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

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Major Antigens of Clostridial Vaccines

<table>
<thead>
<tr>
<th>Clostridium Type</th>
<th>Antigen Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl. perfringens type A</td>
<td>Cytopathic toxin</td>
</tr>
<tr>
<td>Cl. perfringens type B</td>
<td>Cytopathic toxin</td>
</tr>
<tr>
<td>Cl. perfringens type C</td>
<td>Cytopathic toxin</td>
</tr>
<tr>
<td>Cl. perfringens type D</td>
<td>Cytopathic toxin</td>
</tr>
<tr>
<td>Cl. novyi type B</td>
<td>Cytopathic toxin</td>
</tr>
<tr>
<td>Cl. septicum</td>
<td>Cytopathic toxin</td>
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<tr>
<td>Cl. haemolyticum</td>
<td>Cytopathic toxin</td>
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<tr>
<td>Cl. sordelli</td>
<td>Cytopathic toxin</td>
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<tr>
<td>Cl. difficle</td>
<td>Cytopathic toxin</td>
</tr>
<tr>
<td>Cl. tetani</td>
<td>Neurotoxin</td>
</tr>
<tr>
<td>Cl. botulinum</td>
<td>Neurotoxin</td>
</tr>
<tr>
<td>Cl. chauvoei</td>
<td>Toxin + cells?</td>
</tr>
</tbody>
</table>
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)

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
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.
These in-process tests for clostridial antigens use 10,000s mice per annum in Europe alone.

The data provided by these in vivo tests i.e. toxicity and antigenicity of the toxins/toxoids are not fully provided by any of the current in vitro tests.
Replacement of In-process In Vivo Tests
A Direct and Simple Approach

<table>
<thead>
<tr>
<th>Test</th>
<th>Indicator</th>
</tr>
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<tbody>
<tr>
<td>MLD</td>
<td>Mice</td>
</tr>
<tr>
<td>TCP</td>
<td>Mice</td>
</tr>
</tbody>
</table>

Mice are used only as an indicator of toxicity

Replace the mice with a different indicator of toxicity – **Toxin-specific cell lines**
Effect of *Cl. septicum* Toxin on VERO Cells

Control cell monolayer

Treated cell monolayer
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

Assay Outline as Developed at MSD AH UK
Cell line assay plate
Advantages of Cell Line Assays

- Greater Sensitivity (up to x16)
- Improved Accuracy (up to x5)
- Reproducibility
- Speed (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
<table>
<thead>
<tr>
<th>Assay</th>
<th>Correlation</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLD</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>TCP</td>
<td>0.99</td>
<td>0.99</td>
</tr>
</tbody>
</table>

- 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?
Purpose:
To promote the acceptance (within Eur. Pharm. monograph guidelines and by Regulatory Authorities) 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 Mouse Lethal Dose (MLD) and the Total Combining Power (TCP) and the potential replacements identified were cell line based assays.
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.
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
Test Materials:

Standard *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)
Participants and Tests Performed:

- There were 11 participants in the study
- These comprised five manufacturers and six 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 dilutions using reference toxin (CSTx) then at 5- and 3-fold. To check sensitivity of different participant’s mice and Vero cells.

- Latent toxicity –
  Each toxoid, diluted 1 in 10, and the standard antitoxin (VI), at 5IU/ml, in one pair of mice and on Vero cells. To check for any residual toxicity.
BSP130 Study Outline

- 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 three occasions or until 3 valid assays are obtained.
BSP130 Results

Sensitivity testing:

- Approximately 6-fold variation in toxin sensitivity of different participant’s mouse strains (one low sensitivity outlier)
- Approximately 3-fold variation in sensitivity of different participant’s vero cell lines (two low sensitivity outliers)
- Vero cells approximately 1,000-fold more sensitive to toxin than mice
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)
- The toxoids showed 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 standard antitoxin
- 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

Figure 13: Concordance plot of the average MLD (in vitro versus in vivo)
BSP130 Results

Figure 14: Concordance plot of the average TCP (in vitro versus in vivo)

- In vitro (IU/mL)
- In vivo (IU/mL)
BSP130 Results

Concordance correlation between the MLD methods is:

0.961 (if the data are log-transformed) and 0.921 (if the data are not log-transformed)

Concordance correlation between the TCP methods is:

0.968 (if the data are log-transformed) and 0.980 (if the data are not log-transformed)
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
- Correlations between the cell line and mouse assays are 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 MNT for vaccine potency
  5. Establish a basis for applying this approach to other relevant toxin antigens
Acknowledgements

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The study participants

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