3Rs methods applicable to control the quality of Diphtheria and Tetanus vaccine components

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Vaccines are of biological origin and have the potential to vary from batch to batch. Consequently, vaccines are tested for batch-to-batch consistency and many of these tests involve animals i.e. it is at the expense of large numbers of animals that are used in quality control tests before vaccines are released onto the market.

In this session, I will discuss the progress and achievements of 3Rs in the development of alternative test methods involved in the testing of D and T components.
The use of "alternative" methods are generally concerned with the Principles of the 3Rs - Replacement, Reduction, and Refinement of animal testing, first proposed by the scientists William Russell and Rex Burch in their book 'The Principles of Humane Experimental Technique' (1959).

The animal models have several limitations in respect of their relevance, reliability, costs, ethical acceptability and also the process is laborious and time consuming. Moreover, the injection of virulent material or manipulation of contaminated animals exposes technicians to additional risks.
The potency tests like • challenge assays • toxin neutralization assays • serological assays etc. are mainly used for quality control evaluation of vaccines. Mostly potency assays based on an immunization-challenge procedure in laboratory animals are still being used in vaccine research and routine lot-release testing.

These potency tests are multi-dose models that include a challenge procedure with virulent micro-organisms. As a result, animals suffer substantial pain and distress during the testing period.

The quality control evaluations of vaccines require high frequency of tests with large number of laboratory animals.
Potency

1. Potency in guinea pigs by lethal challenge: The challenge potency test for diphtheria vaccine (adsorbed) is determined by comparing the dose of the vaccine to that of a reference preparation required to protect guinea pigs from lethal toxin challenge. It is a multi dilution assay.

2. Single dilution test: It is a one dilution assay and is performed: 1. when the potency of the test vaccine consistently and significantly exceeds minimum requirements i.e. the potency of the test vaccine is significantly greater than the minimum requirement per human dose for the product under test and 2. when parallelism between test and reference vaccine has been demonstrated over time.
3. Potency in guinea pigs by serology:
Serology is an alternative procedure to the guinea pig challenge method, antibody responses to diphtheria toxoid induced in guinea pigs after 5 to 6 weeks are compared relative to the antibody response induced by the reference vaccine.

WHO recommends that functional end-point assays such as serology using Vero cells can be used as an alternative procedure to the challenge tests in guinea pigs for determination of diphtheria vaccine potency.

4. Potency in mouse by Vero cell assay:
The mouse potency assay involves the detection of functional antibodies against diphtheria toxoid induced in mice by observing neutralizing effect of sera on diphtheria toxin in a Vero cell culture model.
  (mice are not sensitive to diphtheria toxin)
Schematic diagram of the Vero cell assay for potency testing of diphtheria vaccine

1. **Immunisation (3 dilutions)**
   - Reference Vaccine (IU)
   - Test Vaccine

2. **5-6 Week Bleed**
   - Tissue culture microplate
     - Serial twofold serum dilutions
     - Add diphtheria toxin
     - Incubate 1h
     - Add Vero cells
     - Incubate 6 days

3. **MTT assay**
   - Calculate end point titre of test and reference sera
   - Vaccine potency calculation by parallel line analysis

   - Blue/purple colour = cell growth (neutralisation of toxin)
   - No colour = cell death (no neutralisation of toxin)
EFFECT OF DIPHTHERIA TOXIN ON VERO CELLS – HEALTHY CELLS
EFFECT OF DIPHTHERIA TOXIN ON VERO CELLS – PARTIALLY AFFECTED CELLS
EFFECT OF DIPHTHERIA TOXIN ON VERO CELLS – IRREVERSIBLE DEATH/METABOLIC INHIBITION
5. In vivo toxin neutralization test:
The *in vivo* toxin neutralization test (TNT) can be performed on the depilated skin of guinea pigs owing to the ability of diphtheria toxin to cause an erythematic reaction when injected intradermally.

**Specific Toxicity**

**In vivo test for absence of toxin and absence of reversion to toxicity in guinea pigs:**
This in vivo assay remains the method of choice for routine testing or validation of production processes.

**Vero cell test for absence of toxin and absence of reversion to toxicity:** A Vero cell culture system may also be used as an alternative to in vivo tests for specific toxicity and reversion to toxicity.
Testing for Tetanus Vaccines

Potency

1. Potency in guinea pigs and mice by challenge (lethal and paralysis):
   The challenge potency test for tetanus vaccine (adsorbed) is determined by comparing the dose of the vaccine to that of a reference preparation required to protect guinea pigs or mice from either a lethal or paralytic toxin challenge.

2. Single dilution test: It is a one dilution assay and is performed (i) when the potency of the test vaccine consistently and significantly exceeds minimum requirements i.e. the potency of the test vaccine is significantly greater than the minimum requirement per human dose for the product under test and (ii) when parallelism between test and reference vaccine has been demonstrated over time.
Testing for Tetanus Vaccines

3. Potency in guinea pigs by serology:
Serology is an alternative procedure to the guinea pig challenge method, antibody responses to tetanus toxoid induced in guinea pigs after 5 to 6 weeks are compared relative to the antibody response induced by a reference vaccine.

WHO recommends that Enzyme Linked Immunosorbent Assay (ELISA) can be used with serology assays to determine the potency of tetanus vaccine for routine lot release after its validation against the challenge assay or the toxin neutralization test.

4. Titration of immune sera by ToBI
WHO recommends that Toxin Binding Assay (ToBI) can be used with serology assays to determine the potency of tetanus vaccine for routine lot release after its validation against the challenge assay or the toxin neutralization test.
Testing for Tetanus Vaccines

5. Potency in mice by serology (ToBI): Potency test for routine lot release can be performed by immunizing mice as well as guinea pigs with appropriate dilutions of the calibrated reference preparation and the test vaccine. Titration of immune sera may be performed in vitro by ToBI test.

Specific Toxicity

In vivo test for absence of toxin and reversion to toxicity in guinea pigs: The purpose of the specific toxicity test for tetanus toxin is to confirm freedom from residual toxin and reversion to toxicity in final bulk vaccines and/or bulk purified toxoid. The in vivo assay remains the method of choice for routine testing or validation of production processes.
Implementation of 3Rs in quality control testing of vaccines at Serum Institute

Serum Institute of India Pvt. Ltd. (SIIPL)-PUNE is India’s largest manufacturer of vaccines and other biotech products.

SIIPL vaccines produce several bacterial, viral and recombinant vaccines. SII also manufactures combination and multivalent vaccines.

SIIPL is committed to the development, introduction, validation, and implementation of 3Rs (Refinement, Reduction, and Replacement) and consistency based approaches.

India Pharmacopoeia has always been supportive and receptive to such initiatives.
Potency testing of conventional vaccines such as DTP group of vaccines involves challenge methods. The methods require large numbers of animals and induce substantial levels of suffering.

One of the most feasible alternatives is serology. Serology allows quantitative (antibody titers) instead of qualitative endpoints (death or survival) and also a significant reduction in the number of animals used.

SII committed itself for development of such serology based alternatives.
DTP GROUP OF VACCINE
(Serological assays): Replacement

CONVENTIONAL METHOD

Lethal Challenge test
Animals used: Guinea pigs / Mice

ASSAYS FOR 3R APPROACH

• Vero cell assay (Potency of Diphtheria component)

• T-ELISA (Potency of Tetanus Component)
The serological are multi-dilution assays and thus imply still the use of great number of animals.

Single dilution assays were considered a reduction approach once production consistency has been established.

Implementation requires an extensive and rigorous comparison with multi-dilution assay.

SIIPL team committed itself on the same and data on large number of batches was submitted to National control laboratory for approval.

SIIPL got the approval for these assays from the NCL, NRA and subsequently from WHO.

SIIPL was successful in implementation of such assays for routine testing and control.
### Single dilution Vero cell and T–ELISA Assay: Regulatory acceptance

<table>
<thead>
<tr>
<th>Steps in process of test development</th>
<th>Timelines (Diphtheria)</th>
<th>Timelines (Tetanus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development of assay</td>
<td>June 2003</td>
<td>2004</td>
</tr>
<tr>
<td>Submission of proposal to National Control Laboratory</td>
<td>October 2005</td>
<td>October 2006</td>
</tr>
<tr>
<td>Final acceptance by National Control Laboratory</td>
<td>March 2006</td>
<td>August 2007</td>
</tr>
<tr>
<td>Approval obtained</td>
<td>1 in 10 lots needs to be tested in challenge test.</td>
<td>April 2017 - Approval for 1 in 25 lots to be tested or once in six months which ever is earlier.</td>
</tr>
</tbody>
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Implementation of 3Rs approaches at SIIPL and annual animal savings

<table>
<thead>
<tr>
<th>Potency</th>
<th>Method</th>
<th>Animal model</th>
<th>Duration of Test (days)</th>
<th>Number of animals per lot</th>
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<tr>
<td>Tetanus Potency</td>
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<td>Guinea Pigs</td>
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<tr>
<td><strong>Total G. Pigs</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>232</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tetanus and Diphtheria potency together In case of DT, DTP and combined vaccines</th>
<th>Method</th>
<th>Animal</th>
<th>Duration (days)</th>
<th>No. of animals</th>
<th>% reduction in animal consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ELISA &amp; Vero cell assay</td>
<td>Std. T – 10</td>
<td>Guinea pigs</td>
<td>44 &amp; 49</td>
<td>~ 85%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std. D – 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Test Vaccine-10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total = 30</td>
<td></td>
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<tr>
<td>Tetanus Potency</td>
<td>*Paralytic challenge test</td>
<td>Mice</td>
<td>33</td>
<td>126</td>
</tr>
</tbody>
</table>

*NCL’s approval obtained to perform Paralytic Challenge Assay in Mice in 2017
Total G. Pigs Replaced with Mice 116
SIIL always aimed for alternatives. Have been partner to various international collaborative studies aimed at 3Rs on Diphtheria, tetanus, and pertussis since 1999.

<table>
<thead>
<tr>
<th>Tetanus Vaccine</th>
<th>BSP035</th>
<th>EDQM: Invitro methods for alternatives to challenge test of tetanus toxoid.</th>
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<tr>
<td>Diphtheria Verocell assay</td>
<td>BSP034</td>
<td>EDQM: Verocell assay as alternative to diphtheria potency test.</td>
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<tr>
<td>Pertussis (PsPT)</td>
<td>Serological assay</td>
<td>Humane Endpoints for Lethal Parameters (HELP) funded by ECVAM.</td>
</tr>
</tbody>
</table>

- Regulatory acceptance: (Non animal methods are being accepted for release of vaccines for example, hepatitis B vaccine, glycoconjugate vaccines. Similar approach may be followed for rabies)
- Mechanisms to introduce such recommendations in regulatory documents, pharmacopeias
- Harmonization of regulatory requirements: Important for global supplier like us.
Reference

WHO Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines

WHO/IVB/11.11
Thank You