Progress in Development of an *In Vitro* Potency Test for *Clostridium haemolyticum*

Teresa J. Yeary, Ph.D.  Science Fellow  
USDA, APHIS, VS, Center for Veterinary Biologics  
Ames, IA  50010  
Tel: (515) 663-7449  
Email: Teresa.J.Yeary@aphis.usda.gov
Center for Veterinary Biologics

• Mission is to ensure that the veterinary biologics available for the diagnosis, prevention, and treatment of animal diseases are **pure**, **safe**, **potent**, and **effective**.

• Accomplishes mission through review of data submitted to support licensing, on-site inspections of manufacturing facilities, and laboratory testing of seeds and finished products.

• Laboratory provides standard clostridial reference materials to support testing and test development (e.g. toxins, antitoxins, spore challenge, MAbs).
# Definitions

## Terms related with quantity and quality of vaccine components

<table>
<thead>
<tr>
<th>Term:</th>
<th>Definition:</th>
<th>Influenced by or dependent on:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity</td>
<td>Molarity</td>
<td>Concentration, Ag integrity</td>
</tr>
<tr>
<td>Antigenicity</td>
<td>Ability to bind to specific Abs</td>
<td>Quantity, Ag conformation, Epitope exposure, Matrix - salts, detergents, etc.</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>Ability to induce a specific immune response</td>
<td>Antigenicity, Species <em>(in vivo)</em>, Status of species (immunogenetic background, sex, age), Cell type <em>(in vitro)</em></td>
</tr>
<tr>
<td>Potency</td>
<td>Ability to induce a protective immune response</td>
<td>Immunogenicity, Susceptibility to the pathogen</td>
</tr>
<tr>
<td>Efficacy</td>
<td>Ability to prevent disease in the target species</td>
<td>Potency, Contacts (type, #) within immunized population, Health status of immunized population, Infection pressure by target pathogen, General infection pressure</td>
</tr>
</tbody>
</table>

Current Issues for Veterinary Clostridials

- Reduce the use of animals in potency and toxicity testing of toxoid vaccines; shift from *in vivo* to *in vitro* methods for measuring response to vaccines – 3Rs Concept.

- Clostridial toxoids accounts for the majority of animal usage for establishing potency of product.

- *In vivo* tests are less reproducible than chemical/physical tests.

- Static market creates fierce competition for market share.

- Limited R&D budgets aimed at gaining market share or decreasing cost of production.
## Economics of Clostridial Veterinary Biologicals

<table>
<thead>
<tr>
<th>Doses sold/yr</th>
<th>Product</th>
<th>~Cost /dose</th>
<th>~Market/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 Mil</td>
<td>7-way toxoid</td>
<td>$0.50</td>
<td>$48 Mil</td>
</tr>
<tr>
<td>35 Mil</td>
<td>8-way toxoid</td>
<td>$0.50</td>
<td>$48 Mil</td>
</tr>
<tr>
<td>27 Mil</td>
<td>7-way + <em>M. bovis</em> or 7-way + <em>H. somnus</em></td>
<td>$0.75</td>
<td>$13.5 Mil</td>
</tr>
<tr>
<td>34 Mil</td>
<td><em>C. perfringens</em> C/D</td>
<td>$0.30</td>
<td>$17 Mil</td>
</tr>
<tr>
<td>12 Mil</td>
<td>Tetanus toxoid</td>
<td>$0.50-1.50</td>
<td>$6-18 Mil</td>
</tr>
<tr>
<td>11 Mil</td>
<td><em>C. botulinum</em> C</td>
<td>$0.10</td>
<td>$1.1 Mil</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>~200-250 Mil</strong></td>
<td><strong>$100-150 Mil</strong></td>
<td></td>
</tr>
</tbody>
</table>

10 firms produce clostridial biologicals for the US market
9 CFR: Animals and Animal Products  
Part 113 - Standard Requirements  
Sec. 113.107  *Clostridium Haemolyticum Bacterin.*

*Clostridium Haemolyticum Bacterin shall be produced from a culture of Clostridium haemolyticum which has been inactivated and is nontoxic. Each serial of biological product containing Clostridium haemolyticum fraction shall meet the applicable requirements in Sec. 113.100 and shall be tested for purity, safety, and potency as prescribed in this section. A serial found unsatisfactory by any prescribed test shall not be released.*

(a) Purity test. Final container samples of completed product from each serial and each subserial shall be tested for viable bacteria and fungi as provided in Sec. 113.26.

(b) Safety test. Bulk or final container samples of completed product from each serial shall be tested for safety as provided in Sec. 113.38.

(c) Potency test. Bulk or final container samples of completed product from each serial shall be tested for potency using the two-stage test provided in this paragraph.

1) Each of at least 8 but not more than 10 guinea pigs, each weighing 300 to 500 grams, shall be injected subcutaneously with a guinea pig dose. A second guinea pig dose shall be injected 21 to 23 days after the first dose. Each guinea pig dose shall be one-fifth of the dose recommended on the label for a calf.

2) *Clostridium haemolyticum challenge material, available upon request from Animal and Plant Health Inspection Service, shall be used for challenge 14 to 15 days following the last injection of the product. Each of eight vaccinates and each of five additional nonvaccinated guinea pigs for controls shall be injected intramuscularly with approximately 100 LD50 of challenge material. This dose shall be determined by statistical analysis of results of titrations of the challenge material. The vaccinates and controls shall be observed for 3 days post challenge and all deaths recorded.*

3) For a valid test, at least 80 percent of the controls shall die within the 3 day post-challenge observation period. If this requirement is met, the results of the potency test shall be evaluated according to the following table:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of vaccinates</th>
<th>Cumulative # of vaccinates</th>
<th>Cumulative total number of deaths for a satisfactory test</th>
<th>Cumulative total number of deaths for an unsatisfactory test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>8</td>
<td>1 or less</td>
<td>3 or more</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>16</td>
<td>4 or less</td>
<td>5 or more</td>
</tr>
</tbody>
</table>

The second stage shall be required only when exactly two animals die in the first stage. The second stage shall be conducted in a manner identical to the first stage.

Clostridial Animal Tests
Numbers of animals used to support licensing and release of serials

9CFR, Part 113, Sections 113.106 - 113.112, and 113.114 describes testing that is required of Clostridial toxoids in animals.

An 8-way, multi-component Clostridial Bacterin/Toxoid product would require at a minimum animal testing as shown below prior to release:

- **C. tetani** → 10 guinea pigs
- **C. chauvoei** → 13 guinea pigs
- **C. haemolyticum** → 13 guinea pigs
- **C. perfringens C** → 20 mice
- **C. perfringens D** → 20 mice
- **C. sordellii** → 20 mice
- **C. novyi** → 20 mice
- **C. septicum** → 20 mice
- **9 rabbits**
- **9 rabbits**

Total # Animals: 36 Guinea Pigs 9 Rabbits 100 Mice
Risk vs Reward:  
Potency Testing of *C. chauvoei* Toxoids

400 tests conducted at CVB-L since 1984

<table>
<thead>
<tr>
<th># 2nd Stage</th>
<th># Retests</th>
<th># Unsatisfactory</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

CVB Costs*:
- 8,000 guinea pigs sacrificed
- $200,000 materials + animal care

*Does not account for testing performed by each firm
Regulations and Guidelines for Antigen Quantification

9 CFR 113.8
Potency can be determined by comparing antigen content of a test serial to a reference using a parallel line immunoassay.

Veterinary Services Memorandum No 800.90
Guidelines for Veterinary Biological Relative Potency Assays and Reference Preparations based on ELISA Antigen Quantification.
Abstract

Goal:
To reduce the amount of animal testing required for testing *C. haemolyticum* in veterinary clostridials products.

Approach to the problem:

- Characterize the protective immunogens of *Clostridium haemolyticum*
- Investigate Surface Plasmon Resonance as an *in vitro* alternative to the guinea pig test for potency testing of *C. haemolyticum* toxoid.
- Establish a national reference antitoxin and antitoxin unit for *C. haemolyticum* beta toxin.
Is C. haemolyticum beta toxin a protective immunogen?

- Purify native toxin by IEF

- Created and characterized MAbs against the beta toxin:
  --Screened for specificity
  --Passive immunization of guinea pigs and mouse bioassay
  --Assay for anti-phospholipase [neutralizing] activity on Egg Yolk Agar plates using beta-toxin and MAb
  --Western blots to confirm MWt of the target protein

- Purified native toxin by immunoaffinity chromatography using the neutralizing MAbs

- Active immunization of guinea pigs
### In Vitro Immunoassays for Characterizing Antigens and Vaccines

- ELISA – Enzyme linked immunosorbant assay
- Non-labelled immunoassays – flocculation, immunoprecipitation
- Immunoblotting
- Immunogold electron microscopy
- Surface Plasmon Resonance

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#### Analytical chemical methods

<table>
<thead>
<tr>
<th>Chromatography</th>
<th>Peptide Mapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoresis</td>
<td>Mass Spectrometry</td>
</tr>
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</table>

#### Spectrophotometric methods

<table>
<thead>
<tr>
<th>Circular dichroism</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>Infrared spectroscopy</td>
<td>NMR</td>
</tr>
<tr>
<td>Electron Microscopy</td>
<td>Dynamic light scattering</td>
</tr>
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</table>
Limitations of *In Vitro* Testing

- Lack of ability for the test to reflect the total protective response of the host; i.e., either humoral immunity or cellular immunity is being measured

- Ability of the test to identify a minimal antigen level which can elicit a protective host response

- Re-qualification of references which constitute the critical component of a valid in vitro test; How will this be done?

Biacore® Surface Plasmon Resonance (SPR) Detection System

Biacore’s SPR technology is designed to investigate the functional nature of binding events.
The gold layer in the sensor chip creates the physical conditions required for Surface Plasmon Resonance (SPR). Essentially, SPR detects changes in mass in the aqueous layer close to the sensor chip surface by measuring changes in refractive index caused by binding/dissociation of analyte and ligand.

The functional analysis of molecular interactions is accomplished in a microfluidics system with flow cell volumes of 0.06µl.
Biacore® Sensor Chip Technology

The sensor chip consists of a glass surface, coated with a thin layer of gold. This forms the basis for a range of specialized surfaces (linker layer) designed to optimize the binding of a variety of molecules, like dextran, that immobilize the ligand.

Quantitative measurements of the binding interaction between one or more molecules in solution (the analyte) are dependent on the immobilization of a target molecule (ligand), such as an antibody, to the sensor chip surface.
Biacore® Sensor Chip Technology

Integrated microfluidics cartridges allow analyte to pass over the sensor surface in a continuous, pulse-free and controlled flow – maintaining constant analyte concentrations at the sensor chip surface at bulk flow rates of 1-100µl/min.
When analyte in the test solution binds to ligand, the increase in mass at the sensor surface causes an increase in refractive index which alters the angle of incidence of polarized light from the optical detection system creating the SPR phenomenon.

The change is measured as a response signal which is displayed in a sensorgram – a continuous, real-time monitoring of the association and dissociation of the interacting molecules.

*The sensorgram provides quantitative information in real time on specificity of binding, active concentration of molecule in a sample, kinetics, and affinity.*
Advantages of SPR

Low sample consumption:
Only \( \mu l \) quantities of analyte and ligand are required. Nanomolar concentrations are measured.

Analyte can be captured from complex mixtures without prior purification as they pass over the chip.

None of the reactants needs to be labelled.

Molecules as small as 100 Da can be studied.

Absence of an air-solution interface where samples can evaporate and proteins can be denatured.

Concentration of free analyte is constant.

A range of surface ligand concentrations & contact times can be analyzed in one experiment – improving kinetic and concentration analysis.

4 channels can be measured simultaneously:
Allows comparison of samples with a blank and automatic reference subtraction; screening of multiple samples is possible.
Advantages of SPR

Interactions are measured in real time:
    Kinetic rate constants and equilibrium affinity constants can be determined.

Sensor chips may be reused up to 100 times:
    The surface sensor is treated with dissociating reagent after each use to remove analyte from immobilized ligand; conformation of the ligand is preserved

SPR technology could replace several *in vivo* assays including:
    Mouse LD50, Lf, L+ and TCP for several clostridial toxins and toxoid antigens.

Analysis may be completed in less than 10-20 min:
    It may reduce animal usage and achieve potency assay results within minutes rather than days which is an important commercial consideration.

May be used to optimize the fermentation processes in vaccine production:
    More consistent harvesting of toxin nearer its peak concentration, thus reducing the number of poor lots which may be the result of hydrolysis of toxin by proteases in the culture.
Project Milestones

• The *Clostridium haemolyticum* beta-toxin gene has been cloned, sequenced, and deposited in GenBank.

• 48% of the deduced amino acid sequences of the active beta toxin have been verified by N-terminal peptide sequencing and with tryptic digestion coupled with MALDI-ToF mass spectroscopy.

• The C-terminal domain shows 46-54% aa homology with other pathogenic Clostridial PLPCs.

• A paper has been submitted for publication to the journal *Anaerobe*: “Cloning and Molecular Characterization of the Beta Toxin Gene (Phospholipase C) of *Clostridium haemolyticum*.“

• Beta toxin-specific and toxin-neutralizing monoclonal antibodies have been developed for use in *in vitro* assays.

• Preliminary studies with guinea pigs indicate that the beta toxin will be a protective immunogen.
Current Activities

- Attempts at expression of a recombinant *C. haemolyticum* beta toxin are underway.

- A bank of toxoids of varying quality are being produced using various amounts of heat and formalin. Toxoid quality will be evaluated in serological tests and SPR. These standard toxoids will be used to validate any tests measuring toxoid quantity and quality.

- Use of SPR: Treat toxin with 5 different concentrations of formaldehyde ranging from low, moderate, to high, and one untreated control; GP studies with toxoids; SPR with toxoids.

- Purified recombinant *C. haemolyticum* beta toxin will be toxoided and used to vaccinate guinea pigs to determine if it is protective. The project involves cooperation with Biacore to assess the potential of SPR for use in evaluating vaccine potency.
Potential Pitfalls

• The *C. haemolyticum* beta toxin may not be the protective antigen. Focus will then have to be diverted to other potential protective antigens, like the flagellae. A time consuming hunt for protective antigens will be required which will slow progress.

• No tests that are capable of measuring both *quantity* and *quality* of antigens currently exist. SPR *appears* to be a great candidate technology for this purpose, *but* implementation of SPR and interpretation of the data as it relates to current animal testing protocols may be difficult.

• *Tests developed as a result of this project may require changes in final product testing policy and/or contract testing policy.* If those policy changes are not made, the project may not have much long term impact.
Anticipated Results

Successful completion of the project will impact each of the primary activities of the Center for Veterinary Biologics-Laboratory (CVB-L) which include:

- prelicense testing;
- test development and standardization (including reagent activities);
- postlicense quality control monitoring.

Similar studies with other clostridials may be pursued.
Questions??????
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The functional analysis of molecular interactions is accomplished in a microfluidics system with flow cell volumes of 0.06µl.