BSP130: Validation of cell line assays for toxicity and antigenicity testing of *Clostridium septicum* vaccine antigens

Part 1 (Phase I & II)

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Presentation by Keith Redhead, given by Marie-Emmanuelle Behr-Gross (supported by Botond Siklodi and Lukas Bruckner)

Simplified Toxoid Vaccine Manufacture

Grow organism in liquid culture

↓ analytical

Remove cells (centrifugation &/or filtration)

↓ analytical, **toxicity and antigenicity**

Chemically inactivate toxins in supernatant

↓ analytical, **toxicity and antigenicity**

Blend with other antigens and adjuvant

↓ analytical, potency and safety

Dispense vaccine

analytical

Current Testing for Clostridial Antigens

In-Process: In vivo

Toxicity of toxin (Minimum lethal dose, MLD)

Residual toxicity of toxoid (MLD)

Antigenicity of toxoid (Total combining power, TCP)

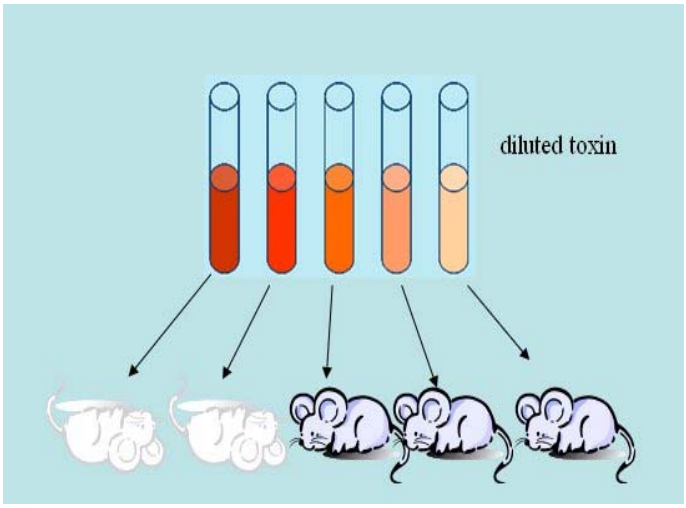
In Process Testing

Toxicity / Freedom from Toxicity

Assessed by the **Minimum Lethal Dose** (MLD) test using mice

How far can the toxin/toxoid be diluted before it is no longer lethal in mice

MLD in mice



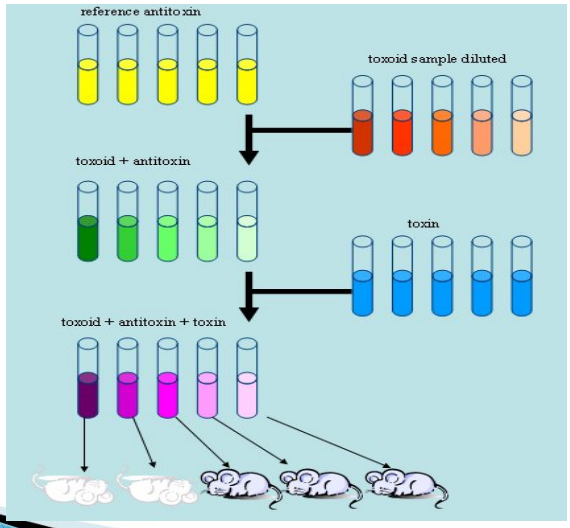
Antigenicity (toxoid)

Antigenicity of the toxoid is assessed by the Total Combining Power (TCP) test using mice

How much reference neutralising antitoxin is bound by the toxoid ?

The amount of active unbound antitoxin remaining is measured on the basis of its ability to neutralise a lethal amount of toxin – assessed in mice

TCP in mice



Summary of regulatory tests considered for BSP130 Part 1

Test	Indicator
MLD	Mice
TCP	Mice

Mice are used only as an indicator of toxicity

Animal welfare aspects

These in-process tests for clostridial antigens use 100,000s mice per annum in Europe alone

The data provided by these *in vivo* tests i.e. toxicity and antigenicity of the toxins/toxoids were not fully provided by any of the available *in vitro* tests by the time BSP130 started

(e.g. ELISA does not measure the overall biological effects)

Replacement of In-process In Vivo Tests

A Direct and Simple Approach

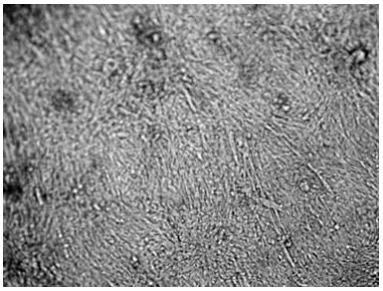
Test	Indicator
MLD	Cell line
TCP	Cell line

As mice are used only as an indicator of toxicity, replace the mice with a different indicator of toxicity :

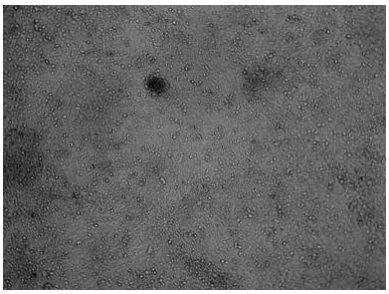
Toxin-sensitive cell lines

Effect of *Cl. septicum* Toxin on VERO Cells

Control cell monolayer



Treated cell monolayer



Assay Outline

Seed microplates with suitable cell line

↓

Incubate to form confluent cell layer

↓

Add dilutions of toxin or toxoid (or toxin+antitoxin etc)

↓

Incubate

↓

Visualise effects (staining of viable cells)

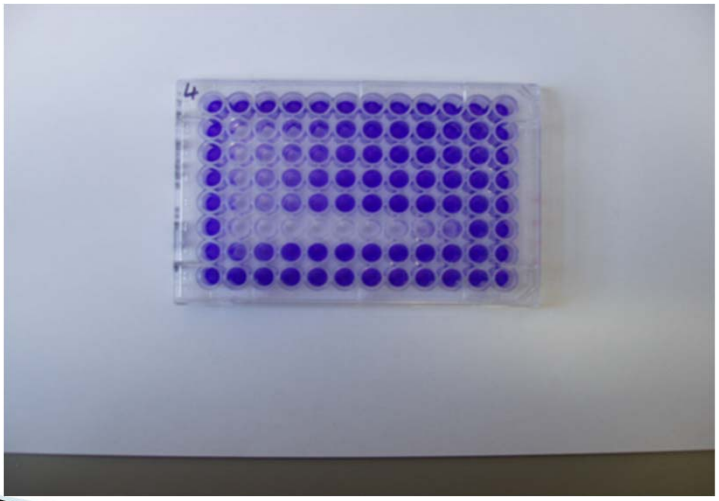
↓

Assess effects (measure staining)

↓

Determine end-points and calculate toxicity or antigenicity

Cell line assay plate



Advantages of Cell Line Assays

Greater Sensitivity (up to x1000)

Improved discriminative power (up to x5)

Precision / Reproducibility

Speed of the assay (24 hours vs 4 days)

Ethics

Cost

In Vitro *Cl. septicum* Cell Line Assays

MSD Animal Health In-house correlations of in-process *in vitro* and *in vivo* assays

MSD AH In-house Correlation Study Results

Assay	Correlation	Linear Regression
MLD	0.99	0.99
TCP	0.99	0.99

- These results are only applicable to *Cl. septicum* toxins and toxoids produced and tested by MSD AH UK
- Can similar levels of correlation be obtained in other laboratories using toxins and toxoids from other sources?

EPAA Clostridial Working Group: Purpose

To promote the acceptance (Ph.Eur. and Regulatory Authorities assessing Marketing Authorisation applications) of *in vitro* alternatives to mouse *in vivo* tests for the in-process control testing of veterinary clostridial vaccine antigens.

The tests selected for replacement were the Minimum Lethal Dose (MLD) and the Total Combining Power (TCP) and the potential replacement identified were cell line based assays

EPAA Clostridial Working Group:Approach

Selection of one species of *Clostridium* for which the toxin and toxoid can be assessed.

Assemble a group of participants from manufacturing and OMCL backgrounds in different countries able to test this type of toxin and/or toxoid in the *in vivo* and/or *in vitro* assays.

Perform an international collaborative study, with toxins and toxoids from various sources and of different strengths, to validate the *in vitro* assays and assess concordance with the *in vivo* tests.

EPAA Clostridial Working Group Participants

Industrial

CEVA	Hungary
MSD AH	UK, USA, NZ
Pfizer AH (CZV)	UK (Spain)
SYVA	Spain

Non-Industrial

Bornova Vet Inst	Turkey
NEBIH	Hungary
PEI	Germany
CVB	USA
EDQM	Europe
IVI	Switzerland

EPAA Clostridial Working Group

Test Materials:

WHO IS *Cl. septicum* antitoxin, VI – NIBSC, UK
Reference *Cl. septicum* toxin, CSTx – CEVA, Hungary

Six batches each of *Cl. septicum* toxins and toxoids of differing strengths. Sourced from:

MSD AH UK (3 toxins and 3 toxoids)
MSA AH NZ (1 toxin and 1 toxoid)
CEVA (1 toxin and 1 toxoid)
MSD AH USA (1 toxin and 1 toxoid)

EPAA Clostridial Working Group

Tests Performed by Participants:

- There were 11 participants in the study (5 manufacturers and 6 OMCLs from Europe and USA)
- One participant performed *in vivo* tests only
- Five participants performed in *vitro* tests only
- Five participants performed *in vitro* and *in vivo* tests

BSP130 Study Outline

- **Sensitivity testing** – MLD at 10-fold then at 5- and 3-fold dilutions using CSTx. To check sensitivity of different participant's mice and Vero cells.
- **Latent toxicity** – Each toxoid, diluted 1 in 10, and the standard antitoxin, at 5IU/ml, in one pair of mice and on Vero cells(to check for any residual toxicity).
- **Preliminary ranging** – **MLD** at 10-fold dilutions on all 6 toxins on one occasion in mice and Vero Cells.
  TCP at 40 unit steps on all 6 toxoids on one occasion in mice and Vero cells.
- **Full testing** – MLD at 5- or 3-fold dilutions on all 6 toxins on three occasions or until 3 valid assays are obtained. TCP at 20 unit steps on all 6 toxoids on 3 occasions or until 3 valid assays are obtained.

BSP130 Results: Latent toxicity

- Standard antitoxin (VI) showed no toxicity
- No toxoids showed latent mouse toxicity
- All toxoids showed some latent Vero cell toxicity (expected due to greater sensitivity) but at different toxicity levels
- Generally the labs ranked the toxoids in the same order of toxicity

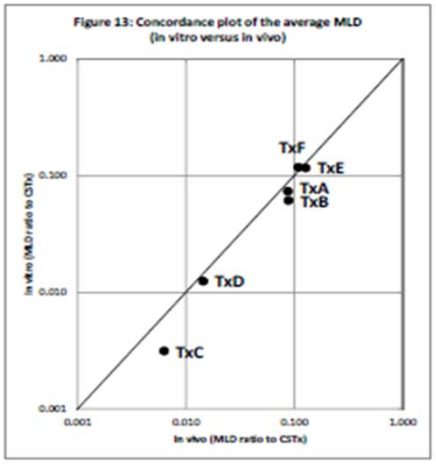
BSP130 Results:MLD

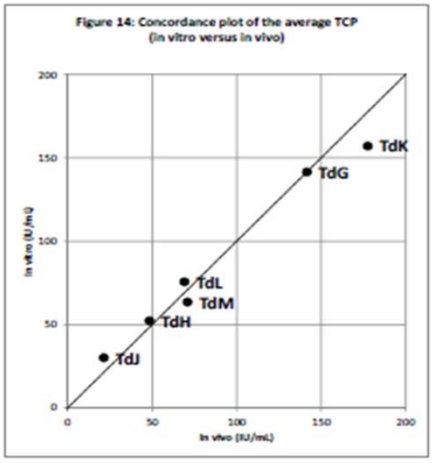
- Ranking of toxins in mice similar in all labs
- Ranking of toxins in Vero cells similar in all labs and similar to the ranking by mouse MLD
- Reported invalid Vero cell MLD assays: 9%
- Toxin/antitoxin neutralisations on Vero cells allowed quantification of toxin in terms of VI
- This method of expressing toxicity of CSTx produced inter-lab GCVs of only 7%

BSP130 Results: TCP

- Ranking of toxoids in mice similar in most labs
- Ranking of toxoids in Vero cells similar in most labs and similar to the ranking by mouse TCP
- Reported invalid Vero cell TCP assays: 4%

BSP130 Results





BSP130 Results

Concordance correlation between the MLD methods is **0.964**

Concordance correlation between the TCP methods is **0.968**

BSP130 Conclusions

- Cell line assays are repeatable and reproducible
- Relatively easily transferable to other laboratories
- More sensitive and reproducible than mouse tests
- Can provide an objective measure of toxicity
- More accurate antigen quantification
- Concordance between the cell line and mouse assays is excellent

BSP130 Outcomes

- Cell line assays are suitable replacements for the mouse MLD and TCP tests for *Cl. septicum* antigens
- Cell line MLD could be the basis for an objective measurement of toxicity
- Cell line TCP gives more accurate quantification of antigenicity than the mouse test
- The *in vitro* assays can give significant savings in animal usage, shorten the duration of QC testing, allow more accurate and reproducible blending of final vaccines and provide a basis for harmonisation

Recommendations

- Vero cell MLD and TCP assays to be promoted as replacements for the *Cl. septicum* mouse tests
- Follow up study, with optimised protocol and assay methods to:

1. Fully exploit the advantages of the *in vitro* assays
2. Assess a modified MLD assay's potential to provide objective measurement of toxicity
3. Increase accuracy of TCP antigen quantification
4. Investigate replacement of TNT on mice for vaccine potency
5. Establish a basis for applying this approach to other relevant toxin antigens

Acknowledgements

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European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

Major Antigens of Clostridial Vaccines

Cl. perfringens type A	Cytopathic toxin
Cl. perfringens type B	Cytopathic toxin
Cl. perfringens type C	Cytopathic toxin
Cl. perfringens type D	Cytopathic toxin
Cl. novyi type B	Cytopathic toxin
Cl. septicum	Cytopathic toxin
Cl. haemolyticum	Cytopathic toxin
Cl. sordelli	Cytopathic toxin
Cl. difficile	Cytopathic toxin
Cl. tetani	Neurotoxin
Cl. botulinum	Neurotoxin
Cl. chauvoei	Toxin + cells?