Validation of the "BINACLE" (Binding and Cleavage) Assay for the *In Vitro* Safety Testing of Tetanus Vaccines

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INTRODUCTION

Tetanus neurotoxin (TeNT) and tetanus vaccines

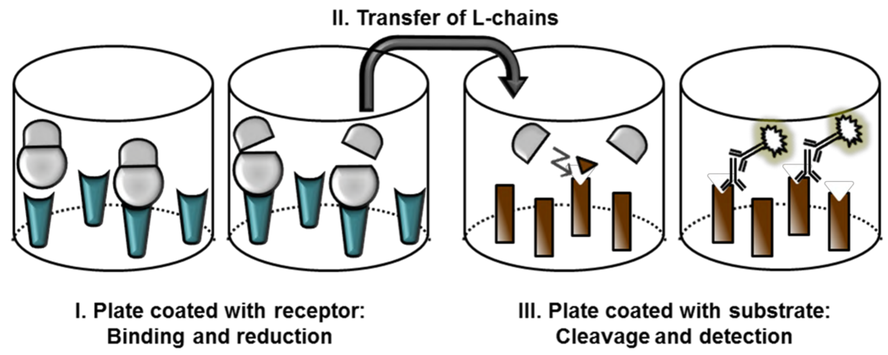
TeNT is one of the most toxic substances known. It causes severe spasms by blocking neurotransmitter release from inhibitory interneurons. Its mode of action is similar to that of botulinum neurotoxins: TeNT consists of two protein subunits connected by a disulfide bond. The heavy (H-)chain binds to gangliosides on the surface of neurons. The toxin is then endocytosed and undergoes retrograde axonal transport to the central nervous system, where it enters an inhibitory interneuron. Using a transmembrane channel formed by the N-terminal region of the H-chain, the light (L-)chain of TeNT is translocated into the cytosol where it specifically cleaves synaptobrevin, a protein related to neurotransmitter release.

Tetanus toxoids are inactivated preparations of TeNT obtained, e.g., by a formaldehyde treatment. After adsorption to mineral adjuvants, such toxoids are used as tetanus vaccines. In accordance with the European Pharmacopoeia, each toxoid bulk has to be tested for the absence of residual toxin using toxicity tests in guinea pigs. These animal tests have been introduced decades ago, and have never been validated according to modern standards. We want to provide a reliable *in vitro* assay for the detection of active TeNT, with the aim to replace the *in vivo* safety test.

The BINACLE strategy for detection of active TeNT

TeNT molecules are only toxic *in vivo* if their H-chain and their L-chain are intact. The BINACLE (binding and cleavage) assay which has been developed in our laboratory specifically detects active TeNT *in vitro* based on the two most important functions of the toxin: The receptor-binding ability of the H-chain and the substrate-cleavage activity of the L-chain.

Compared to *in vitro* methods relying on only one function of the toxin (e.g. protease activity), false-positive results caused by fragmented or partly inactivated TeNT molecules are minimized with the BINACLE approach.



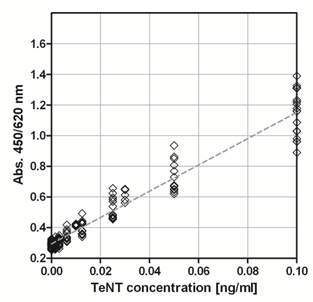
[Figure adapted from: Behrensdorf-Nicol, Weisser and Krämer (2015), ALTEX 32, 41-46]

Principle of the BINACLE assay. (I.) TeNT molecules (grey) bind to immobilized ganglioside GT1b (green) via their H-chains. Then the TeNT L-chains are released by reduction of the disulfide bonds. (II.) The supernatant containing the liberated L-chains is transferred to a plate containing immobilized synaptobrevin (brown). (III.) TeNT L-chains cleave the synaptobrevin, and the cleavage fragment is detected using a specific antibody.

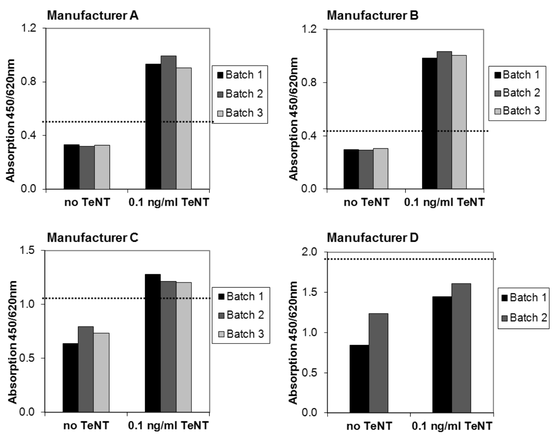
VALIDATION STUDIES

Results from an in-house validation study

- The BINACLE assay detects active TeNT with a detection limit of ~0.03 ng/ml, which is comparable to the *in vivo* test.
- The assay can sensitively detect active TeNT which has been spiked into vaccine toxoids.
- In most cases, a cutoff-based strategy can be used to discriminate between TeNT-positive and TeNT-negative samples.



Determination of the detection limit. Dilution series of pure TeNT were tested in three BINACLE assays. Each sample was measured sixfold per assay. Shown are the raw data from all three assays and the resulting regression line (slope: 8.58, R-square: 0.945).

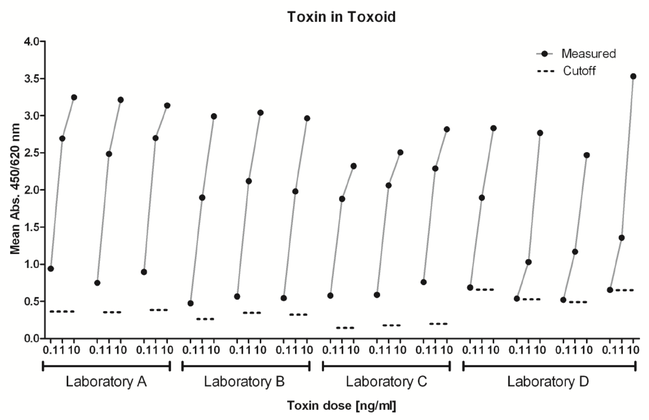


Cutoff-based detection of TeNT in tetanus toxoids. Toxoid batches from 4 vaccine manufacturers were tested in the BINACLE assay in pure form and spiked with 0.1 ng/ml TeNT. Shown is the mean absorbance from a 3-fold measurement of each sample. Dotted lines represent the cutoffs (mean absorption of the non-spiked batches plus 3-fold residual standard deviation).

[Figures taken from: Behrensdorf-Nicol et al. (2013), Vaccine 31, 6247-53]

Results from a transferability study

- 4 international laboratories were provided with a detailed test protocol and specific assay reagents.
- Each laboratory performed 3-4 test runs to measure TeNT diluted in buffer or in a reference toxoid.
- All participants were able to successfully perform the assay. 0.1 ng/ml TeNT (which corresponds to the estimated detection limit of the animal test) was detectable in most of the test runs.
- Intra-assay variability was usually below 15%, inter-assay variability below 20%.



Cutoff-based approach for detection of TeNT. Each curve represents the mean absorption values obtained in one test with the TeNT concentrations indicated on the x-axis (0.1, 1, and 10 ng/ml) diluted in toxoid. The short dotted lines represent the corresponding cutoffs (mean absorption of the blank sample + 3-fold standard deviation) for each test.

[Figure taken from: Behrensdorf-Nicol et al. (2014), Biologicals 42, 199-204]

Ongoing international collaborative study

- The BINACLE collaborative study is part of the Biological Standardization Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM).
- The aim of the study is to characterize the suitability of the BINACLE assay for the safety testing of tetanus toxoids and to pave the way for regulatory acceptance of the method.
- Each of the 19 participants was provided with a detailed assay protocol and the specific reagents needed for the assay.
- Samples contained 3 different TeNT concentrations spiked into various tetanus toxoids. The lowest spike concentration (0.1 ng/ml TeNT) represents the estimated detection limit of the animal test.
- All tests have been completed by now; data analysis is ongoing.

Design of the BINACLE collaborative study (BSP136)	
Participants	19 international laboratories (National Control Laboratories; vaccine manufacturers)
Test samples	TeNT (0.1 ng/ml, 0.5 ng/ml, 5 ng/ml) added to: - WHO reference tetanus toxoid - Toxoids from 3 vaccine manufacturers
Controls	Positive control: TeNT diluted in buffer Negative controls: Buffer/toxoids without TeNT
Number of tests	3 independent tests per participant ⇒ 57 test runs in total

CONCLUSION/OUTLOOK

- The validation studies performed to date indicate that the BINACLE assay for the *in vitro* detection of active TeNT may represent an alternative to the prescribed animal safety tests for tetanus toxoids from several relevant vaccine manufacturers.
- The assay is highly sensitive: It is capable of detecting active TeNT already in concentrations below 0.1 ng/ml, i.e. its detection limit is equivalent to that of the *in vivo* test.
- Based on the outcome of the collaborative study, we aim to replace the guinea pig safety test for tetanus vaccines described in the European Pharmacopoeia by the BINACLE assay.

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