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Alternative potency tests for quality control of immunobiologicals: a critical review of the validation approach

Testes de potência alternativos para controle de qualidade de imunobiológicos: revisão crítica da abordagem de validação

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ABSTRACT

Introduction: In addition to low reproducibility, *in vivo* potency tests used in the quality control of immunobiological products require too many animals, causing them significant pain and suffering. In the last decades, many studies have been conducted to validate alternative methods for quality control and batch release of products such as vaccines and other immunobiologicals, especially for potency tests. **Objective:** To discuss validation studies on alternative methods proposed for replacing the *in vivo* potency tests and the used statistical approach, as well as to propose harmonization of terminology and to design validation studies for alternative potency methods. **Method:** A review of scientific databases was carried out to compile the products, data on the validation procedures and to verify their inclusion in the pharmacopeias. **Results:** Four trials were incorporated into the pharmacopeias. Statistical approaches included mainly regression assessment, ANOVA and Chi-square test. **Conclusions:** It is a challenge to conduct appropriate validation studies that are widely accepted by regulatory authorities, especially where validation centers have not yet been established. A clear indicator of this difficulty was the low number of methods for biological products incorporated into the guidelines.

KEYWORDS: Alternative Methods; Immunobiologicals; Potency Test; Validation Approach

RESUMO

Introdução: Os ensaios de potência *in vivo* utilizados no controle da qualidade de imunobiológicos requerem o uso de muitos animais, e além da baixa reprodutibilidade, causam dor e sofrimento significativos. Nas últimas décadas, muitos estudos foram desenvolvidos para validar métodos alternativos para o controle da qualidade e liberação de lotes de produtos como vacinas e outros imunobiológicos, especialmente para os testes de potência. **Objetivo:** Discutir os estudos de validação sobre métodos alternativos para substituir ensaios de potência *in vivo*, a abordagem estatística utilizada e propor a harmonização da terminologia e o desenho para os estudos de validação de métodos alternativos de potência. **Método:** Uma pesquisa de revisão foi realizada em bases de dados científicos para compilar os produtos e dados dos procedimentos de validação, verificando sua inclusão nas farmacopeias. **Resultados:** Quatro ensaios foram incorporados em farmacopeias. As abordagens estatísticas incluíram principalmente a avaliação da regressão, ANOVA e teste de Qui-quadrado. **Conclusões:** É um desafio realizar estudos de validação adequados que sejam amplamente aceitos pelas autoridades reguladoras, especialmente onde os centros de validação ainda não foram estabelecidos. Um indicador claro dessa dificuldade foi o baixo número de métodos para produtos biológicos incorporados nas diretrizes.

PALAVRAS-CHAVE: Métodos Alternativos; Imunobiológicos; Teste de Potência; Abordagem de Validação

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INTRODUCTION

Several tests required by the regulatory agencies and guidelines for ensuring product efficacy and safety use too many animals causing them significant pain and suffering[1,2,3,4,5,6,7,8,9,10,11,12,13]. It has been estimated that about 10 million laboratory animals are used in the industry and quality control of biologicals worldwide yearly, of which 80% is required for testing safety and potency in batch release[14].

In the traditional batch release approach, the general basis for quality testing consists of demonstrating the consistency of production using analytical methods. However, originally, a different paradigm was applied to vaccines, which are complex immunobiological products containing antigens, adjuvants, excipients, and preservatives, and the batches were considered as unique products. For this reason, regulators required extensive quality control testing of each batch of a licensed vaccine so that lot-to-lot safety and potency were tested usually on animals[15].

Unlike vaccines with viable agents that are evaluated by *in vitro* titration, *in vivo* potency assays are required for each batch of inactivated vaccines. Generally, the classical potency tests of inactivated vaccines are based on vaccination followed by a lethal challenge against a standard agent or toxin[16,17,18,19]. These tests are well known for the large number of animals needed, long duration, high variability, and the issues to reach the assay acceptance criteria[20,21].

The consistency of production approach proposed as a new quality control concept for vaccines is considered a paradigm shift[14]. In this approach, quality control consists of using a set of parameters to determine a product profile, which is monitored during production, ensuring that each batch is similar to the manufacturer-specific vaccine of proven clinical efficacy and safety[22].

In the last decades, many studies have been designed to validate alternative methods applied to control and batch release of biological products, especially for potency tests. Despite the possibility of using alternative assays, vaccination-challenge (VC) assays are still widely used[12]. The academic and compendia literature proposes several *in vitro* assay alternatives to analyze the efficacy and safety of immunobiologicals that require analytical validation. Among their advantages are test duration, better reproducibility, low-cost animal tests, and the fact that they are subject to methodological validation, which has a positive impact on the quality control routine.

Validation of alternative methods

Validation is a study whereby the reliability and relevance of a method or process are established for a specific purpose[1,9,23,24]. Vaccine potency assays are typically based on: 1) tested vaccine type and 2) specific details of the analytical procedure, in which potency may be expressed as antigen content or, more typically, as biological activity. The methods available include assays based on animals, cell cultures, biochemistry, and receptor-ligand binding in some cases[14,25].

Similar approaches have been applied to the vaccines available in the market, especially those purified and within the scope of the Q6B guidelines[26], while vaccines consisting of proteins or well-characterized peptides are explicitly included in the Q5C guidelines[27] of the International Council for Harmonisation (ICH).

Good manufacturing practices (GMP) and validation

The industry must demonstrate product safety and effectiveness before regulatory agencies such as the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) approve the new drug[28]. Additionally, manufacturing regulations such as the US Current Good Manufacturing Practice (cGMP), which introduced process validation requirement back in the 1980s, also stipulate that modern standards be adopted in the design, monitoring, and control of manufacturing processes as well as facilities to ensure a consistent supply of high-quality products[29].

The FDA reviewed risk analysis, adequacy, and policy inspections so that chemical analytical, manufacturing, and control (CMC) reviews are focused on risk issues. The initiative was guided by a vision of a more efficient, agile and flexible pharmaceutical industry while producing high-quality drugs without regulatory overstatement[30]. The most notable rules resulting from these changes were the publication of ICH-guide documents (ICH Q8 [R2], 8, 9 and 10) related to pharmaceutical development, quality of both risk management and pharmaceutical system[31,32,33]. Other guides establish the requirements to verify compliance with GMP and, provided they are validated, may adopt alternative actions. Validation studies are an essential part of GMP and should be conducted according to pre-defined approved protocols[34].

The methods validated to reduce, refine or replace animal use (3Rs) generally involve those validated by collaborative trials performed by the manufacturer for a particular product, or validated and published by another laboratory. These tests are carried out under the aegis of organizations with this objective while such alternative methods for consistency testing are expected to be accepted by the official control laboratory and manufacturers[8,35,36,37]. The validation process is generally accepted to facilitate and/or accelerate the international (regulatory) acceptance of alternative test methods/approaches[9] since empirical information is generated and/or assessed on reliability and relevance of a test method/approach under standardized and controlled conditions.

Main international organizations involved

The main institutions involved in validation studies, whether by issuing validation guidelines or coordinating studies, include: the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), European Union Reference Laboratory for Alternatives to

Animal Testing – European Center for the Validation of Alternative Methods (EURL-ECVAM), the European Directorate for the Quality of Medicines & Health Care (EDQM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, USA), the Japanese Center for the Validation of Alternative Methods (JaCVAM), the Johns Hopkins Center for Alternatives to Animal Testing (CAAT, USA), the World Health Organization (WHO), and in Brazil, the Brazilian Center for the Validation of Alternative Methods (BraCVAM) and the National Council for the Control of Animal Experimentation (CONCEA).

The EDQM, JaCVAM, BraCVAM, and *Rede Nacional de Métodos Alternativos ao uso de animais* (RENAMA) performed studies to include the successfully validated methods into the pharmacopeia monographs and guidelines. Meanwhile, other studies aim at achieving greater harmonization worldwide to ensure the development and registration of safe, effective, and high-quality medicines, although not directly involved with the validation process, such as ICH.

Also, institutions such as the European Partnership for Alternative Approaches to Animal Testing (EPAA, EU), the Fund for Replacement of Animals in Medical Experiments (FRAME), the Doerenkamp-Zbinden Foundation for Animal-Free Research (GM), the European Consensus Platform for Alternatives - ECOPA), and the Netherlands Knowledge Center on Alternatives to Animal Use (NKCA) are dedicated to research funding and adopting alternative methods.

Validation procedure

In 1998, the ECVAM/EPAA workshop published a report that presented the basic aspects of the validation of alternative methods of vaccine potency tests[38]. After two decades, EMA just published a helpful guideline for implementing 3Rs validated *in vitro* assays[39] and EDQM has included a chapter in the European Pharmacopoeia (Eur. Ph.). The Eur. Ph. Commission added a fourth "R", "Removal", as a strategy to end the unnecessary use of animals by removing the need for regular performance of animal testing proven no longer relevant, which can be deleted without being replaced by another test[40] after scientific scrutiny.

Validation should be considered when developing or modifying validated methodologies. Several studies in the literature report on validating methods for potency determination[1-8,10-13,22,35-38,41-54], describing the approaches to designing validation, acceptance criteria, data analysis and interpretation, and even performance monitoring through quality control[9,38,55,56,57,58,59,60,61,62]. ICH Q2 (R1) is considered the primary reference for recommendations and definitions of validation characteristics of analytical procedures for pharmaceuticals for human use. Typically, accuracy (trueness in International Vocabulary of Metrology - IVM), precision (repeatability and intermediate precision), specificity, detection and quantification limits, linearity, and interval are evaluated. Furthermore, the method must have been qualified[25] before doing a multicenter validation study.

Collaborative studies usually follow a stepwise approach. The number and breakdown of steps depend on the individual case but, generally, include pre-validation steps such as proof of concept and transferability, including rationale and protocol development, and optimization to obtain sufficient specificity, sensitivity, repeatability, and reproducibility. The method is transferred to at least one additional laboratory[63] after establishing proof of concept.

The method is considered validated after determining its reliability and relevance for a particular purpose[38,39,64]. In the study design, the candidate assay procedures are described to allow the necessary conditions for reproducibility and for achieving results within the proposed acceptance criteria[60].

The large-scale collaborative study stage involves many laboratories and includes a range of representative products. At this stage, the protocol, reagents, controls and reference materials should be defined or at least clearly proposed[63].

This study aimed at discussing published validation studies and their statistical approach on alternative methods for potency estimation applied to batch release of biologicals.

METHOD

A review survey on validation studies for potency tests of immunobiologicals was conducted in the following scientific databases: Pubmed, Scientific Eletronic Library Online (SciELO) and *Biblioteca Virtual em Saúde* including Medline, *Literatura Latino-americana e do Caribe em Ciências da Saúde* (LILACS), and Cochrane Library, until November 2018, using the keywords "alternative methods" and "validation" or "validation approach". Papers describing the validation approach for alternative potency tests were included in this study and the other articles were excluded. After finding the scientific articles and validation guidelines, an MS-Excel® spreadsheet was created for compiling the products and validation procedure data, verifying their inclusion in pharmacopeias.

RESULTS

Potency assays

Usually, potency is defined as the ability of a product to generate a particular biological activity that can be quantified[26]. Therefore, potency tests should be designed to measure the relevant biological activity or product-specific property, including the use of a reference standard for comparing and demonstrating batch-to-batch consistency and stability, for the batch release purpose[65]. Of the 2,909 papers found, the selected 22 describe a validation approach for alternative potency tests that replaced, refined or reduced the use of laboratory animals for quality control of immunobiological products. The remaining articles were excluded from the study. Several models were developed as possible alternative methods (Table 1).

Table 1. Studies evaluated according to product, assay, approach, alternative method and validation parameters.

Scientific articles	Product	Assay		Validation			
		Traditional	Candidate	Approach	Parameters	Statistical analysis	Relevant aspect
Hendriksen et al., 1988[1]	NA	VC	ToBI		Reliability Relevance Sensitivity Reproducibility	Linear correlation	It calls reproducibility, which is current repeatability.
Sigoillot-Claude et al., 2015[54]	Anti-rabies veterinary monovalent or combined vaccine	VC	ELISA	Product-specific validation	Feasibility	Linearity (Cochran's and Bartlett's tests) Linear regression	The monitoring during all phases of the product cycle.
Lin et al., 2017[46]	Anti-rabies vaccine	VC	TRFIA		Sensitivity Precision Recovery Linearity Feasibility	Correlation	Preliminary validation of this novel TRFIA for rabies vaccines and the method demonstrated satisfactory results.
Hendriksen et al., 1991[2]	Tetanus toxoid vaccine	VC	ToBI		NI	Correlation Chi-square test	Additional validation is required.
van der Ark et al., 1994[3]	Pertussis whole-cell vaccine	VC	ELISA		Reproducibility	Parallel lines Chi-square test ANOVA Correlation	It calls reproducibility, which is current repeatability. Promising substitute, requiring validation and functional validity studies. Reduces animal use by more than 25%.
Krämer et al., 2009[11]	Anti-rabies vaccine	VC	RFFIT		Correlation study	Pearson's correlation Lin's concordance correlation coefficient	The serological method can be recommended.
Krämer et al., 2013[13]	Anti-rabies veterinary vaccine	VC	Modified RFFIT	*In house* validations	Reliability	Linearity Parallelism Confidence limits	The number of test animals is reduced by up to 85%. The assay is less expensive, easier and faster.
Korimbocus et al., 2016[46]	highly purified F(ab)2 fragments from equine rabies immunoglobulin	NT	Competitive ELISA		Accuracy Precision Linearity Interval	Correlation	Competitive ELISA demonstrated the potential to replace NT and possibly RFFIT for the quantitation of rabies immunoglobulin.
Moreira et al., 2019[66]	Anti-rabies vaccine	VC	Modified RFFIT		Relevance Reliability	Cochran C test Student t-test ANOVA simple linear regression correlation Cohen Kappa coefficient Lin correlation coefficient confidence intervals	The assay was able to distinguish between potent and sub-potent lots. The SPT is a viable candidate for validation and inclusion in pharmacopeias as a reduction and refinement for the NIH test.
Hendriksen et al., 1994[4]	Tetanus toxoid veterinary vaccine	VC	indirect ELISA ToBI HA	Collaborative study	Intra- and Inter-laboratory variation	Correlation Linearity	VC can be replaced by ELISA and ToBI. It is necessary to standardize the HA.
de Kappelle et al., 1997[6]	Pertussis whole-cell vaccine	VC	VC		Reproducibility interlaboratory variation	ANOVA	The international standardization of the protocols is required, variability can be attributed to strain of mice.

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Hunolstein et al., 2008[37]	Pertussis whole-cell vaccine	VC	CHO cell assay ELISA	Collaborative study phase I	Repeatability (intra-assay precision) and intermediate precision (intra-laboratory variation)	Correlation	Promising alternative method for the batch release potency testing of vaccines for which consistency of production has already been demonstrated by the classical VC.
Morgeaux et al., 2017[45]	Anti-rabies vaccine	VC	ELISA		Relevance	Specificity Linearity Accuracy Precision Repeatability	ELISA can discriminate between potent and sub-potent batches. Inherent variability or lack of transferability was not found.
Gross et al., 2009[10]	Human tetanus immunoglobulin	NT	EIA TIA	Collaborative study phase II	Precision, Repeatability and Reproducibility	EIA - parallel lines TIA - 4-parameter logistic curve Lin's correlation ANOVA	Evaluation of parallelism and linearity deviations together with the weighted correlation coefficient.
Rosskopf-Streicher et al., 2001[51]	Erysipela vaccine	VC	ELISA	Collaborative study phase III	Reliability Reproducibility (inter-laboratory variation) Repeatability (intra-laboratory variation)	Correlation ANOVA	Substantial refinement and reduction of the animal number by 80%. It has been included in the European Pharmacopoeia.
RossKopf-Streicher et al., 1999[50]	Erysipelas vaccine	VC	ELISA		Reproducibility	NI	Method is a strong candidate for validation. It uses intra-laboratory reproducibility term as repeatability.
van der Ark et al., 2000[7]	Pertussis whole-cell vaccine	VC	ELISA		Reproducibility Reliability Relevance	Intra- and inter-assay and inter-laboratory precision Correlation	The model is valid for estimating the pertussis potency.
Winsnes et al., 2003[8]	Combined vaccine - diphtheria toxoid component	VC	ToBI *In vitro* Vero cell toxin neutralisation indirect ELISA	Collaborative study phase I, II	Repeatability	ELISA and ToBI - multiparameter logistic curve Cell assay Vero and ELISA or ToBI - parallel lines Correlation	Serological assays may be less problematic than challenge ones. The results obtained recommended to proceed with the study to investigate the reliability of the *in vitro* assays.
Krämer et al., 2010[12]	Anti-rabies veterinary vaccine	VC	RFFIT		Reproducibility Reliability Transferability Adequacy	Wilcoxon-Mann-Whitney's exact test	Significant 3R improvement in the number of animals and refinement. Reduces test time.
Winsnes et al., 2006[36]	Combined vaccine - diphtheria toxoid component	VC	ToBI *In vitro* Vero cell toxin neutralization indirect ELISA	Collaborative study phase III	Repeatability and reproducibility	Correlation	The replacement and possibility of testing both diphtheria and tetanus toxoid potencies serum from the same animals.
Winsnes and Hendriksen, 2000[35]	Tetanus toxoid vaccine	VC	ToBI ELISA	Collaborative study phase I, II, III	intra- and inter-laboratory variation	Pearson correlation	Refinement and reduction the number of animals for batch release.
Hendriksen, 1995[5]	Tetanus toxoid vaccine	VC	ToBI	Case report	NA	NA	Support the investigation of alternatives. Development of guidelines for the validation procedure.

NA: Not applicable; VC: Vaccination-challenge assay; NI: Not identified.
TOBI: Toxin-binding inhibition test; ELISA: Enzyme-linked immunosorbent assay; TRFIA: Time-resolved fluoroimmunoassay; RFFIT: Rapid fluorescent focus inhibition test; NT: Mouse neutralization test; SPT: Serological potency test; NIH: National Institute of Health; HA: Passive hemagglutination test; CHO: Chinese hamster ovary; EIA: Enzyme-linked immunoassay; TIA: Toxoid inhibition assay.

Tetanus toxoids models

Human vaccines

Previous studies have reported a higher correlation degree between the toxin-neutralizing (TN) test in mice and the toxin binding inhibition test (ToBI) than the toxoid enzyme-linked immunosorbent assay (ELISA)[1,2]. These non-TN serological assays have advanced from the results of a collaborative study to determine the potency of tetanus toxoid for veterinary vaccines[4]. Correlations between *in vitro* tests and VC were very good, and have been somewhat better in ToBI than in ELISA.

Information on the intra-laboratory variation of the *in vitro* serological assays (SA) was based on assessing test repeatability and distribution of intra-laboratory precision. The intra-laboratory variation analysis showed that ELISA has better repeatability than ToBI while preliminary information on inter-laboratory variation was considered acceptable. The results justified the extension to a collaborative study for determining the intra- and inter-laboratory variation of *in vitro* assays and conclusion on robustness. Finally, these serological tests concluded to be important to ensure batch consistency, but cannot be used to replace the VC tests for the licensing of new vaccines or the confirmation of potency after modifying significantly the manufacturing processes[35].

In vitro methods were evaluated in a collaborative study for the SA for potency validation of vaccines against diphtheria combined with tetanus toxoid for human use. ELISA or ToBI assays for tetanus serology were performed and compared with the *in vivo* VC assay. In general, the ToBI generated higher potency than the ELISA did as vaccine doses were optimized for the diphtheria component. Unsurprisingly, serum activities vary widely among multipurpose vaccines, raising questions on the use of a parallel line model. The correlation coefficients were considered acceptable and the potency estimated in the challenge assay was similar to that of the ELISA. The data obtained showed that the potency of antitoxin obtained by Vero cell assay and ELISA were highly correlated with potency by neutralization[8].

Although the word "validation" was included in the study title, the authors indicated the need to investigate the reliability of *in vitro* assays, demonstrating that validation itself was not actually performed. However, the clearly reported pre-validation included a detailed description of the study design and its development. In this step, a correlation study evaluated the relevance of the candidate tests. It would also be possible to obtain preliminary reliability data[8].

In the collaborative study on vaccines with diphtheria and tetanus toxoid components, a clear regression can be observed in ELISA and ToBI results. This observation is important because the vaccine doses were optimal for the diphtheria toxoid component. The results revealed that the same sera could be used to determine the potency of both components. The repeatability and reproducibility were generally higher for toxoids ELISA compared to ToBI. The study considered both ELISA and ToBI as valid methods for routine batch release testing of combined tetanus vaccines[36].

Veterinary vaccines

The suitability of *in vitro* SA for testing tetanus toxoid potency of veterinary vaccines was verified by an inter-laboratory validation study. Serum antibody titers from immunized guinea pigs and rabbits were estimated by indirect ELISA, ToBI and passive hemagglutination (HA) assay, compared to NT. Estimated potency showed good agreement, but a significant inter-laboratory variation for the HA, and acceptable for ELISA and ToBI. The results allowed concluding that ELISA and ToBI are valid alternatives, but not the HA test[4].

Tetanus immunoglobulin

Two other *in vitro* alternative models for determining the potency of human immunoglobulin against tetanus were validated. An enzymatic immunoassay (EIA) and a toxin inhibition assay (TIA) showed good reproducibility, precision, and repeatability in an international collaborative study. The methods discriminated between low, medium and high potency were, therefore, considered adequate for the quality control of human tetanus immunoglobulin[10].

The EIA and TIA were submitted to a complementary collaborative study to be validated in high potency products. The assays were able to recognize between low, medium and high potency samples using a precision concept that was understood as reliability since it determined the intra-(repeatability) and inter-(reproducibility) laboratory variations[10].

Pertussis models

A serological ELISA was developed to evaluate the humoral response induced by the whole-cell vaccine as an alternative to the intracerebral challenge model, verifying that mice survival could be predicted by the antibody titers on the challenge day. The vaccine potency results were similar, but reproducibility was better in ELISA. Animal distress levels were lower while reducing by 25% the number of animals used[3].

The Chi-square test was applied to verify the homogeneity of the results; the variance analysis and the regression correlation coefficient were calculated to estimate what the authors defined as reproducibility. Currently, the applicable concept would be repeatability, since it is an intra-laboratory validation[25,67].

A collaborative study was conducted to establish the precision and accuracy of five assay systems. The toxicity and potency of the whole-cell pertussis vaccine were evaluated in mice by the VC test, which showed significant variation and low ability to discriminate different potency levels. Reproducibility was determined by potency estimation in the VC assay. The system data were tested for homogeneity among laboratories by analysis of variance (ANOVA), using mean estimates of the products tested and the mean standard deviation. Intra-laboratory

consistency was also evaluated by ANOVA, as well as the ability of each test system to distinguish between products with different toxicity or potency[6].

Another collaborative study verified the correlation and compared the relevance and reliability of the serological potency test for *Bordetella pertussis*. In the ELISA validation, the intra- and inter-assay, and inter-laboratory precisions were determined by the coefficient of variation (CV%) of antibody concentrations. Intra-assay precision (repeatability) evaluated the differences within or between the plates. The variation within the laboratories was expressed by the intermediate precision (inter-assay variation). The reproducibility (inter-laboratory precision) was calculated by the variation among the participating laboratories. The intra- and inter-laboratory tests had a good correlation in the homogeneity test by Chi-square. Potency values were similar, but ELISA was more reproducible, with a reduced possibility of re-testing due to the smaller confidence intervals. Reproducibility and reliability were determined by the estimated potency analysis (geometric mean, mean variance, and Chi-square p-values). The precision and accuracy concepts were applied as synonyms for reliability (intra- and inter-laboratory precision) and relevance, respectively[7].

A study evaluated two serological methods for potency testing of whole-Cell pertussis vaccines[37], an ELISA to pertussis toxin (PT-ELISA), whole-Cell ELISA (wC-ELISA), and neutralizing antibodies by the Chinese Hamster Ovary (CHO) cell assay. The CHO cell assay was considered reliable due to good repeatability (intra-assay precision) and intermediate precision (intra-laboratory variation). The potency estimated by the VC showed no correlation with PT-ELISA but correlated very well with the wC-ELISA of most of the study samples, showing good intra- and inter-laboratory reproducibility. The assay has shown a good transferability. The SA based on the wC-ELISA is a promising alternative method for potency testing in batch release of Pertussis vaccines for which consistency of production has already been demonstrated by the classical challenge test. However, additional validation data are needed for establishing it as a compendial alternative method.

Diphtheria models

A collaborative study used SA for potency validation of vaccines against diphtheria combined with tetanus toxoid for human use. *In vitro* toxin neutralization assays in Vero cells and ELISA for diphtheria serology were compared with the *in vivo* VC assay in guinea pigs or intradermal challenge[68]. The data showed that the antitoxin potency obtained by Vero cell assay and diphtheria ELISA were highly correlated with potency in the neutralization test. The performed experiments compared potency estimates, and the calculated correlation coefficients demonstrated the similarity between the SA and VC tests. Although the word validation (not performed) was included in the study title, the authors indicated the need to investigate the reliability (intra- and inter-laboratory variation) of the *in vitro* assays[8].

Aiming to complement the previous collaborative study, the relevance and reliability of the Vero cell assay and ELISA were assessed for potency testing of diphtheria combined with tetanus toxoid-containing vaccines. It was also investigated whether sera from the same animals could be used for potency determination of both diphtheria and tetanus toxoid components to reduce the number of animals used. The reliability of the serological assays was investigated by determining test repeatability and reproducibility, which were generally higher in ELISA compared to the Vero cell assay[36].

Rabies immunobiologicals

Vaccines for human use

The challenge test in mice applies to potency testing of inactivated veterinary and human use vaccines[69,70]. Quantification models for the total replacement of animals measure the amount of vaccine antigen or immunogen, including the antibody binding test for rabies (ABT)[71], some ELISA procedures[42-44], and the single radial Immunodiffusion assay (SRD)[72], which is accepted in vaccine batch released for human use[73]. The Eur. Ph. proposes the VC method for human vaccines and, alternatively, a validated immunochemical or serological potency assay[70], including the ELISA to quantify the viral G-glycoprotein in human rabies vaccines without adjuvant[45].

As part of overall efforts to reduce animal testing, three different ELISA for quantifying rabies glycoprotein were evaluated as an alternative to the National Institute of Health potency (NIH) test, and the chosen one is based on monoclonal antibodies specific for the viral G-protein native form. The method, considered specific, linear, accurate and precise, was able to distinguish between potent and sub-potent vaccine batches while agreeing with the VC test satisfactorily. The repeatability, specificity, linearity, and accuracy were evaluated in the pre-validation study. The correlation study showed a good agreement with the NIH test. This ELISA was considered a good candidate and, therefore, selected for a collaborative study[45] that should generate scientific data for supporting the regulatory steps needed for replacing *in vivo* potency tests[47].

Time-resolved fluoroimmunoassay (TRFIA) using specific monoclonal antibodies that only recognized the native, trimeric and immunogenic form of rabies virus glycoprotein preventing the detection of non-immunogenic, soluble glycoprotein in vaccines, was designed to estimate the potency of human rabies vaccines as the glycoprotein content and may be useful for replacing the NIH test. TRFIA showed excellent precision, higher sensitivity, and a much wider detection range compared to traditional ELISA. Although high sensitivity may not be very important for detecting the rabies virus protein, the much wider detection range, excellent precision, and simple operation of TRFIA can greatly save time and workload with many precise and accurate determinations[67].

A SA using the modified Rapid Fluorescent Focus Inhibition Test (mRFFIT) was developed and pre-validated, demonstrating its

relevance, reliability, and good agreement with the potencies determined by the NIH test. The assay was able to distinguish between potent and sub-potent vaccine lots, being a viable candidate for validation as a refinement for the NIH test[66].

Rabies veterinary vaccines

Researchers developed and validated a single-dose serological potency test for the veterinary vaccine that reduced the number of animals and promoted considerable refinement. The comparative study showed a good correlation between the VC test and SA results based on the rapid fluorescent focus inhibition test (RFFIT). The equivalence was evaluated by the Lin correlation coefficient while SA reliability was demonstrated by identifying the vaccines that did not meet the minimum potency specifications[11]. A collaborative study validated this single-dose SA, demonstrating the test reproducibility, reliability, and good intra- and inter-laboratory variation. The proposed method provides a significant improvement over assay repeatability and reproducibility while result transferability and test suitability complemented the validation[12].

The single-dose serological alternative method was included in the Eur. Ph. Monograph 0451 as a refinement method to reduce VC testing on the batch release of veterinary rabies vaccines in mice[69]. This test provides qualitative results but not a potency value. The reliability concept has been applied, even though repeatability was evaluated only when it was not combined with reproducibility[12].

Subsequently, the same research groups developed a serological method in a multi-dose format that allowed determining the potency of vaccines, and provided reliable and more accurate results than the VC test[13].

An ELISA using characterized monoclonal antibodies able to quantify only the trimeric native type of glycoprotein G, the target of neutralizing antibodies, was developed. This assay was shown to work in different steps of the manufacturing process, including the live or inactivated virus and the formulated antigen in the final product. It could be used to follow the batch-to-batch consistency between the various steps of the manufacturing process. A key advantage of this ELISA is its specificity, robustness, and precision[54].

Rabies immunoglobulin

According to international regulations, the quality control of highly purified F(ab)2 fragments produced from Equine Rabies Immunoglobulin (F(ab)2 – ERIGs) requires evaluation of the *in vivo* potency by the NT or RFFIT. A competitive ELISA method (c-ELISA) was developed, validated and evaluated in commercial product lots. The c-ELISA validation has the potential to replace NT and possibly RFFIT for anti-rabies immunoglobulin quantification. A correlation study compared c-ELISA and NT using regression analysis, including ANOVA[46].

Erysipelas model

An ELISA was developed for refining and reducing the challenge-based model for veterinary erysipelas vaccines[49] and, after a pre-validation study[50], an international collaborative study was conducted to determine reproducibility and intra-laboratory precision. The ELISA adequately replaced the VC test while reducing by 80% the number of animals in the potency test[51]. During the pre-validation and validation phases, the transferability was demonstrated by the precision, repeatability, reproducibility and robustness parameters. The validation study confirmed the usefulness of the proposed method for a wide range of inactivated erysipelas vaccines. In 2004, the SA was included in the Eur. Ph.[74].

DISCUSSION

Considerations on validation studies

Lack of Statistics terminology harmonization

Although the main guidelines indicate the same parameters, the used validation terminology differs[75] in several official documents[25,55,56,57,58,59,60,61,62,67,76,77], possibly causing issues in validation studies. Additionally, heterogeneous terminology can be found in the same document sometimes, depending on the section where a particular term is mentioned[75].

ISO 3534-2 clearly differentiate measurements from tests. Measurement is restricted for determining quantities (mass, length, time, velocity), whereas test is used in a broader sense when determining characteristics by measurement or other means such as quantifying, classifying or detecting the presence or absence of a characteristic. ISO defines a test as a technical operation that consists of determining one or more characteristics[77].

The third edition of the IVM states that the basic measurement principles in physics, chemistry, laboratory medicine, biology, and engineering are not fundamentally different. The IVM[67], ISO[75,77], and the European network of analytical chemical measurements (EURACHEM)[62] refer to accuracy as trueness while the best definition is provided by ISO[75] and EURACHEM[62], as the combination of trueness and precision. Trueness is understood as the closeness of agreement between an average value obtained from a large series of results and an accepted reference value, usually expressed in terms of bias[78]. The ICH Q6 (R2) document establishes the bases for validation in the pharmaceutical area but presents misguided terms, and other guides (United States Pharmacopeia; EMA; Brazilian Health Regulatory Agency - Anvisa) follow both the guide and the terms.

Test sensitivity is defined as the ratio of positive samples with a positive result and the specificity to the proportion of negative samples with a negative result. The positive predictive value (PPV) of a test is the proportion of positive results that really are, and negative predictive value (NPV) is the proportion of true negative results[79].

Test accuracy is defined as the ability to provide a measure as close as possible to the assessed substance against the reference value and is associated with a systematic error. Precision

describes the dispersion degree of replicate test results, regardless of reference value and is related to random error[67].

Assay precision expresses the proximity of agreement (dispersion degree) in a measurement series, considered at three levels: repeatability, intermediate precision, and reproducibility. It is usually expressed as variance, standard deviation or coefficient of variation. To determine the intermediate precision, the effects of random events on the analytical procedure precision, such as days, analysts, equipment, among others are evaluated. Reproducibility is tested by inter-laboratory testing, indicated, for example, to include procedures in pharmacopoeias[25,67].

Final analysis

Several studies have been designed to validate alternative methods. The challenge, however, is to conduct an appropriate validation study for providing its regulatory acceptance.

The studies found in this survey are multicenter (collaborative study), internal (in-house validation) and product-specific validation. The goal of these collaborative studies included assessments of test suitability as a valid and reproducible measure of potency, to demonstrate test relevance, reliability, and transferability, aiming at eventual inclusion in pharmacopeias[4,6,78,10,12,51]. Intra-laboratory validation surveys consisted of comparing candidates and traditional methods. The alternative assay validity was evaluated, checking its adequacy and development[1,2,3,11,13,45,46,50,66]. A single survey article discussed the development, validation, and acceptance of an alternative method for vaccine quality control[5]. After being validated in a collaborative study, the alternative method still requires further validation before being transferred/implemented in specific products/laboratories, which has not been mentioned by most of the studies.

Here, we classified the raised studies and identified this transfer step as in-house validation, which varied on a case-by-case basis, supported by data generated by the new method and/or the collaborative study[5,10,14,25,60,62,75].

The need for validating the new methods has become evident through internationally accepted procedures[25] due to barriers for international acceptance and harmonization when applying universal methods for controlling and batch releasing of immunobiologicals. The validation needs to ensure that the method meets the requirements of analytical applications, ensuring the reliability of the results[57].

Often the new methods need to be compared with already established parameters, making the validation procedure very difficult. As an example, some discussions and attempts have already been made to replace the NIH potency test for rabies vaccine[80], however unsuccessfully, mainly due to conservative approaches that require a correlation between alternative and traditional methods that rarely occurs[59,81,82]. A key consideration is that when an *in vivo* test is to be replaced with an *in vitro* test for a given product, the attributes of the product are likely to be assessed differently[63]. Full compliance of potency values cannot be expected due to the high variability inherent to animal experiments, because the tests are based on different readings (for example: survival-death against antibody titers)[13]. Nevertheless, almost all statistical models of the validation studies apply correlation with linear regression to evaluate the similarity of the results and to determine the reliability and relevance of the new methods.

For the Organisation for Economic Co-operation and Development (OECD), the development and validation of toxicity assays have well-known steps. Despite this, differences in the validation processes still hinder the acceptance of the new methods[9], which also appears to occur with immunobiologicals. But the recent publication of guidelines on validating alternative methods with 3Rs published by EMA[39] and Council of Europe[63] should bring logic to the planning of future studies in this area.

Still, no universal agreement has been achieved on the definition of some of the terms used in method validation, but the IVM[67] has been substantially revised, taking into account chemical and biological measurements, and a change to an uncertainty approach in the error estimation measurement[62]. This Vocabulary is intended to promote global harmonization of the terminology used in metrology[67] and its use should be stimulated.

Of the 22 validation approaches, four potency assays were included in pharmacopeial compendia. To compare the results, the statistical studies covered regression evaluation to correlate the methods[1,2,3,4,5,6,7,8,9,10,11,13,46,47,66], including ANOVA[1,3,4,6,7,46,66] and Chi-square test[2,3,7,13].

Proposal for harmonization of terminology and appropriate statistical methods

Some validation processes are performed under the aegis of the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare, and to improve international harmonization, wherever possible, collaborative studies are coordinated with those planned and carried out by the WHO and the FDA[83]. This configuration of the validation process of the alternative methods could be adopted globally to enable the international acceptance of the new methods, including a local/regional organization, a global organization such as the WHO and a third, external one, where the process is centralized. The standardization of testing methods for the quality control of immunobiological products and the development of alternative methods is also a facilitator for the activities of the ICH and the International Cooperation in Harmonisation of Technical Requirements for Registration of Veterinary Medicines (VICH).

In general, collaborative studies are organized as an extension of a previous study, consisting of two parts (parts 1 and 2) that may be subdivided into three consecutive phases (phases I, II and III) allowing interim evaluation of test results and monitoring of study progress. Part 1 includes phases I and II. Pre-validation (phase I) study, performed in few laboratories, indicated that comparable results of the alternative and traditional tests

should be obtained. In phase II, a greater number of other laboratories are involved and more information regarding the traditional assay and the alternative assay is explored. In the light of results obtained in the first two phases, it is recommended to proceed to phase III to investigate reliability of the *in vitro* assays, which includes the collaborative study part 2[8], and sometimes referred to as the feasibility phase[10,12]. However, there is no consensus about organizing the validation phases, since there are studies describing 4 phases, separating the pre-validation step from phase I, which is also called pilot phase[7,35].

Another key point to consider is an appropriate application of statistical methods for validation procedures of alternative potency tests. Table 2 was organized to help designing collaborative studies for validation processes.

Concerning alternative validation methods for the 3Rs purposes, the objectives would be the same as the validations in the ICH framework, since relevance can be the method ability to determine a result that agrees with a reference value, and reliability, a measurement of the dispersion of the results, determining the precision.

When transitioning from an *in vivo* to an *in vitro* based quality control assay system, it is important to understand what *in vivo* assays can and cannot offer. This can be a challenge in some cases as repeated efforts through multicenter international collaborative studies can fail due to the variability inherent to *in vivo* methods. In addition, although having the potential to measure complex functional responses for demonstrating proof of concept, *in vivo* potency assays do not necessarily predict the actual responses in the target population[63].

Potency tests for convencional vaccines are specific to one type of product and use a specific reference that reflects their nature. Nevertheless, having a moderate range, for example: a reference human rabies vaccine produced in cell culture, can be used to test the main classes of products available using the NIH potency assay, whether produced in human diploid cells, Vero cells or chick embryo cells. On the other hand, modern vaccines are increasingly purified and characterized, being tested by *in vitro* potency tests designed during their development and, therefore, dependent on inputs and standards specific for that preparation produced by the manufacturers, hampering evaluation by the National Control Laboratories. Therefore, vaccine potency assays become relatively different with a more restricted approach during the validation of alternative methods, including the generally limited number of products and manufacturers of the same classical vaccine type[23].

Precision is obtained in the reliability test by determining repeatability and reproducibility. This evaluation should include a statistical analysis of intra- and inter-laboratory variability or coefficient of variation analysis. When the proposed assay is mechanically and functionally similar to a validated method with established performance standards, the reliability of the two methods should be compared[9].

Potency must be correlated with efficacy but a potency test does not necessarily need to measure efficacy directly. The test must, however, be capable of detecting batches with activity different from that of a batch, or batches, for which efficacy has been demonstrated. Therefore, two basic validation aspects need to be considered: validation of the correlation with efficacy and of the method itself[38].

The procedures used to demonstrate specificity depend on the intended purpose of the assay. For potency tests, they shall ensure a result that allows an accurate statement of the content or potency of the analyte in the sample. Appropriate identification tests should be able to discriminate positive results compared to a reference material together with negative results[25]. Tests based on specific immunological reactions or effects of inherently specific microorganisms do not need to be assessed for specificity[84].

Linearity should be assessed by visual inspection of a graph as a function of analyte concentration or content. In some cases, before regression analysis, the test data need to be mathematically transformed to obtain linearity between assays and sample concentrations. A minimum of five concentrations is recommended to obtain linearity[25]. In general, potency tests for products such as vaccines do not need to demonstrate the characteristic linearity since the same dose is used always, regardless of age, weight, among others[84].

Table 2. Proposal for designing collaborative studies: validation and stages of the development of potency tests.

Parts	Phases	Development stages	Data-sheet
1	I - Pre-validation	Design	Definition of test characteristics. Description and validation proposal rationale - Regulatory support
	II	Development and Refinement	Reliability - Precision (intra and interlaboratory variation, repeatability and reproducibility) Relevance (accuracy - sensitivity and specificity) - performance evaluation
2	III - Validation	Assay qualification	Determine reliability and relevance Animal welfare considerations (reduction, refinement and replacement) Practical considerations (critical evaluation of strengths and limitations test) Quality considerations (test implementation process)

Source: Adapted from BSP and OECD[9,83].

The specified range is usually derived from linearity studies and established as the doses or concentrations of the tested interval in which the assay has an acceptable degree of trueness and precision or better saying, accuracy[25].

CONCLUSIONS

The configuration of the validation studies should include a local/regional organization, a global organization such as the WHO and a third external one, where the process is centralized with competence in the standardization of biological products. This configuration could be adopted globally to provide harmonization and international acceptance of the new methods.

It is challenging to carry out appropriate validation studies that are widely accepted by regulatory authorities, especially where validation centers have not yet been established. The terminology used is critical and requires global harmonization as well as the application of appropriate statistical methods.

The research, development, validation, and harmonization of alternative control procedures may lead to the reduction, refinement or even replacement of animal use in potency tests of immunobiologicals.

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Authors' Contribution

Moreira WC- Conception, planning (study design), acquisition, analysis, interpretation of results, writing of the work. Machado NS - Acquisition, analysis, writing of the work. Freitas JFS- Acquisition, analysis, writing of the work. Almeida AECC- Conception, analysis. Moura WC- Planning (study design), acquisition, analysis, interpretation of results, writing of the work. All authors approved the final version of the work.

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.