# Outline of BSP130 Phase III:
## Method optimisation and their principles (TNE +, TCP)

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## Background – the drivers of Phase III

1. BSP130 Phase II study established a convincing *in vivo-in vitro* correlation in C.septicum toxin and toxoid assays
2. The dispersion of the data was high
3. The toxicity and antigenicity values were not standardized

# BSP 130 Phase III - Goals

- **Refinement** of the assay setup based on the results of Phase II to increase assay precision
- **Standardization** of the toxicity/immunogenicity values ⟶ linked to antitoxin's IU/mL

  - **Toxin: MLD ⟶ TNE + (the concept of *in vivo* L+50)**
  - **Toxoid: TCP ⟶ TCP (refined)**

# BSP 130 Phase III – Study design

- **Logistics** was coordinated by Ceva – materials were offered by the manufacturers
- **Materials**
  - 6 test toxins: TxR, TxS, TxV, TxW,TxY, TxZ
  - 6 test toxoids: TdA, TdC, TdD, TdN, TdO , TdP
  - NIBSC reference antitoxin
  - Each participant used its own line of Vero
- **Sequence**
  - Test item preliminary dilution ranges were determined by Ceva
  - Test items distributed to participants
  - Participants were asked to perform a trainig session (due to complex assay design)
  - Participants performed testing of test items
- **Assay development**
  - Joint and iterative procedure driven by Ceva-Phylaxia team and Study statistician
  - Supported by the project leaders
- **Data collection**
  - Reporting sheet (locked!) with automatic calculations

# Steps - overview

- **Step 0:** Training session
- **Step I:** confirmation of sensitivity of the participants' Vero cell lines
- **Step II:** latent toxicity testing of the standard antitoxin and test toxoids.
- **Step III:** measurement of the Toxin Neutralization Equivalence plus (TNE+) of the detector toxin (CSTx2)
- **Step IV:** testing of sample panels by TNE+ and TCP

# Steps – details

| Step | Assay | Test materials | Purpose |
|---|---|---|---|
| 0. | TNE+ | CSTx2 | Training (1.3-fold dilution, 1 assay) |
| 0. | TCP | TdA | Training (20 increment steps, 1 assay) |
| I. | MLD | CSTx2 | Determination of Vero cell sensitivity (2-fold dilution) |
| II. | MLD | Test toxoids | Determination of latent toxicity (2-fold dilution) |
| II. | MLD | Antitoxin | Determination of latent toxicity (2-fold dilution) |
| III. | TNE+ | CSTx2 | Initial ranging of CSTx2 TNE+ (1.3-fold dilution, 1 assay) |
| III. | TNE+ | CSTx2 | Determination of CSTx2 TNE+ (1.1-fold dilution, 3 assays) |
| IV. | TNE+ | Test toxins | Initial ranging of test toxins' TNE+ (2-fold dilution, 1 assay) |
| IV. | TNE+ | Test toxins | Determination of test toxins' TNE+ (1.3-fold dilution, 3 assays) |
| IV. | TCP | Test toxoids | Initial ranging of test toxoids' TCP (20 increment steps, 1 assay) |
| IV. | TCP | Test toxoids | Determination of test toxoids' TCP (10 increment steps, 3 assay) |

# Assay procedure – overview

(method developed by MSD in Phase I)

- **Day 0**
  - plating of Vero cells onto a 96-well TC plate – incubate overnight in $CO_2$ incubator
- **Day1**
  - replacement of growth medium with serum-free assay medium (assay plate)
  - preparation of test item dilutions/mixtures in a 96 deep-well plate (dilution plate)
  - pipetting of test items onto the assay plate
  - incubate overnight in $CO_2$ incubator
- **Day2**
  - staining for viable cells
  - reading OD

# Assay validity criteria and end-point determination

- $CV_{OD\ neg\ ctrl} < 20\%$
- Parallels on a plate : (MLD, TNE+) must not differ by more than one dilution step
- Parallel plates: the mean end-point values of the replicate plates must not differ by more than two dilution steps
- Cut-off: 50% of the median value of the negative controls
  - Negative control OD median = 0.804, i.e. the cut-off = 0.402
- End-point: the well with the highest dilution of material which has an OD below the cut-off value.

# I. Vero cell sensitivity - plate setup

| | Col 1 | Col 2 | Col 3 | Col 4 | Col 5 | Col 6 | Col 7 | Col 8 | Col 9 | Col 10 | Col 11 | Col 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Untreated (no cell, 100 µL medium/well) | | | | | | | | | | | |
| B | Neg. | Neg. | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 | 1/512000 | 1/1024000 | 1/2048000 | 1/4096000 |
| C | Neg. | Neg. | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 | 1/512000 | 1/1024000 | 1/2048000 | 1/4096000 |
| D | Neg. | Neg. | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 | 1/512000 | 1/1024000 | 1/2048000 | 1/4096000 |
| E | Neg. | Neg. | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 | 1/512000 | 1/1024000 | 1/2048000 | 1/4096000 |
| F | Neg. | Neg. | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 | 1/512000 | 1/1024000 | 1/2048000 | 1/4096000 |
| G | Neg. | Neg. | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 | 1/512000 | 1/1024000 | 1/2048000 | 1/4096000 |
| H | Neg. | Neg. | 100 µL NBS | | | | | | | | | |

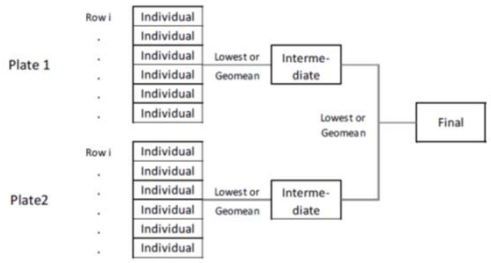
# II. Residual toxicity - plate setup

| Toxoid | | Col 1 | Col 2 | Col 3 | Col 4 | Col 5 | Col 6 | Col 7 | Col 8 | Col 9 | Col 10 | Col 11 | Col 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | Untreated (no cell, 100 µL medium/well) | | | | | | | | | | | | |
| TdA | → B3 | Neg. | Neg. | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | Dilutions |
| TdC | → C3 | Neg. | Neg. | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | |
| TdD | → D3 | Neg. | Neg. | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | |
| TdN | → E3 | Neg. | Neg. | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | |
| TdO | → F3 | Neg. | Neg. | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | |
| TdP | → G3 | Neg. | Neg. | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | |
| Antitoxin | → H3 | Neg. | Neg. | 5 | 2,5 | 1,25 | 0,625 | 0,3125 | 0,15625 | 0,07813 | 0,03906 | 0,01953 | 0,00977 | => IU/mL |

# III. TNE+ - plate setup

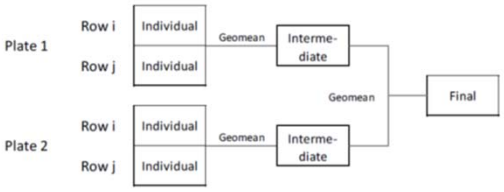
- CSTx, Initial assay, 1.3-fold dilution

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | UT (No cells) | | | | | | | | | | | |
| B | Neg | Neg | CSTx2, 525 | 683 | 887 | 1153 | 1499 | 1949 | 2534 | 3294 | 4283 | 5567 |
| C | Neg | Neg | CSTx2, 525 | 683 | 887 | 1153 | 1499 | 1949 | 2534 | 3294 | 4283 | 5567 |
| D | Neg | Neg | CSTx2, 525 | 683 | 887 | 1153 | 1499 | 1949 | 2534 | 3294 | 4283 | 5567 |
| E | Neg | Neg | CSTx2, 525 | 683 | 887 | 1153 | 1499 | 1949 | 2534 | 3294 | 4283 | 5567 |
| F | Neg | Neg | CSTx2, 525 | 683 | 887 | 1153 | 1499 | 1949 | 2534 | 3294 | 4283 | 5567 |
| G | Neg | Neg | CSTx2, 525 | 683 | 887 | 1153 | 1499 | 1949 | 2534 | 3294 | 4283 | 5567 |
| H | Neg | Neg | NBS | | | | | | | | | |




- Test toxins

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | UT (No cells) | | | | | | | | | | | |
| B | Neg | Neg | Tx1, 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 | 5120 |
| C | Neg | Neg | Tx1, 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 | 5120 |
| D | Neg | Neg | Tx2, 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 | 5120 |
| E | Neg | Neg | Tx2, 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 | 5120 |
| F | Neg | Neg | CSTx2, 1020 | 1122 | 1234 | 1358 | 1493 | 1643 | 1807 | 1988 | 2186 | 2405 |
| G | Neg | Neg | CSTx2, 1020 | 1122 | 1234 | 1358 | 1493 | 1643 | 1807 | 1988 | 2186 | 2405 |
| H | Neg | Neg | NBS | | | | | | | | | |



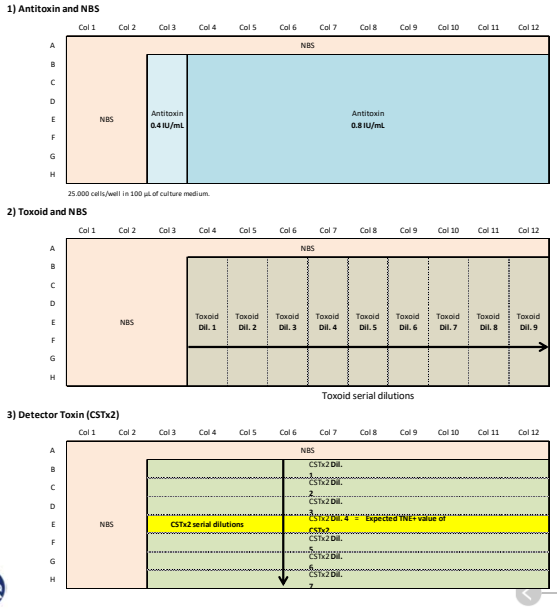

# TNE+ calculation

2 fold dilution of toxin in reaction mixture

**Endpoint dilution x 2 x 0.1** ← to give IU/mL, since 0.1 IU/mL standard antitoxin is in equilibrium with the toxin

e.g. **1280** x 2 x 0.1 = **256 IU/mL.**

# IV. In vitro TCP – crosswise dilution setup!




Antitoxin: FIXED concentration

Toxoid: horizontal dilution

CSTx2: vertical dilution

# TCP calculation

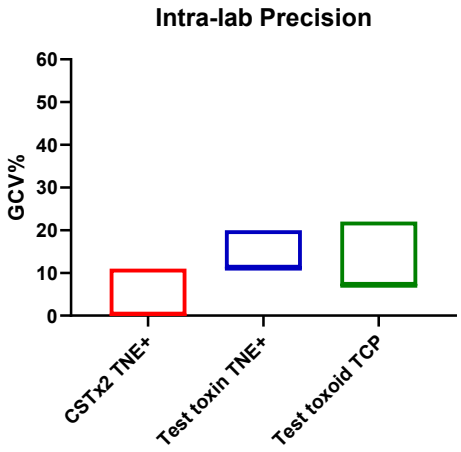
4 fold dilution of toxoid in reaction mixture

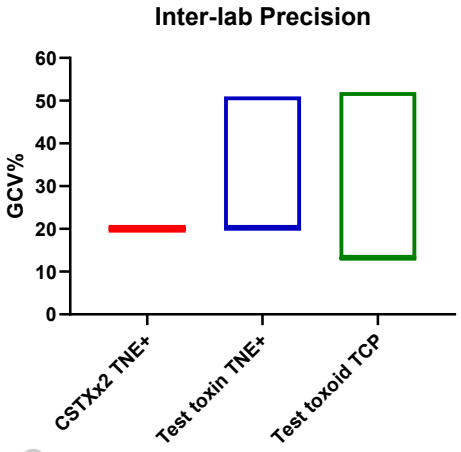
**Endpoint dilution x 4 x 0.1** ← to give IU/mL, since 0.1 IU/mL standard antitoxin holds equilibrium with the toxoid

e.g. **160** x 4 x 0.1 = **64.**

# Key performance parameters

- Invalidity rate: 15%




# Back-up slides

# Recommended dilution ranges as determined at Ceva-Phylaxia

| Sample code | Type of sample | Initial TNE+ dilution range | Initial TCP dilution range Expressed as reciprocal of dilution step |
|---|---|---|---|
| **CSTx2** | Detector Toxin | 525 - 5570<br>1.3-fold serial | N/A |
| **TxR** | Toxin | 10 – 5120<br>2-fold serial | N/A |
| **TxS** | Toxin | | |
| **TxV** | Toxin | | |
| **TxW** | Toxin | | |
| **TxY** | Toxin | | |
| **TxZ** | Toxin | | |
| **TdA** | Toxoid | N/A | 40 to 200 in 20-increment steps* |
| **TdC** | Toxoid | | 10 to 90 in 10-increment steps** |
| **TdD** | Toxoid | | 20 to 180 in 20-increment steps |
| **TdN** | Toxoid | | 20 to 180 in 20-increment steps |
| **TdO** | Toxoid | | 100 to 260 in 20-increment steps |
| **TdP** | Toxoid | | 140 to 300 in 20-increment steps |

| Step | Assay | Test materials | Purpose | Details |
|---|---|---|---|---|
| Pre-I | TNE+ | CSTx2 | Training | 1.3-fold dilution series, as detailed in Table 1 |
| Pre-I | TCP | TdA | Training | 20-increment step dilution range, as detailed in Table 1 |
| I | MLD | CSTx2 | Determination of Vero cell sensitivity | 2-fold dilution series from 1/8,000 to 1/4,096,000 |
| II | MLD | All test toxoids | Determination of latent toxicity | 2-fold dilution series from 1/5 to 1/2,560 |
| II | MLD | Antitoxin (VI) | Determination of latent toxicity | 2-fold dilution series from 5IU/mL to 0.00975IU/mL |
| III | TNE+ | CSTx2 | Determination of TNE+ value | Ten step 1.3-fold dilution series, as detailed in Table 1 |
| III | TNE+ | CSTx2 | Determination of TNE+ value | Ten step 1.1-fold dilution series, as appropriate |
| IV | TNE+ | All test toxins | Determination of TNE+ values | Ten step 2-fold dilution series, as detailed in Table 1 |
| IV | TNE+ | All test toxins | Determination of TNE+ values | Ten step 1.3-fold dilution series, as appropriate |
| IV | TCP | All test toxoids | Determination of TCP values | 20-increment step dilution range, as detailed in Table 1 |
| IV | TCP | All test toxoids | Determination of TCP values | 10-increment step dilution range, as appropriate |