3Rs Updates

Replacement of the Rabbit Pyrogenicity Test

Replacement of NIH for Rabies

Laura Viviani

29 April 2020

E-workshop on Regulatory Pathways and changes in Vaccine Testing

Agenda

1. Replacement of the Rabbit Pyrogenicity Test
    1. Pyrogens
    2. RPT
    3. Bacterial Endotoxin Test (or LAL)
    4. Monocyte Activation Test
    5. Recombinant Factor C
    6. Advantages: Use of Alternative Methods
2. Replacement of NIH for Rabies
    1. The NIH Test
    2. History of Replacement Opportunities
    3. G-protein ELISA
    4. Updates on the BSP-148

1.1 Pyrogens

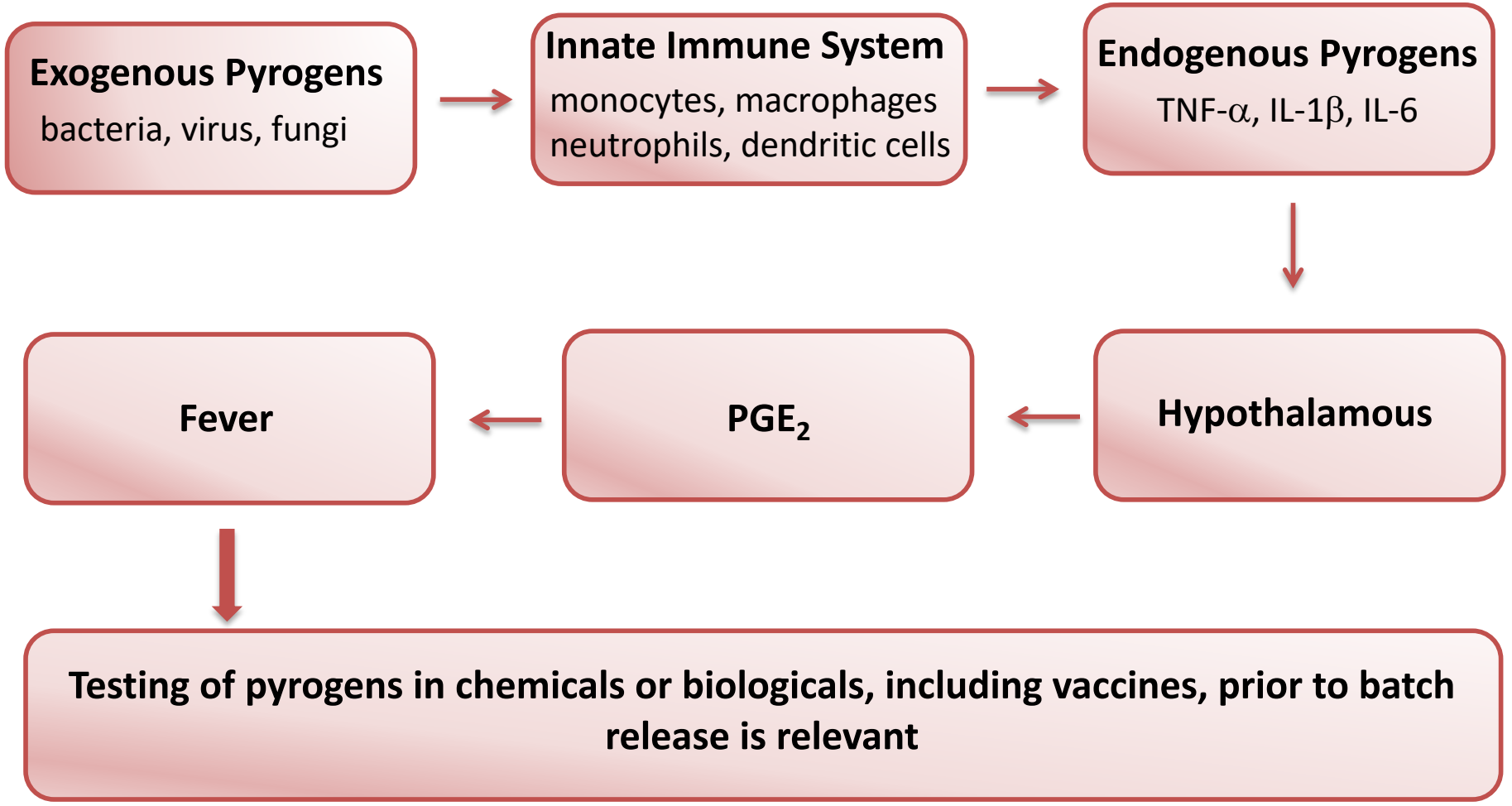
Pyrogen are fever inducing agent - metabolic products of Microorganisms.

- Soluble and Filterable
- Thermostable and non Volatile

- Endogenous (inside body) - produced by the immune cells that are activated by the presence of infectious agents (e.g. bacteria, viruses). Endogenous pyrogens are usually cytokines, such as interleukin-6, interleukin-1, tumor necrosis factor, interferon-alpha, gp130 Receptor Ligands, etc.

- Exogenous (outside body) - foreign substance that are derived outside the host (lipopolysaccharides and other substances produced by pathogenic microorganisms). These substance becomes pyrogens when they are administered parenterally to the host.

1.1 Pyrogens: what they trigger



Courtesy of Dr. Marilena Etna – ISS (Italy) – 1st 3Rs WG Meeting, Bangkok, 2019

1.2 Rabbit Pyrogenicity Test

<u>Qualitative measurement of endotoxin and non-endotoxin pyrogens</u>

"The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined" (Chapter 2.6.8 Eur. Ph.).

The development of the test for pyrogens dated back to the 1920s

A pyrogen test was introduced into the USP XII (1942)

1.2 Rabbit Pyrogenicity Test

Other species not predictable

Rabbit chosen for economic purposes

Similar threshold pyrogenic response to humans

Reproducible (often) pyrogenic response

1.2 Rabbit Pyrogenicity Test

- Rabbits must be healthy and mature New Zealand or Belgian Whites
- Either sex may be used
- Must be individually housed between 20 ° and 23°C (no variations more than ± 3º C).

- Free from disturbances likely to distress them.
- Equipment and material used in test (glassware, syringes, needles etc), free from Pyrogens by heating at 250 °C for not less then 30 minutes or any other method.

- Retaining boxes
- Thermometers or thermistor probe (standardized position in rectum, precision of ± 0.1°C).

1.2 Rabbit Pyrogenicity Test

Preliminary test (Sham Test)

- Intravenous injection (rabbit's ear) of sterile pyrogen-free saline solution
- Warm the pyrogen free solution up to 38.5ºc to exclude any animal showing an unusual response to the trauma of injection
- Any animal showing a temperature variation greater than 0.6°C is not used in the main test

1.2 Rabbit Pyrogenicity Test

Main test

- group of 3 rabbits
- preparation and injection of the product:
  - warming the product
  - dissolving or dilution
  - duration of injection: not more than 4 min
  - injected volume: not less than 0.5 ml per 1 kg and not more than 10 ml per kg of body mass
- determination of the initial and maximum temperature
  - all rabbits should have initial temperature: from 38.0 to 39.8°C
  - the differences in initial temperature should not differ from one another by more than 1°C

1.2 Rabbit Pyrogenicity Test

Interpretation of the results

- the test is carried out on the first group of 3 rabbits; if necessary on further groups of 3 rabbits to a total of 4 groups, depending on the results obtained

- intervals of passing or failing of products are on the basis of summed temperature response

Minor differences depending on the various Pharmacopoeias

- https://apps.who.int/phint/pdf/b/7.3.5.3.5-Test-for-pyrogens.pdf

1.3 Bacterial Endotoxin Test

Also known as Limulus amebocyte lysate test (LAL)

It measures the concentration of endotoxins of gram-negative bacterial origin reagent: amoebocyte lysate from horseshoe crab, *Limulus Polyphemus or Tachypleus tridentatus).*

It became an alternative to RPT in the 80s (USP 1980, FDA 1987, EU. PH. 2.6.14 - 1988, BP 1989).

There are 3 techniques for this test:

- gel-clot technique, which is based on gel formation;
- turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate;
- chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen complex.

https://www.who.int/medicines/publications/pharmacopoeia/Bacterial-endotoxins_QAS11-452_FINAL_July12.pdf

1.3 Bacterial Endotoxin Test

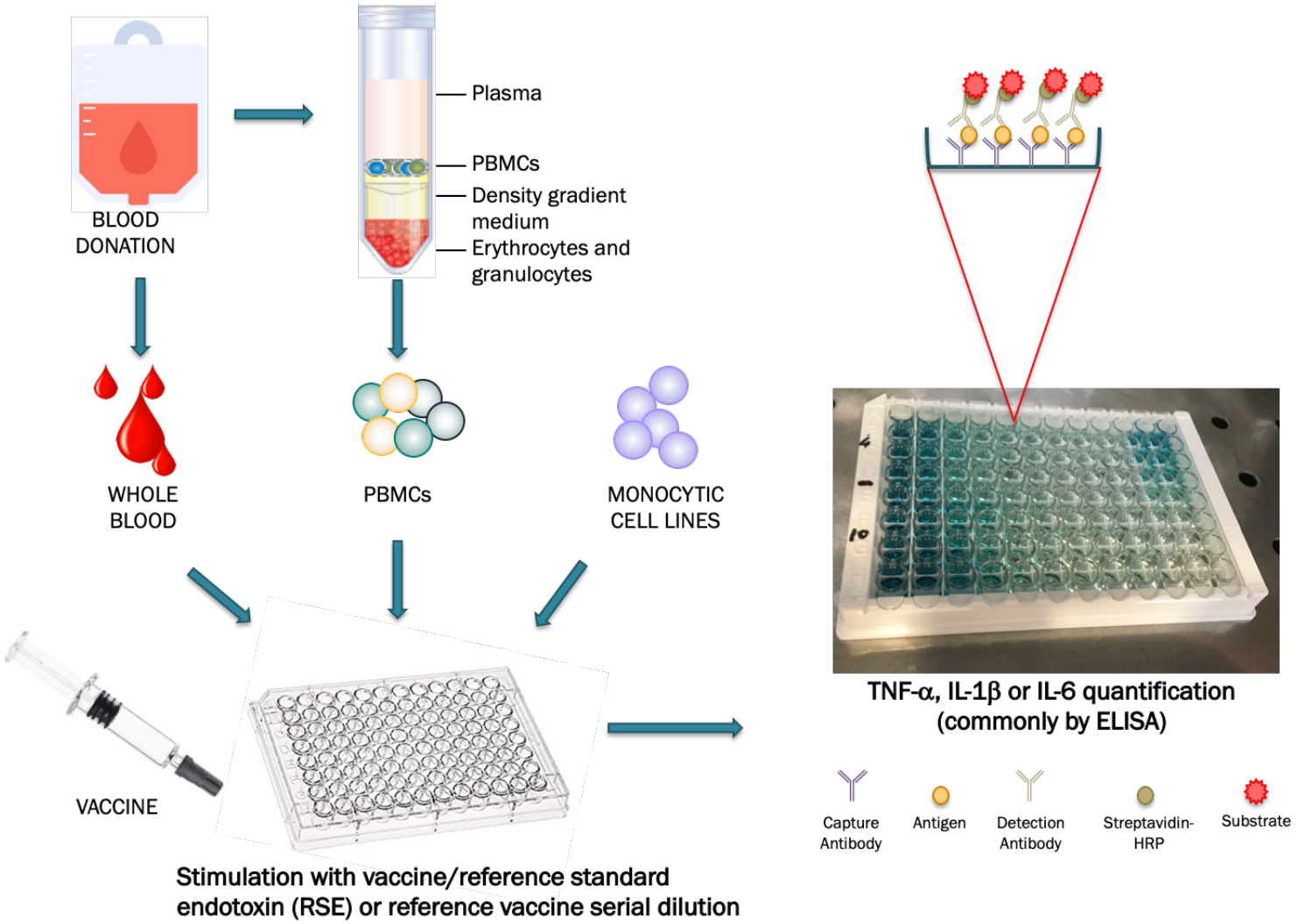
- The addition of solution containing endotoxin to a solution of lysate produce turbidity.
- The rate of reaction depends upon concentration of endotoxin , the pH and the temperature.
- The endotoxin reference standard is the freeze dried.
- The test is based on the primitive blood-clotting mechanism of the horseshoe crab

1.4 Monocyte Activation Test

Semi-quantitative/quantitative measurement of pyrogens.

"The MAT is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example TNF-α, IL-1β and IL-6." (Chapter 2.6.30 Ph. Eur.).

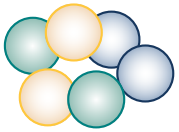
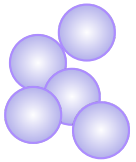
1.4 Monocyte Activation Test



Courtesy of Dr. Marilena Etna – ISS (Italy) – 1st 3Rs WG Meeting, Bangkok, 2019

1.4 Monocyte Activation Test

CELL SOURCE FEATURES

 WHOLE BLOOD	 PBMCs	 MONOCYTIC CELL LINES
[POLIMORFONUCLEAR AND MONONUCLEAR CELLS]	[MONONUCLEAR CELLS]	[MONO-MAC-6 AND THP1]
Donor variability	Donor variability	Very low variability
For unspecified pyrogens	For unspecified pyrogens	For known pyrogens
Presence of cytokines and antibodies in plasma	Basal activation due to PBMC isolation procedures	

1.4 Monocyte Activation Test

EU.PH 2.6.30 Chapter – Currently under review

METHOD A QUANTITATIVE TEST →

Method A involves a **comparison of the product examined with standard endotoxin dose-response curve**. The contaminant limit concentration (CLC) of the preparation being examined is to be less than the contaminant limit concentration to pass the test.

METHOD B SEMI-QUANTITATIVE TEST →

Method B involves a **comparison of the product examined with standard endotoxin**. The contaminant concentration of the **product** is to be **less than the CLC** to pass the test. The highest product concentration must be chosen for the pass decision, unless otherwise justified and authorized.

METHOD C REFERENCE LOT COMPARISON →

Developed to address extreme donor variability in response to certain product containing high level of pyrogen contaminants. Method C involves a **comparison of the preparation being examined with a validated reference lot of that preparation**.

1.4 Monocyte Activation Test

RPT: multivalent DTwP-HepB vaccine, vaccines against HepB, rabies, TBEV, pneumococcal and meningococcal polysaccharide vaccine;

MAT: *Neisseria meningitidis* group B vaccine (BEXSERO®); Salmonella vaccine (Typhim Vi® - ANSM communications to OMCL annual meeting – Sarajevo 2018);

<u>MAT is not applied so far for the batch release of other vaccines</u>

1.4 Monocyte Activation Test

Although the MAT has been well accepted in Europe, FDA and USP position remains vague (c.f. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-pyrogen-and-endotoxins-testing-questions-and-answers)

MAT acceptance by other pharmacopoeias is not too far:

- China has announced MAT implementation in the Pharmacopeia for 2020;
- Health Canada and the National Institute of Health Science in Japan are on the way.

1.4 Monocyte Activation Test

MAT is a non-animal alternative to the RPT, and it allows the testing of human vaccine in human setting. It requires a product-specific validation.

Since RPT was originally developed to test pyrogens in parenterals the method is not appropriated for testing pyrogens in intramuscularly or subcutaneously administered vaccines (dilution is needed).

MAT execution (from purchase of material/animals to data report) is quicker with respect to the RPT. Although MAT incubation time (22 ± 2 hours) is longer than RPT one (3 hours), it allows the detection of delayed inflammatory response.

MAT sensitivity could be adjusted to face the heterogenicity of vaccine formulation: ranging from the possibility to chose between primary cell or monocytic cell to three different methods of analysis. MAT could be a useful tool during development of the production process (R&D), manufacturing process or for batch release to rule out the presence of endotoxin and non-endotoxin pyrogens in vaccines.

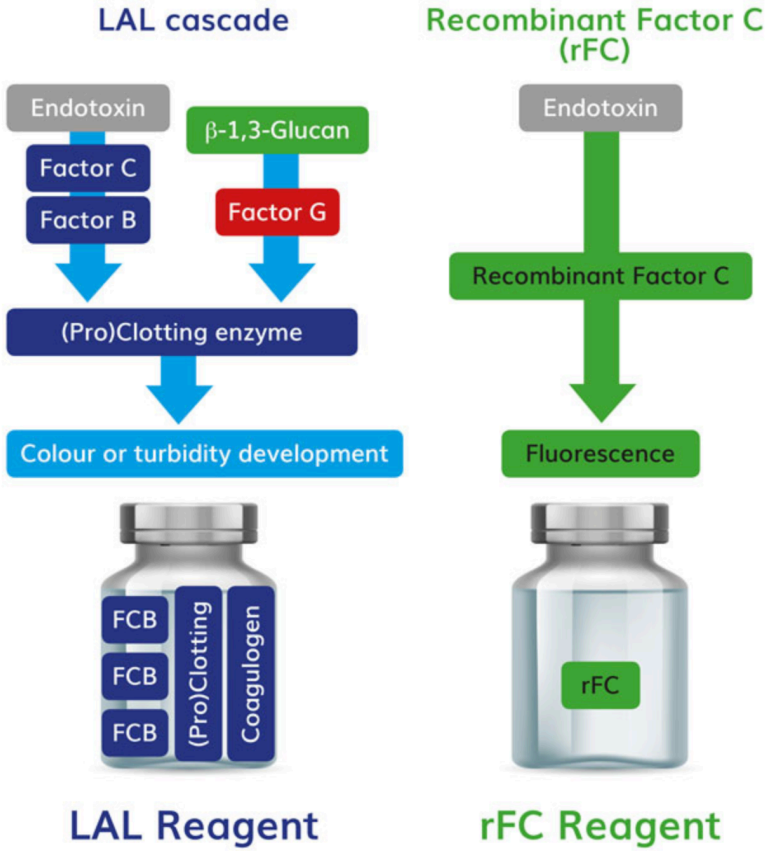
1.5 Recombinant Factor C

Quantitative measurement of endotoxin.

The test is used to quantify endotoxin from gram-negative bacteria by mean of a non-animal-derived reagent namely Recombinant Factor C (coming soon as chapter 2.6.32 Ph. Eur.).

1.5 Recombinant Factor C

- rFC is is a recombinantly manufactured protein used for the detection of bacterial endotoxins
- It is the synthetic alternative of the horseshoe crabs blood that eliminates the need for animal products in endotoxin detection.
- Commercially available rFC tests detect endotoxins with results equivalent to or better than LAL, regardless of which company manufactured it.
- EU and US approved some commercial kits.



Saving the horseshoe crab: A synthetic alternative to horseshoe crab blood for endotoxin detection. Tom Maloney, Ryan Phelan , Naira Simmons. PLOS Biology | https://doi.org/10.1371/journal.pbio.2006607 - October 12, 2018

1.6 Advantages: Use of Alternative Methods

- The BET or LAL requires the blood of horseshoe crabs (30% of their of the total hemolymph). The test is fast, circa 2 hours. However the mortality rates of horseshoe crabs is within 10-15% and the increase of the demand is endangering the specie in both USA and China (Hermanns et al., Proceedings of WC8).
- BET/LAL is unable to detect non-endotoxin pyrogen contaminations, so it is not a complete alternative.
- The MAT is the true alternative, and the one of least cost for pyrogen testing (Hartung et al., 2001), detecting LPS, NeP (Nakagawa et al., 2002; Hermann et al., 2001), and mixtures (Kikkert et al., 2007).
- Despite scientific value and lower costs, MAT still sees limited use – even in Europe, where it's part of the EU PH since 2009.
- Recombinant Factor C is another alternative methods for BET/LAL that could reduce by 90% the use of reagents derived from horseshoe crabs by using the synthetic alternative for the testing of water, other common materials used in the manufacturing process, but also biologicals (quantify endotoxins from gram-negative bacteria).

2. Replacement of NIH for Rabies

1. NIH Test
2. History of Replacement Opportunities
3. G-protein ELISA
4. Updates on the BSP-148

2.1 The NIH Test

- Developed by EB Seligmann, Jr. and published in Laboratory Techniques in Rabies (2nd Edition, 1966)

- Adopted for use as the potency assay for first licensed rabies virus vaccines – neural tissue derived
- Immunization of groups of mice (16 – 20 mice per group) with dilutions of test and reference vaccines on days zero and seven followed by inter-cranial challenge with live rabies virus on day 14 after the initial immunization
- ED50 is calculated and potency is determined relative to the standard at day 28

- EU. PH. 0216, WHO TRS 941

2.1 The NIH Test

Reasons for the Replacement

- Time consuming 28 days to complete the test (up to 6 weeks)
- 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. 150/300 animals per test
- Availability of CVS (challenge virus strain)

2.2 History of Replacement Opportunities

- 1966 – NIH potency test defined
- 1984 – Collaborative Study: SRD/NIH potency test – 14 labs, 7 countries
- 1985 – Workshop on NIH potency test – Geneva, Switzerland
- 1991 – Workshop on rabies vaccine potency testing – Malzeville-Nancy, France
- 1992 – Collaborative study: in vitro assays/NIH potency test – 4 labs, 49 lots of vaccine
- 1992 – Collaborative study: calibration of the 5th IS
  - Performed using all available assays – SRD, ABT, EIA, NIH
  - First reference defined using multiple test modalities

c.f. Robin Levis, US FDA/CBER, Implementing non-animal approaches to human and veterinary vaccine testing. Achieving scientific and regulatory success for Rabies and beyond. Bethesda, Maryland : 16-17 October 2018

2.2 History of Replacement Opportunities

- 2000 – Workshop to reinitiate discussion on alternate test development – Bethesda, Maryland
- 2005 – Creation of the EPAA – European Partnership for Alternative Approaches to Animal Testing
- 2010 – Workshop on the consistency control of vaccines – Strasbourg, France. Rabies vaccines were one of the topic vaccines
- 2011 – Workshop on alternate rabies virus vaccine potency test development – Ames, Iowa
  - NIH test should be replaced as soon as possible
  - Extensive discussion on the need (or not) for correlation between NIH potency test and any alternate assay

c.f. Robin Levis, US FDA/CBER, Implementing non-animal approaches to human and veterinary vaccine testing. Achieving scientific and regulatory success for Rabies and beyond. Bethesda, Maryland : 16-17 October 2018

2.2 History of Replacement Opportunities

- 2012 – Workshop to define alternate potency assay, Arcachon, France. Creation of an International Working Group to define a replacement for the NIH Potency Test
  - Sponsored by EPAA and ECVAM with international experts in human rabies vaccines from government, industry and academia.
  - Mission is to define the roadmap and coordinate the replacement of the NIH Potency Test by an in vitro glycoprotein assay

  Outcomes:
  - A sandwich, direct ELISA method was chosen as method to develop as an alternative potency assay
  - Selection of the most appropriate ELISA reagents and methodology were to be determined by a well defined, pre-collaborative study

c.f. Robin Levis, US FDA/CBER, Implementing non-animal approaches to human and veterinary vaccine testing. Achieving scientific and regulatory success for Rabies and beyond. Bethesda, Maryland : 16-17 October 2018.
c.f. Achieving scientific and regulatory success in implementing non-animal approaches to human and veterinary rabies vaccine testing: A NICEATM and IABS workshop report. Biologicals 60 (2019) 8–14

2.2 History of Replacement Opportunities

- 2015 –Workshop #2 to define alternate potency assay, Arcachon, France
  - Review results from the pre-collaborative study and define an implementation strategy for the selected ELISA
  - Working group determined that the Sanofi Pasteur assay was appropriate for further development

- Sanofi Pasteur ELISA mAbs bind to conformational epitopes located on well defined antigenic sites.
- Sanofi Pasteur assay clearly discriminates potent from sub-potent vaccine lots.
- Pre-validation study to work towards goal of defining a replacement assay.

Replacement of in vivo human rabies vaccine potency testing by in vitro glycoprotein quantification using ELISA - Results of an international collaborative study. Morgeaux S, et al., Vaccine. 2017 Feb 7;35(6):966-971.

2.3 G-Protein ELISA

- The EPAA workshop in 2015 selected a quantitative sandwich ELISA method
- 2 monoclonal antibodies
  - for coating/capture : 1112-1
  - for detection : D1-25 *biotinylated
- a reference standard (calibrated in IU)

(in-house reference calibrated vs. WHO 6th IS)

- Recognises most vaccine strains used worldwide for human rabies vaccines (PV, Flury-LEP, PM – from different manufacturers, aGV, CTN, Vnukovo)
- Biological Standardization Program BSP 148

c.f. Jean-Michel Chapsal. DCVMN Workshop: Optimization of vaccines' manufacturing, containers and testing for global supply. Hyderabd, 2018

2.4 Updates on BSP-148

Objective

Evaluation of the transferability and robustness of the Rabies G protein ELISA method that was selected through the EPAA Rabies Working Group Study.

- Propose to the Ph. Eur. Group 15 (human vaccines) to revise the Ph. Eur. texts to include a standardized ELISA

- Propose a global replacement of the in vivo test used for the QC of human rabies vaccines by a standardized ELISA

2.4 Updates on BSP-148

Phase 1: preparatory phase (2019-2020)

- Logistical support for the procurement/testing of additional vaccines (11)
- Commercial distribution of the antibodies
- Capture antibody o Detection antibody
- Production of batches of both mAbs for the exclusive use of BSP148 (2019/2020)

c.f. Jean-Michel Chapsal. IABS Conference Animal Testing for Vaccines. Implementing Replacement, Reduction and Refinement: Challenges and Priorities. Bangkok, 2019

2.4 Updates on BSP-148

Phase 2: collaborative study (2020)

- 11 manufacturers and 20 officials laboratories
- Central distribution: test vaccines, WHO 7th IS, study protocol including ELISA SOP and standard reporting sheets
- Study design: 3 independent assays with selected ELISA
- Optional: “in house” ELISA method (in addition to selected ELISA)
- Central statistical analysis of the datasets

Phase 3: reporting phase (2020-2021)

Data collection and analysis

Symposium (2022)

EU. Pharmacopoeia revision (2022-2023)

c.f. Jean-Michel Chapsal. IABS Conference Animal Testing for Vaccines. Implementing Replacement, Reduction and Refinement: Challenges and Priorities. Bangkok, 2019

THANK
YOU

THANK
YOU