments, consumables, travel);

VLA: mass spectrometry; supply of several peptides; no additional input.

SVA: 3 k€ (shipments and travel); mass spectrometry equipment and labour.

Evaluation	
Deliverables met?	
Cooperation	
Value	
Further dissemination	
Advice towards new calls / new projects	