Rabies Batch Release Testing

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First DCVMN 3Rs Experts Working Group Meeting
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Overview

- Introduction
- Epidemiology and burden of disease
- Current Vaccines and Process
- 3R Strategy
- Development of ELISA method (G-Protein)
- Conclusion
Introduction

• Rabies causes an estimated 59,000 mortalities every year
• That’s approximately 1 death every 15 minutes
• Human Rabies is present in 150 countries and territories and on all continents, except for Antarctica
• 80% of cases occur in individuals living in rural populations- most of which are children
• Domestic dogs are the most common reservoir of the virus, with more than 95% of human deaths caused by dogs
• 95% of cases are reported in Asia and Africa
Epidemiology and burden of disease

A. Human Deaths form Rabies; B. Death rates per capita (per 100,000 population); countries shaded in grey are free from canine Rabies

https://www.who.int/rabies/epidemiology/en/
Rabies virus (RABV)

- Rabies virus (RABV) is the prototype virus of the genus *Lyssavirus* (from the Greek *lyssa* meaning ‘rage’) in the family *Rhabdoviridae* (from the Greek *rhabdos* meaning ‘rod’).
- RABV is a highly neurotropic virus in the infected mammalian (animal and human) host, invariably causing a fatal encephalomyelitis.
- Organization of the rabies virus genome has Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Gcoprotein (G) and large RNA-polymerase protein (L) genes are separated by intergenic di- and penta-nucleotide sequences and the long pseudogene (Ψ) sequence and are flanked by the leader (Le) RNA and trailer (Tr) RNA sequences at the 3’ and 5’ ends.
Rabies Virus Life Cycle

History of strains of rabies viruses used as vaccine seeds

PAS, Pasteur; PM, Pitman-Moore; PV, Pasteur virus; SAD, Street Alabama Dufferin

Ref. Plotkin’s - Vaccines
Brief Manufacturing Process (Zydus)

1. SPF Chicken Egg receipt & Incubation
2. Candling, Embryo collection and pooling
3. Trypsinization and Preparation of cell suspension
4. Rabies Virus Infection and Adsorption
5. Virus Harvest
6. Pooling and Filtration of Virus Harvest
7. Purification and Concentration of virus Harvest
8. Inactivation of Bulk
9. Final Bulk Vaccine preparation and blending
10. Filling and Lyophilisation
11. Finished Product
Rabies NIH Potency assay (Finished Product)

Sample Dilution

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>1/25</td>
<td>1/625</td>
</tr>
<tr>
<td>1/125</td>
<td>1/3125</td>
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Day 0
- Primary Dose

Day 7
- Booster Dose

Day 14
- Intracerebral Challenge

Counting and euthanasia of rabid mice

Day 19

Day 28

- Worldwide used release test, specification ≥ 2.5 IU per single human dose
- Ph.Eur. 0216, WHO TRS 941
Issues and challenges with the *in vivo* challenge (NIH) test

- Time consuming 28 days to complete the test
- Very high variability: 25-400%
- Hazardous - Safety issues - Need for BSL3 containment due to the use of live rabies virus
- Purity of the animal strain/breed
- Animal usage – Approx. 150 animals per test
- Availability of CVS (challenge virus strain)
- Regulators: in most of the Regulatory Guidelines, NIH is mandatory for final lot release
Alternatives to Animal Experiments

3R

- **Replace**: Replace animal studies with other methods
- **Reduce**: As many trials as required, as few as possible
- **Refine**: Minimize stress of study animals

W. M. S. Russell and R. L. Burch in 1959
Global Scenario on alternate strategy to NIH

- The *in-vitro* ELISA, as an alternative to the NIH test, is:
  - in accordance with the Ph. Eur. 3Rs strategy: replacement
  - already used by some manufacturers for blending and monitoring of the consistency of production

- EPAA (European partnership for alternative approaches in animal testing) has already started a study with industrial collaborative partners to replace Human Rabies potency test (Project Code BSP148) which is been very well supported by all vaccine manufacturers and WHO for Harmonization and make it a release test
In-vivo Assays:
- Early 1900s Lethal Challenge in Animal models (Animal challenge study)
- High cost, time, labour, resources yet high variability.

In-vivo/In-vitro Assays:
- In-Direct ELISA
- Neutralization Assay
- PRNT

In-vitro Assays:
- SRID (Influenza)
- ELISA: In-vitro Potency assay to check antigen using specific monoclonal antibody
Development of in-vitro potency assay

- Zydus being one of the vaccine manufacturers of human rabies vaccine has developed an *in-vitro* potency assay based on G-Protein
- Validation of the method with the different batches are under progress
<table>
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<td>assay of inactivated tissue culture rabies vaccines: determination of</td>
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<td>the rabies virus glycoprotein with polyclonal antisera.</td>
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<td>monoclonal antibody.</td>
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<td>test for human rabies vaccine batch release.</td>
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<td>vitro glycoprotein quantification using ELISA - Results of an</td>
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<td>international collaborative study.</td>
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<td>rabies vaccines: Agreement between the NIH assay and a G-protein</td>
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<td>based ELISA.</td>
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G-protein based ELISA as a potency test for rabies vaccines

Martine Chabaud-Riou, Nadège Moreno, Fabien Guinchard, Marie Claire Nicolai, Elisabeth Niogret-Siohan, Nicolas Sève, Catherine Manin*, Françoise Guinet-Morlot, Patrice Riou

Sanofi Pasteur, Campus Mérieux, 1541 Avenue Marcel Mérieux, 69280, Marcy l’Etoile, France

Abstract

The NIH test is currently used to assess the potency of rabies vaccine, a key criterion for vaccine release. This test is based on mice immunization followed by intracerebral viral challenge. As part of global efforts to reduce animal experimentation and in the framework of the development of Sanofi Pasteur next generation, highly-purified vaccine, produced without any material of human or animal origin, we developed an ELISA as an alternative to the NIH test. This ELISA is based on monoclonal antibodies recognizing specifically the native form of the viral G-protein, the major antigen that induces neutralizing antibody response to rabies virus. We show here that our ELISA is able to distinguish between potent and different types of sub-potent vaccine lots. Satisfactory agreement was observed between the ELISA and the NIH test in the determination of the vaccine titer and their capacity to discern conform from non-conform batches. Our ELISA meets the criteria for a stability-indicating assay and has been successfully used to develop the new generation of rabies vaccine candidates. After an EPAA international pre-collaborative study, this ELISA was selected as the assay of choice for the EDQM collaborative study aimed at replacing the rabies vaccine NIH in vivo potency test.

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Fig. 2. Comparative analysis of different lots of rabies vaccine at the Filled Product step. The horizontal bar corresponds to the threshold for conformity. Triangle and right-hand y axis: NIH test; Square and left-hand y-axis: ELISA assay.

Riou et al., 2017
G Protein

• G Protein, which is a trimer of approximately 67kDa, is the major antigen responsible for inducing production of VNAs and for conferring immunity against lethal infection with rabies virus.
**In-vitro Potency assay**

- Serum Antibody Assay
  
  G-Protein ELISA (In-direct ELSIA or c-ELISA)

- Antigen ELISA
  
  - Sandwich ELISA
    - Polyclonal Sera
    - Monoclonal ELISA
    - Polyclonal and Monoclonal ELISA

Challenge can be avoided

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In-vitro Assay Platform

Sandwich ELISA

Critical Reagent:

1. Capture antibody
2. Detection antibody
3. Reference standard
1. M777-16-3 ($\text{IgG1}$) binds to Site IIa

2. 62-71-3 ($\text{IgG2b}$) binds to Site III
Technical Information of the Monoclonal antibody

Development of a Mouse Monoclonal Antibody Cocktail for Post-exposure Rabies Prophylaxis in Humans

Thomas Müller¹, Bernhard Dietzschold², Hildegund Ertl³, Anthony R. Fooks⁴, Conrad Freuling⁵, Christine Fehlner-Gardiner⁵, Jeannette Kliemt⁶, Francois X. Meslin⁶, Charles E. Rupprecht⁷, Noël Tordo⁸, Alexander I. Wanderler⁵, Marie Paule Kieny⁹

1 WHO Collaborating Centre for Rabies Surveillance and Research, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Wusterhausen, Germany, 2 WHO Collaborating Centre for Neurovirology, Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, Pennsylvania, United States of America, 3 WHO Collaborating Centre for Reference and Research on Rabies, Wistar Institute, Philadelphia, Pennsylvania, United States of America, 4 WHO Collaborating Centre for the Characterization of Rabies and Rabies-related Viruses, Veterinary Laboratories Agency, Department of Virology, New Haw, Addlestone, Surrey, United Kingdom, 5 WHO Collaborating Centre for Rabies Control, Pathogenesis and Epidemiology in Carnivores, Canadian Food Inspection Agency (CFIA) Centre of Expertise for Rabies, Ottawa, Ontario, Canada, 6 Neglected Zoonotic Diseases (NZD), Department of Neglected Tropical Diseases (NTD), Cluster HIV/AIDS, Malaria, Tuberculosis and Neglected Tropical Diseases (HTM), World Health Organization, Geneva, Switzerland, 7 WHO Collaborating Centre for Reference and Research on Rabies, Rabies Section, Division of Viral and Rickettsial Diseases, Viral and Rickettsial Zoonoses Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America, 8 Unit Antiviral Strategy, CNRS URA-3015, Institut Pasteur, Rabies Unit, Paris, France, 9 Initiative for Vaccine Research, Vaccines & Biologicals, Health Technology & Pharmaceuticals, World Health Organization, Geneva, Switzerland
## Technical Information of the Monoclonal antibody

### Table 1. Available technical information for candidate MoMAbs.

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<tr>
<th>History of hybridomas</th>
<th>E559.9.14</th>
<th>1112-1</th>
<th>62-7-13</th>
<th>M727-5-1</th>
<th>M777-16-3</th>
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<tbody>
<tr>
<td>Mouse strain providing B-cells</td>
<td>BALB/c mice</td>
<td>BALB/c mice</td>
<td>BALB/c mice</td>
<td>BALB/c mice</td>
<td>BALB/c mice</td>
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<tr>
<td>Antigen</td>
<td>ERA G protein</td>
<td>ERA G protein</td>
<td>whole ERA</td>
<td>whole ERA, #167-169</td>
<td>whole ERA, #167-169</td>
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<tr>
<td>Reference</td>
<td>[35]</td>
<td>[50]</td>
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<td>no</td>
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<tr>
<td>Number of donating steps</td>
<td>4</td>
<td>Not known</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Purity/homogeneity of cell line</td>
<td>Not known</td>
<td>Not known</td>
<td>Sub-cloned 2x, single IgG peak</td>
<td>Isotype as pure IgG 2a</td>
<td>Isotype as pure IgG 1</td>
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<tr>
<td>Origin of FCS used</td>
<td>New Zealand</td>
<td>USA</td>
<td>USA (GIBCO)</td>
<td>USA (Sigma), Canada (Wisten)</td>
<td>USA (Sigma), Canada (Wisten)</td>
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<td>Culture conditions</td>
<td></td>
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<tr>
<td>Medium</td>
<td>Iscove's DMEM 1</td>
<td>DMEM (modified)</td>
<td>Iscove's DMEM 2</td>
<td>HY-HT (10% FCS)</td>
<td>HY-HT (10% FCS)</td>
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<tr>
<td>Cell concentration</td>
<td>$10^5$-10$^6$</td>
<td>$10^4$-10$^5$</td>
<td>2 x $10^3$</td>
<td>$6 \times 10^4$ - $3 \times 10^5$</td>
<td>$7 \times 10^4$ - $3 \times 10^5$</td>
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<td>Serum-free culture medium</td>
<td>CD HM or PFHM II protein-free</td>
<td>Not tested</td>
<td>tested but no specification</td>
<td>Ultraloma-PF</td>
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<tr>
<td>Type of immunoglobulin</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>IgG subtype</td>
<td>IgG 1 (ELISA)</td>
<td>IgG 1 (ELISA)</td>
<td>IgG 2b (ELISA)</td>
<td>IgG2a (FCA)</td>
<td>IgG 1 (FCA)</td>
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<tr>
<td>Heavy/light chains cDNAs</td>
<td>Yes</td>
<td>Yes</td>
<td>no</td>
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<td>Antigenic site recognized on G</td>
<td>II</td>
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<tr>
<td>Method for determining epitope</td>
<td>sequencing</td>
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<td>cross neutralisation</td>
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<tr>
<td>Escape mutants</td>
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<td></td>
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<tr>
<td>derivation</td>
<td>SAD B19</td>
<td>CVS-11</td>
<td>not available</td>
<td>not available</td>
<td>ERA</td>
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<tr>
<td>aa substitutions in G</td>
<td>aa 57 (Leu to Arg)</td>
<td>aa 53 (Gly to Glu)</td>
<td></td>
<td>aa 198 (Lys to Gln)</td>
<td>aa 286 (Ala to Thr)</td>
</tr>
<tr>
<td>Production yield</td>
<td>Yield in IU/ml (crude hybridoma)</td>
<td>62.5</td>
<td>3</td>
<td>30-60</td>
<td>22-32</td>
</tr>
</tbody>
</table>

Legend: aa–amino acid, CVS 11–Challenge virus standard 11, DMEM–Dulbecco’s minimum essential medium, ELISA–enzyme linked immunosorbent assay, ERA–Evelyn Rokitskii Abelseth SAD derived RABV strain, FCA–Fluorion-C A Assay, H8–hybridization medium, SAD–Street Alabama Dufferin strain of RABV. Media specification: Iscove’s DMEM 1 = Iscove’s modified DMEM + HAM F12 (1:1) + 10% FCS; Iscove’s DMEM 2 = Iscove’s modified DMEM + ITS + antibiotics/antimycotics + L-glutamine + 5% FCS.
Development of G-Protein SW-ELISA method for Rabies vaccine testing

- In-house highly characterized mAbs binding to the G protein of Rabies antigen

1. M777-16-3 (IgG1) binds to **Site II a** (Capture Antibody)

2. 62-71-3 (IgG2b) binds to **Site III** (Detection Antibody)
Sandwich ELISA Procedure

**Step 1:** Coating of Rabies capture antibody (Site II)
Incubate the ELISA plate at 2-8°C for overnight

**Day 1**

**Step 2:** 200µL of blocking solution to each well of the ELISA plate and incubate for 1 hrs ± 5 min at 37 °C± 2° C

**Step 3:** Add standard and sample in different dilutions

**Step 4:** Add Detection antibody (Site III)

**Step 5:** Add anti antibody HRP conjugated

**Step 6:** Substrate, stop reaction and read plate at 450nm

**Day 2**
18 Batch Data of NIH Potency Vs SW-ELISA
## Cost of Animal Potency

### Rabies Vaccine Testing

<table>
<thead>
<tr>
<th>Current Method</th>
<th>Method</th>
<th>No of Animals used per Batch</th>
<th>Cost per animal (Approx. on the lower side) in Rs.</th>
<th>Husbandry cost for 28 Days per animal in RS.</th>
<th>Total cost for a 28 day batch release study in Rs.</th>
<th>No of Rabies batches taken in 2017-18</th>
<th>Total Animal Cost for releasing the batches in Rs.</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH Animal Potency</td>
<td>136</td>
<td>200</td>
<td>300</td>
<td>68,000</td>
<td>60</td>
<td>40,80,000</td>
<td>Handling of challenge virus, Facility to do challenge study</td>
<td></td>
</tr>
</tbody>
</table>

**Total No. of Animals Approximately Consumed- ~5000- 8000 Nos.**
## Cost of Sandwich ELISA

<table>
<thead>
<tr>
<th>Alternate Method</th>
<th>Method</th>
<th>Time for Test</th>
<th>Cost of the Assay per plate (Approx. for testing 5 batches) in Rs.</th>
<th>No of Rabies batches taken in 2017-18</th>
<th>Total Cost for testing 60 batches in Rs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house Developed Sandwich ELISA (EDQM harmonizing to approve)</td>
<td>1 Day</td>
<td>300</td>
<td>60</td>
<td>18,000</td>
<td></td>
</tr>
</tbody>
</table>

**No Animal Usage**
Zydus approach to Development of *in-vitro* potency assay
G-Protein ELISA method for Rabies vaccine testing

- In-house highly characterized mAbs binding to the G protein of Rabies antigen

1. M777-16-3 (IgG1) binds to Site II

2. 62-71-3 (IgG2b) binds to Site III

- Developed sandwich ELISA and made the standard curve
- Assay range is from 1.25IU/mL to 0.01IU/mL
- Screened 18 batches of Rabies vaccine by ELISA method
- Correlation between NIH and ELISA was studied
- **More validation under QC is under progress to understand the potent and low potent batches**
Questions to be answered for Replacing the in-vivo NIH with in-vitro assay

• Are all manufacturers interested in moving from in-vivo NIH potency to in-vitro (ELISA) method?
• Are the critical reagent available to implement the assay?
• Method validation/correlation for the assay performed?
• Are enough lots tested or Is it possible to get more manufactures share the final lot for validating the Assay?
• Can a harmonized training provided to all manufacturers?
Conclusion

• Zydus Cadila as one of the leading vaccine manufacturers of Human Rabies vaccine is interested to collaborate and validate the assay platform through DCVMN network program

• Further Validation of method is in Progress
Thank You