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Substituting *In Vitro* for *In Vivo* Potency and Safety Assays: Science Versus the Fear Factor

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AFSA-HealthforAnimals Webinar
October 8, 2024

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Acknowledgments

European Directorate for the Quality of Medicines (EDQM) Vaccines Working Group (Groups 15 and 15V) for *In Vitro* Substitution (Ph. Eur. 5.2.14) Initiated 2012 implemented in 2018

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Center for Biologics Evaluation and Research (CBER), USA/FDA
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Overview

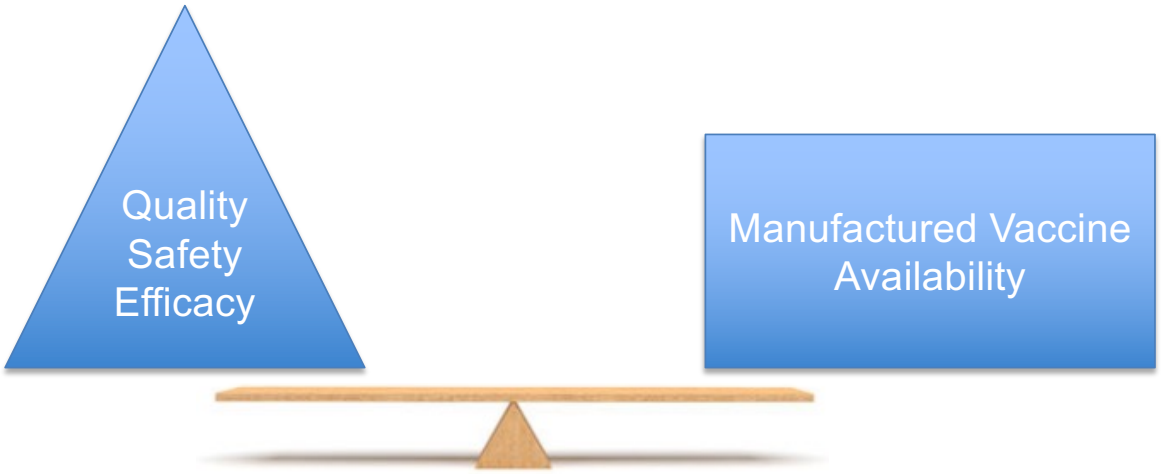
- Barriers to the transition from *in vivo* to *in vitro* assays for key legacy public heath vaccines include the stories we as regulators believe that creates a negative feedback loop with industry.

- If regulators believe that *in vivo* assays are essential to maintain the safety of vaccines, and they are perceived as non-supportive of change, industry will not invest in innovation and new assays, and no change can occur.

- The path forward then is a more science-based, less fear-driven, risk-aware mindset that Ph. Eur. 5.2.14 approaches support.

Key Message: The *in vivo* assay dilemma is resolvable through data driven critical thinking by regulators, as well as by industry, to developed stories that better reflect reality to eliminate the negative feedback loop.

What is the goal … what are the hurdles?



Requires:

- Innovative thinking by regulatory authorities and industry
- Science-based decision making to support manufacturing and testing strategies that can lead to global regulatory harmonization / convergence

Vaccine QC without *in vivo* testing

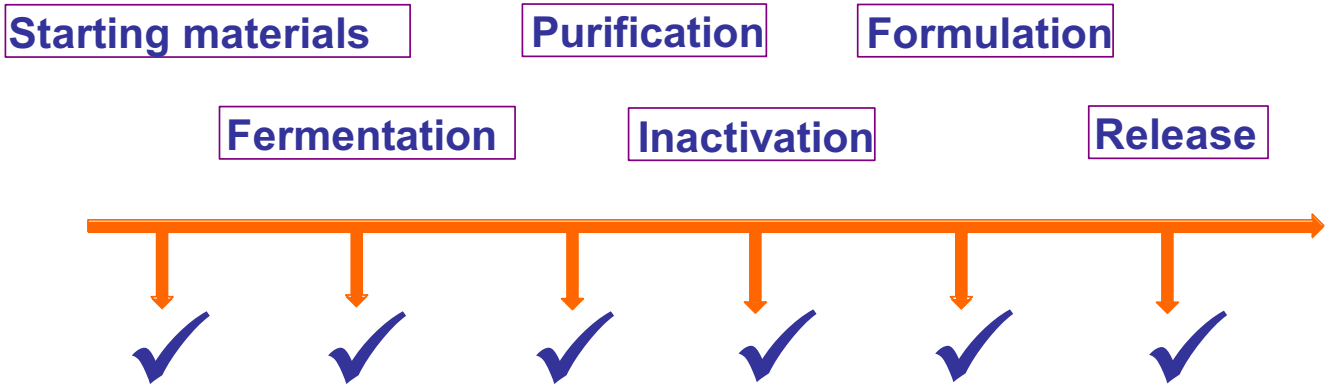
Many vaccines are controlled through production, lot release & stability testing without the use of *in vivo* assays:

- Human Papilloma Virus (HPV) Vaccines; recombinant viral-like particles (VLP) plus adjuvant(s), controlled with physical chemical methods and ELISA
- Meningococcal and Pneumococcal Bacterial Conjugate Vaccines; defined polysaccharides conjugated to carrier proteins, controlled with physical chemical methods
- EMA and North American authorized COVID-19 Vaccines; exclusively use *in vitro* QC assay control strategies regardless of platform (e.g., mRNA, subunit, viral vector, etc.), animal use is restricted to proof of concept and other preclinical studies

Key Message: Modern QC control strategies for vaccines involve a combination of physical chemical & *in vitro* methods to monitor the critical quality attributes (CQA) to maintain the efficacy, safety profile and product shelf-life profile established at licensure.

Quality is built into the process

- Design, development, in-process controls, cGMP
- Consistency monitoring; a vaccine may be tested > 300 times before release



- Approved rabies vaccines in North American and Europe have been manufactured and formulated using *in vitro* assays for decades, and then potency tested with NIH animal assay
- Typically conjugate bacterial vaccine label claims are in µg/mg of defined components (i.e., specific polysaccharides or adjuvant)

Yet *in vivo* testing for vaccines persists

Ph. Eur. 5.2.14 project initiated 2012, because EU 3R regulations did not prevent delays with *in vitro* assay implementation, or result in the deletion scientifically irrelevant *in vivo* tests:

Rabies NIH test	40 yrs. of assay development (e.g., Single Radial Immunodiffusion (SRID), *in vitro* neutralization & stability indicating glycoprotein (GP) ELISA) but no implementation
GST / Abnormal Toxicity	20 yrs. of effort in EU (PEI) for GST deletion but, the GST was still in human. vaccine licences in EU, NA & in WHO / national vaccine guidance worldwide
Pertussis vaccine HIST[1]	20 yrs. no implementation of alternative *in vitro* assays for HIST for Eur. Ph., WHO or national guidance
Toxoid irreversibility tests	Decades of stability data for vaccine toxoid stability, yet *in vivo* irreversibility testing still generally required
DPT[2] potency & safety tests	Lack of progress with over decades with the implementation of *in vitro* methods *via* conventional pathways
Rabbit pyrogenicity	Preferred by most authorities over a monocyte activation test (MAT)

Key Message: A scientific, less animal-centric mindset was needed at an international level to implement alternative *in vitro* assays, required for world markets by vaccine manufacturers.

[1]Histamine Sensitization Test (HIST): to demonstrate absence of pertussis toxin in human pertussis toxoid vaccines

[2]Diphtheria (D), Pertussis (P) and Tetanus (T) combination vaccines

Key limitations of *in vivo* assays

Decades of failed reform efforts prompted the EDQM Group 15 & 15 V to challenge the myths perpetuating the use *in vivo* assays in vaccine QC:

- It's the variability of *in vivo* assays that has resulted in multiple failures of multi-centre international collaborative studies requiring one-to-one comparison, not the more precise and consistent *in vitro* methods (e.g., the fit for purpose alternatives to NIH rabies test).

- Most *in vivo* assays predate ICH Q2 (R1) or VICH GL2 guidelines, yet considered validated since they are compendial. Hence, one-to-one comparisons are challenging, or not possible in some cases because precision, reproducibility, limits of detectability, etc., not established for the *in vivo* method or would be unethical or against EU conventions to do so retrospectively.

Key limitations of *in vivo* assays cont'd

Challenging the myths continued:

- While properly established *in vivo* methods have the potential to measure complex functional responses for demonstrating proof of concept, they **do not** predict the responses in the target population. They are merely, highly variably bioassays.

- Fit for purpose *in vitro* alternative assay QC strategies, using one or more new methods, will likely assess the same quality attribute differently. Hence, the expectation of a one-one agreement between *in vitro* and *in vivo* assays may not be scientifically justified.

- A fit for purpose *in vitro* test strategy can and must provide at least the same confidence regarding the control of the key quality attributes, and case studies have supported this expectation.

A new approach for human and veterinary vaccines

Substitution as an alternative approach for *in vitro* assay implementation:

- Replacement: Involves a one-to-one comparison and establishment of a correlation between the two methods (e.g., *in vitro* to *in vitro* or *in vivo* to *in vivo*).

- Substitution-5.2.14: To facilitate the implementation of *in vitro* methods as substitutes for existing *in vivo methods, in cases* where a typical one-to-one assay comparison is not appropriate for reasons unrelated to the suitability of one or more *in vitro* methods (Ph. Eur. 5.2.14). Assays must be fit for purpose.

- Stability Indicating: Quality parameters (direct or indirect indicators of vaccine efficacy or safety) that are sensitive to storage conditions. These parameters are used in stability studies to assure product quality throughout the shelf-life. Determination of these parameters should result in quantitative values with a detectable rate of change (WHO TRS 999, Annex 5, Vaccine Stability Guidance Definition).

2015 EPAA Workshop impact on Ph. Eur. 5.2.14

EPAA Workshop followed the 2015 FDA deletion of the General Safety Test: Biologicals, 48 (2017), pp. 55-65.

- Industry (**Sanofi**) stressed resource drain with multiple versions of *in vivo* potency and safety assays for several DPT vaccines: month long lot release delays with *in vivo* assays / invalid tests / repeat testing impacting otherwise compliant lots and causing vaccine shortages.

- Germany's authority (**PEI**) presented the clear case for no scientific rationale to retain the so-called General Safety Test (GST). Designed as a phenol test for tetanus antitoxins in the early 1900s, lost scientific relevance for in QC for vaccines decades ago.

- UK's national control laboratory (**NIBSC, now MHRA**) presented *in vitro* (ELISA) results versus *in vivo* potency assays for diphtheria (D) and tetanus (T) products. Demonstrated higher sensitively and improved stability indicating potential of the *in vitro* methods.

The key GST and DT *in vitro* potency conclusions from the EPAA Workshop were presented to Group 15 and greatly helped drive the 5.2.14 development and GST deletion efforts to completion.

Key Message: Joint work by industry and innovative regulators was central to the development of Ph. Eur. 5.2.14, and VAC2VAC (EU industry, academic and regulatory consortium to develop *in vitro vaccine* QC assays.)

What Eur. Ph. 5.2.14 states

- All QC methods "*should ensure comparability of the quality attributes between commercial batches and those batches originally found to be safe and efficacious in clinical studies or, for veterinary vaccines, in the target species*."

- However, "*the inherent variability of in vivo assays can make them **less suitable** than appropriately designed in vitro assays for monitoring consistency of production and for assessing the potential impact of manufacturing changes. As a result, it is essential to continually challenge the scientific value and relevance of these in vivo test methods*."

- "*The use of appropriate in vitro methods … enhances the predictability of the release of safe and effective vaccine lots for use*."

Key Message: Group 15 and 15V moved past the fear related to the loss of animal assays. This was the result of an evidence-based discussion, where long standing beliefs (myths) were challenged and put aside.

Key elements of the Ph. Eur. 5.2.14 approach

- The primary focus for the implementation of any proposed *in vitro* method within a QC system should be the scientific relevance of the *in vitro* assays for control of the critical quality attributes.

- While in the Ph. Eur., *in vivo* assay replacement with *in vitro* assays is typically achieved following multicentre collaborative studies, this should not be a prerequisite for individual products.

- While it may be desirable to have assays that are widely applicable to a class of products, this should not be a requirement.

- In some cases, an existing *in vivo* method may need to be substituted by more than 1 *in vitro* method to characterise the critical qualitative and quantitative attributes measured by the existing test.

Ph. Eur. 5.2.14 approach cont'd

Approaches with specific types of assays are presented in Ph. Eur. 5.2.14

- Potency assays:
  - Design of stability indicating assays, or combinations of alternate methods to capture critical quality attributes (CQA) related to potency is discussed
  - General fit for purpose principles are also discussed

- Safety assays:
  - Considerations for different types of assay are presented for:
    - Specific Toxicity
    - Molecular consistency by Next Generation Sequencing (NGS) versus the neurovirulence test
    - Detection of viral extraneous agents by molecular methods, such as NGS

Progress post-Ph. Eur. 5.2.14 implementation

Rabies NIH Test*	2023 GP ELISA for human vaccine approved to substitute (5.2.14) for NIH test
GST / Abnormal Toxicity*	Removed Ph. Eur., WHO discontinues test from future vaccine & biologics
Pertussis (P) HIST*	Removed Ph. Eur., controlled at DS, *in vitro* test with validation of stable toxoid
PT Irreversibility of toxoid	Removed Ph. Eur., toxoid stable & test not scientifically relevant
Tetanus (T) Specific Toxicity	Removed Ph. Eur., controlled at DS, GP test & validation of stable toxoid
T Irreversibility of Toxoid	Removed Ph. Eur., toxoid stability confirmed, toxin loss at 37º C test condition
Diphtheria (D) Specific Toxicity	Removed Ph. Eur. with validation of stable toxoid (no *in vivo* test for toxicity)
Rabbit Pyrogenicity Test (RBT)	Draft Gen. Ch. 5.1.13 Pyrogenicity, supports suppression of RBT, “suitable” tests for BET in Ph. Eur. 2.6.14 or 2.6.32, or non-BET MAT Ph. Eur. 2.6.30
MAT-Inherently pyrogenic vac.	Ph. Eur. Gen. Ch. 2.6.40

Progress post-Ph. Eur. 5.2.14 Implementation cont'd

Adventitious Agent Testing*	Ph. Eur. Gen. Ch. 2.6.16 Tests for extraneous agents in viral vaccines for human use
	Ph. Eur. Gen. Ch. 5.2.3 Cell substrates for production of vaccines for human use
	Draft Ph. Eur. Gen. Ch. 2.6.41 High-Throughput sequencing for viral extraneous agents
DT Potency & Safety Tests*	*In vitro* assays in development through VAC2VAC* consortium in consultation with EDQM and EMA in process
QC for COVID-19 Vaccines	Currently authorized vaccines in North American and EU use only *in vitro* QC methods (while not linked to Ph. Eur. 5.2.14, but consistent with the same principles)
WHO "5.2.14-like" TRS*	WHO Drafting Group initiated by WHO ECBS, based on recommendation of NC3Rs report and the success of Ph. Eur. 5.2.14

Post-5.2.14 updates: Histamine Sensitization Test (HIST)

A brief history of the HIST for the detection of residual pertussis toxin (PTx) bacterial vaccines

- Introduced: Japanese Pharmacopeia in 1981 and in 1991 to the Ph. Eur..
- Basis of the HIST test: Mice naturally resistant to histamine, but exposure to *B. pertussis* decreases LD50 to histamine up to 300 fold, purified PTx acts similarly.
- Lethal end point and temperature change methods protocols: Groups of mice injected with different doses of PTx or test vaccine, after 4 to 5 days animals are challenged with histamine, mortality or temperature change is the read out. HIST LOD is 1-2 IU / dose. At least 6 different international protocols are described for various jurisdictions (e.g., Canada, China, EU, Japan, USA and WHO).

Situation: As with all *in vivo* tests, the HIST is a highly variable assay, that can result in false positive and inconclusive tests, which can delay lot release, and cause product shortage. A more sensitive and reliable *in vitro* currently is currently in use, yet the HIST persists due the belief by regulators that the *in vivo* test is central to maintaining the safety of pertussis toxoid childhood vaccines.

HIST cont'd

Considerations for *in vitro* assays to the HIST:

- Several methods developed for adjuvanted final bulk / final product
- One considered for Ph. Eur. with LOD like HIST (1-2 IU/dose)
- However, if pertussis toxoid is stable (no reversion), has a consistent manufacturing record, is final bulk / final product testing required?
- PTx has no defined LD50 in humans, unlike T & D toxins
- Rat and Mouse PTx LD50 2,000 -17,000 IU/kg body weight & PTx in whole cell pertussis vac 100-350 IU/mL
- Validated drug substance (DS) in process test with lower LOD than the HIST is in general use

Chinese hamster ovary (CHO) cell PTx *in vitro* DS test: Described in 1983, CHO cells cluster (but do not die) in the presence of PTx. LOD approximately 0.006 IU/dose. Manufactures use validated CHO method for PTx detection at DS post-detoxification, prior to adjuvant addition (adjuvant toxic to CHO cells)

Key Message: For non-reverting pertussis toxoids, validated CHO PTx *in vitro* assay at DS post-detoxification is sufficient. Hence, HIST could be deleted if the above conditions were met.

Detailed HIST presentation by Richard Isbrucker: "Testing for pertussis toxin in aP containing vaccines: a bit of HISTory", NC3Rs Workshop: Implementing the 3Rs in WHO biologicals guidelines, September 19-20, 2023: https://vimeo.com/873737045/14addd2473?share=copy

Sanofi's rabies G protein 1112-1 mAb ELISA is a more robust stability indicating assay relative to the NIH test

- **Out of Specification (OOS) vaccine obtained by thermal degradation**
  - Batch status regarding respective acceptance criterion

VERORAB™ The Rabies Vaccine You Can Trust

Method	Acceptance criteria	Assay	Intact vaccine	Vaccine treated	50/50 Spiked vaccine
ELISA UI/dose (IC 95 interval) 	[1,9 – 4,3] UI/dose	#1	**3,3** (3,1 – 3,6) ✓	< LLOQ ✗	**1.6** (1,5 – 1,7) ✗
		#2	**3,2** (3,1 – 3,3) ✓	< LLOQ ✗	**1.6** (1,5 – 1,7) ✗
NIH UI/dose (IC95 interval) 	≥ 2,5 UI/dose	#1	**8,1** (3,6 - 19,9) ✓	< LLOQ (*) ✗	**7,5** (2,8 - 21,9) ✓
		#2	**11,7** (5,5 - 24,0) ✓	< LLOQ (*) ✗	**4,9** (2,4 - 10,1) ✓

(*) some mice survived in both NIH assay. ***Data from:*** *A. Toinon – Sanofi Pasteur –- WC11 conference – NIH replacement for human Rabies vaccine*

The proposed acceptance criteria for the *in vitro* potency test allows more precise & accurate discrimination of OOS batches than NIH *in vivo* potency test. Hence, there is no scientific rationale to maintain *in vivo* tests for post-manufacturing change re-validation.

Next steps

- As per Ph. Eur. 5.2.14, Group 15 and 15V will continue to examine the scientific rationale for existing *in vivo* potency and safety assays with a more informed and science-based approach.

- With the increasing acceptance of the Ph. Eur. 5.2.14 principles by regulators and WHO, manufacturers are more likely to develop *in vitro* methods for vaccine characterization, in process control and QC release assays, as substitutes for existing *in vivo* methods for legacy vaccines.

- Global acceptance of appropriately developed fit for purpose *in vitro* QC control strategies is essential, given the word market for vaccines.

- The use of non-animal vaccine characterization and QC strategies for rapid development of the COVID-19 pandemic vaccines further demonstrates the value of this approach.

Key Message: Ph. Eur. 5.2.14 approaches will continue to accelerate of the transition from *in vivo* QC assays to more effective and robust *in vitro* alternative methods for vaccines.

Thank-You!

Questions?