Quality control of vaccines-A journey from classical approach to 3Rs.

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Abstract

Most vaccines for human and veterinary use are complex biological preparations having one or more antigens along with an adjuvant product to improve the immune response against the specific disease. Conventional vaccines have used numerous animals for production and quality compliance before the release of the final product. Animal based potency tests were required to help in ensuring that each batch of vaccine is consistently safe, pure, potent and effective by providing a level of protection as determined in the original efficacy studies. As a result, the levels of pain and suffering in animals were compromised as compared to other purposes of animal experimentation. Therefore, there is a need to identify viable options to replace these methods of vaccine production and quality check without affecting their quality, potency, and efficacy. This review article will highlight the progress and breakthroughs about the achievements of 3Rs (replace, reduce, and refine) principles especially in the development of alternative test models including physio-biochemical, immunochemical and *in-vitro* methods using so- called "consistency approach" in the area of human and veterinary vaccinology. This article also provides a critical review on the various methods used for potency assays and the factors affecting the accuracy of these methods.

Keywords: Potency test, Vaccine, 3Rs.

Accepted on March 4, 2018

Introduction

Vaccines have been proved a wonderful tool of immunization in modern medicine, which saved more, lives worldwide than any other medical product or procedure in past 50 years. Their importance will further increase in coming the days due to more outbreaks of contagious diseases with varied strain and serotypes. According to literature, history of vaccination can be traced back to ancient Greece as far as 429 BC. The Greek Thucydides noticed that people who survived the smallpox plague in Athens did not become re-infected with the disease. Later on, in 900 AD, Chinese were the first to discover and use a primitive form of vaccination called variolation. The aim was to prevented smallpox by exposing healthy people with tissue from the scabs of diseased person. In 1796, a British physician Dr. Edward Jenner discovered vaccination in its modern form and proved to the scientific community that it worked. Subsequently, Royal Jennerian Institute founded in 1803 for the production of the vaccines [1].

Different types of vaccines, classified on the basis of the antigen used in their preparation have been shown in the Table 1 below:

Table 1. *Examples of vaccines by types* .

S No	Type of vaccine	Method of Production	Examples of Vaccines
1	Inactivated (Killed antigens)	Vaccines containing killed microorganisms	Hepatitis A, flu, cholera, plague, Whole-cell pertussis (wP), Inactivated polio virus (IPV)
2	Live Attenuated Vaccines (LAV)	Vaccine contains live organisms that have been weakened to disable their virulent properties	Oral polio vaccine (OPV), Measles, Rotavirus, Yellow fever, rubella, measles, mumps, typhoid, tuberculosis, Bacillus Calmette Guerin or BCG
3	Toxoids (Inactivated toxins)	Vaccines containing inactivated toxic compounds secreted by the organisms	Diphtheria toxoid, Tetanus toxoid (TT)
4	Subunit vaccines (Purified antigens)	These contain part of the virus is responsible for creating disease	Hepatitis B (Hep B), Human papillomavirus, Acellular pertussis (aP), Haemophilus infuenzae type b (Hib), Pneumococcal (PCV-7, PCV-10, PCV-13).

Potency assay is one of the main methods used for assuring the quality of vaccine which is based on the measurement of one or several parameters that have been shown to be related directly or indirectly with product efficacy (the ability to produce an effective level of protection in the target species) [2]. The quality control tests of vaccines have their roots in the work of 19th century scientists Pasteur, Koch, Behring, and Ehrlich. The multi-dilution test design assay with the use of reference preparation which depended upon ED_{50} was introduced between 1930s-1950s. Subsequently, the current *In-vivo* quality control tests for established vaccines have been developed during 1950s-1970s (Kendrick test, NIH, etc.) [3].

The main types of potency tests performed by vaccine manufacturers includes *in-vitro* titration of live organisms), enzyme-linked immunosorbent assays (ELISAs) and *in-vivo* methods involving immunization of small laboratory animals (e.g., mice, rats & guinea pigs) followed by challenge with a toxin/virus/bacteria or titration of immune sera to measure the antibody response. For live, attenuated vaccines, *in-vitro* potency assay is mainly used but it is not commonly used for inactivated vaccines [4]. Since, production of vaccine occurs in batches, there is an obviousness of variation in their characteristics if strict controls are not ensured. Therefore, all the manufacturer and regulatory bodies has duty to formulate quality procedures for potency testing using various *in-vivo* or *in-vitro* assays.

The use of "alternative" methods are generally concerned with the Principles of the 3Rs,- Replacement, Reduction, and Refinement-of animal testing, first proposed by the scientists William Russell and Rex Burch in their book 'The Principles of Humane Experimental Technique' (1959). Subsequently, various regulatory bodies encourages the development of alternative methods with appropriate relevance, supporting data and test method validation to reduce, refine and replace the animal use for vaccine potency testing. The present review article will provide a deep insight in the progress and achievements of 3Rs in the development of alternative test methods along with discussions on the relevant factors that might be responsible for slow progress in the introduction of alternatives particularly test validation and harmonization of Guidelines.

Quality Control of Vaccines

Some methods in biomedical research and testing are used in the modern era of science without undergoing any major change from their early development onwards. This is well applied to potency measurement, especially in vaccines. A test for potency is one of the required listed tests (21CFR610.10) which shall consist of either *in-vitro* or *in-vivo* tests, or both, that have been specifically designed for each product so as to indicate its potency in an adequate manner to satisfy the interpretation of potency given by the definition in 600.3(s). As per 21CFR600.3(s), “potency” is defined as “the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to affect a given result.” [5].

Vaccines are derived from living organisms which can be easily distinguished from chemical pharmaceuticals due to their complex physical, chemical and molecular compositions [6]. This indicates that their characteristics may vary from batch to batch, therefore, each batch is considered to be unique. Moreover, the inherent variability of living organisms, the potential of contamination coming from starting materials and the environment must require strict control measures to ensure product consistency, safety and efficacy of each batch [6].

As per norms, safety, quality, and efficacy of the products are the prime legal responsibility of the manufacturer in the countries where vaccines are manufactured and marketed. These bodies are responsible for the review of licensing applications, lot release, and monitoring the performance of product. Therefore, details of processes by which the vaccine is produced and tested including the in-process and final product testing are the primary goal of the manufacturer [6]. However, accomplishing this goal depends largely on the quality control tests conducted at various critical steps during the production process and application of Good Manufacturing Practices [7,8].

Previously established concepts and methods of quality control were based on the uniqueness of each individual batch of vaccine. The consistency in the production of vaccine means that each batch of the product is of the same quality and is within the same specifications of the batch which has been shown to be safe and efficacious in human trials or in the target animal species. However, a shift in emphasis away from reliance on final product testing will require the development of a control scheme for each product or product class. Therefore, the development and validation of alternative methods based on the principles of 3Rs for potency testing of vaccines to establish consistency in different batches is crucial and of prime importance before the product moves to international markets [6].

Importance of 3Rs in the Field of Vaccinology

The potency test is mainly used for quality control evaluation of vaccines based on an immunization-challenge procedure in laboratory animals. Many attempts have been made to modify these animal models to improve its relevance and statistical significance. However, the principle of immunization-challenge procedure in laboratory animals is still being used in vaccine research and routine lot-release testings. However, these models have proven to be instrumental in scientifically underpinning the correlation between protection of selected vaccine antigens and their efficacy [9]. The quality control evaluations of vaccines require high frequency of tests with large number of laboratory animals. These potency tests are multi-dose models that include a challenge procedure with virulent micro-organisms [10]. As a result, animal suffer substantial pain and distress during the testing period.

Generally, use of laboratory animal can be scientifically justified if the study benefits for public or veterinary health versus distress to animals and costs of experiment. In spite of that, there is a strong feeling in public circles that how the laboratory animals can be replaced, reduced or refined of their use in biomedical research and testing. Although, for the time being, few of the animal models in vaccine research and development are inevitable and irreplaceable, a significant progress has been made in using *in-vitro* pre-screening tests to evaluate immunological parameters. For a long time, *in-vivo* potency tests have been used for routine vaccine lot-release and most of the pharmacopeia relied on direct or indirect-challenge procedure in laboratory animals [9].

Major limitations of the challenge model are that the procedure is expensive and time consuming. It usually takes 2 months for results to come. Moreover, most of the vaccines have shelf life of about 2 years and after this test the vaccine reaches market with only 22 months of life. Potency tests have been designed

to measure the ability of the vaccine to protect against subsequent challenge with the active component responsible for pathogenicity. Besides, use of virulent microorganisms or toxins in *in-vivo* potency assays poses a potential risk to those working in the laboratory. Furthermore, there is striking evidence that some surrogate models for potency testing poorly predict the efficacy of the vaccine in the target species [11]. Therefore, the routine use of these methods has not resulted in the on time release of effective vaccines. Use of *in-vitro* methods might limit the use of *in-vivo* models for quality evaluation of vaccines. Additionally, the animal models have several limitations in respect of their relevance, reliability, costs and moral acceptability.

The 3Rs approach is driven by the scientific limitations of the animal models used in potency test of vaccines. There are few examples like the rabies vaccine potency assay (or NIH test) which has a poor reproducibility and wide confidence intervals [12]. Moreover, these tests have to be repeated to get the valid results. The safety test in quality control of veterinary vaccines is often criticized for its doubtful relevance [13]. In addition, animal use in viral vaccine production is strongly discouraged for safety reasons as the animal cells might be a source for transmission of pathogens. Finally, all alternative approaches have one thing in common that they have ultimately resulted in refinement, reduction or replacement of animals use. Now, there is an increased interest in the development of alternatives to the current *in-vivo* potency tests. Newer models are mainly based on the use of serology instead of the challenge, use of humane endpoints or *in-vitro* antigen-quantification tests. This new avenue in the quality control of vaccines is called the "consistency approach" which is state-of-the-art in quality control of the new-generation vaccines. In this promising approach, a set of parameters are used to constitute a product profile [9]. These parameters are monitored throughout production and aim to demonstrate that each new batch of vaccine produced is of a similar quality to a vaccine batch of the same provenance, and is of proven efficacy and safety. The consistency in this new approach relies heavily on the implementation of quality systems, such as good manufacturing practice and quality assurance. Further, this strategy involves demonstrating consistency by using a battery of immunochemical, physicochemical and *in-vitro* methods [14].

Assessment of the Potency Assay

The safety and potency assessment are the main tests used during vaccine development. Usually a set of 15-20 assay results have to be made available for making quality control and monitoring chart. Therefore, the availability of fast and reliable methods further benefits the existing vaccines by using *in-vitro* alternatives for *in-vivo* safety tests. For example, the use of a PCR based method to measure a virulent poliovirus in oral polio vaccine preventing neuro virulence testing in monkeys [15]. Another example is a very sensitive *in-vitro* vero cell assay for the detection of residual diphtheria toxin described in the Ph Eur. Monograph [16]. Further, safety of a vaccine is ensured by consistency in production and effective post-marketing surveillance.

The available potency assays can be divided into four different categories: challenge test, toxin neutralizing test, cell-based assays and titration assays. The test of potency for live-attenuated vaccines is generally determined by measurement of the number of viable particles, either by colony counting or by virus titration. The use of quantitative PCR (qPCR) has also appeared to be feasible in some cases instead of performing virus titration [17]. The main advantage of qPCR is that it is faster, accurate and reproducible.

Challenge Test

The challenge test has been used for decades for the quality testing of vaccines, still inferior products arrive in the market. Therefore, the interest in new approaches is increasing especially for lot release testing. In safety point of view, it is easy to manage the risks in cell culture lab as compared to Animal Facility. Although all procedures can be performed in safety cabinets and animals can be housed in containment systems (e.g., isolators or individually ventilated cages) but the injection of virulent material or manipulation of contaminated animals exposes technicians to additional risks. Moreover, animal testing is also laborious, time consuming and expensive.

As mentioned earlier most of the vaccines have a shelf life of less than 2 years and a routine potency test requires around 8 weeks, which is undesirable. Nowadays, due to strict ethical regulations, specific housing conditions such as barrier systems and ventilation equipments, the maintenance cost of animals are rising. In most western countries, majority of scientific community is inclined towards non-animal experimentations, especially for those experiments which inflicts severe pain and suffering [18]. These tests mainly based on large numbers of animals, usually over 100 animals per test and in the majority of cases, the animals will show severe clinical signs. As a result policy makers often emphasize the development of new methods based on 3 Rs. Apart from the economic and ethical reasons, there are also scientific reasons to overlook animal based potency tests. In rabies vaccine and whole-cell pertussis vaccine, the results have poor intra- and inter laboratory reproducibility, resulting in repeat tests by manufacturers [19,20].

In challenge test, determining an immune response in animal model as a correlate for protection against a disease would facilitate the rational development of an effective vaccine. However, finding such a correlate can be difficult. These animal models only assessed the interactions of the organism with the innate, humoral and cellular immune system. However, it is extremely difficult to develop animal models strictly for human pathogens such as Neisseria meningitis, due to the specificity of a range of surface proteins that interact with the host receptors [21]. One of the critical steps required for developing an animal model is identification of molecules that play important role in attachment of pathogens to host cells.

Further, the potency tests are questioned in a term of their clinical efficacy like in case of tetanus vaccine. Moreover, studies have demonstrated a lack of correlations with efficacy

in clinical studies [22]. Additionally, some animal models do not mimic the human situation like whole cell pertussis potency testing. In regulatory requirement animals are challenged by the intra-cerebral route and not by the intranasal route, which is the natural port of entry for Bordetella pertussis. Moreover, the potency testing does not provide any information about the effect of booster immunizations or the time interval for weaning of immunity [23].

Transgenic mice expressing human target receptors such as CD46 or CD66 have been developed by using genetic methods and used for measles virus or Neisseria infections [24,25]. In addition, the 'humanized immunodeficient mice', in which human tissue retaining immunological functions is transplanted into mice, can be used. In mice transplanted with human umbilical cord blood CD34+ cells, human immune functions could be reconstructed and studied in the presence of HIV infections [26]. However, the infection induced immune responses (direct challenge procedure) may not always match the protection appeared after vaccination in the laboratory animal model [27].

New version of the in-vivo potency tests

During 1980's, the animal numbers per vaccine was strictly followed as per monograph of pharmacopeia due to prevailing widespread variation in response of individual animals. In the meantime, many factors especially health status, microbiological status of the animals, skills of the technicians, food quality and overall conditions of the Animal facility have been optimized. As a result, there was a reduction in variation in response of testing animals which further allowed a significant in cutback in number of animals per vaccine dilution which was revealed in many studies [28-30].

Based on the outcome of these studies, the WHO and Ph.Eur. have made a slight modification in traditional classical challenge test. These agencies have revised their guidance by indicating that the number of animals to be used should meet the confidence interval criteria specified. Moreover, a single dose potency approach used for diphtheria and tetanus toxoid vaccine in the monograph of Ph. Eur. in place of multi-dose potency test has been allowed further to reduce the number of animals. If there is consistency in vaccine production, the single dose approach is only an option which indicates that the vaccine under study meets the minimum requirement in IU/ml [31].

Humane endpoint and in-vivo potency test

A humane endpoint is the point at which an experimental animal's pain and/or distress can be terminated, minimized, or reduced by actions such as killing the animal humanely, terminating a painful procedure, or providing treatment to relieve pain and/or distress (CCAC 1998) [32]. Humane endpoint can be realized by replacing crude general endpoints such as death or severe clinical findings with more specific local end without compromising of the scientific outcome of the test. The 3R methods have been developed for several vaccines that include a nonclinical endpoint, ultimately resulting in the reduction in number of animals and significant decline in pain and distress. An example is the use of serology in potency testing of tetanus and diphtheria toxoid vaccines. Humane endpoint might be considered as the best approach to limit the level and duration for several potency assays in which replacement of animal is not possible [33].

As per regulatory guideline, new batches of vaccines must be tested to ensure that they are safe and can provide protective immunity. In general, the routine testing typically involves immunization of several groups of animals with different dilutions of the vaccines followed by exposure of the animals to the infectious agent of interest. Animals with insufficient protective immunity develop induced infections. Unprotected animals often develop the disease which is frequently lethal. Although regulatory authorities have in the past typically required death as an endpoint for such studies, some authorities now allow the humane killing of moribund animals (USDA1998b) [34].

Now, most of the pharmacopoeias or guidelines on vaccine quality accept the concept of establishing humane endpoints in vaccine potency testing based on the scientific applicability. According to 9 CFR 117.4 (e) (USDA), "test animals that show clinical signs of illness that are due to the test may be treated humanely, destroyed if the illness has progressed to a point when death is certain to occur without therapeutic intervention." [35,36]. Severe clinical signs or death are the endpoint of potency test in most of the pharmacopeia. The object behind humane endpoints is to reduce the time interval of pain and distress an animal have to suffer by killing the animal in an early stage of disease without compromising the scientific objective of the test.

In May 2012, the USDA Center for Veterinary Biologics (CVB) issued guidance on the use of humane endpoints and methods in animal testing of biological products. CVB Notice No. 12-12 includes specific guidance regarding the use of humane endpoints in biological products testings, including the rabies challenge test. These guidelines emphases on the use of the anaesthesia for intra-cerebral inoculation of mice during rabies vaccine testing and encourages the use of analgesics in animal studies and potency testings when it can be shown that the study outcome is not affected [37].

To set the humane endpoints in vaccine potency testing, identification of parameters which are predictive of death or severe clinical signs are required. Generally, two types of humane endpoints based on clinical signs and pathophysiological parameters (body weight and body temperature) are used. The use of humane endpoints during the potency testing of whole cell pertussis vaccine, human inactivated rabies, tetanus, and diphtheria vaccines has been shown in the Table 2 below:

Table 2. *Examples of earlier humane endpoint for vaccine potency assays*.

S No	Test	Traditional Methods	Traditional clinical observations	Earlier humane end point	Reference
1	Whole cell pertussis vaccine potency testing	Assay in mice uses a virulent challenge by intracerebral route 14 days after immunization with a lethal dose of virulent B pertussis microorganisms.	Piloerection, hunched back posture, apathy, and convulsions to moribund condition and finally death.	Specific decreases in body temperature are effective early predictors of eventual death and increased levels of acute phase proteins resulting from cytokine production during infections	[38]
		Animals are observed for 14 days, and the number of mice per dose group surviving this period is used for probity analysis and estimation of potency.		Hind limb paralysis and a decrease in body temperature to less than 34.5°C, which are predictive of impending death for Pertussis-infected animals,	[39,40]
2	Rabies vaccine testing	The regulatory requirement for a mouse lethality test with a 14-day survival period to be performed on each batch of rabies vaccine [39].	A surrogate endpoint was established for death	Specific weight loss and the presence of specific neurological signs, which are predictive of eventual death in unprotected rabies-infected mice.	[40]
3	Tetanus toxoid vaccine (*Clostridium tetani*) and tetanus component in combined vaccines	Multidilution vaccination challenge on guinea pigs or mice.	Guinea pig or mouse lethal challenge test	Hind leg paresis	[41]
4	Diphtheria component in combined vaccines (*Corynebacteri umdiphtheria*)	Multidilution vaccination challenge on guinea pigs with around 20 control animals.	Guinea pig lethal challenge test	Erythema score following intradermal challenge in guinea pigs	[42]

The use of humane endpoint has been widely accepted in most pharmacopeia, still, regulatory authorities in individual countries might adhere to results obtained by the lethal challenge test. There might be several reasons for reluctance. However, the obstacles are very serious in nature. Therefore, the International acceptance of humane endpoints will require harmonization or mutual recognition of test requirements. The motto of the implementation of humane endpoints in vaccine quality control does not result in scientific benefits or economic profits but is a matter of humane care and ethics. Now, it is time for regulatory authorities to play a key role in encouraging implementation of humane endpoints when reviewing manufacturer's submissions for lot release based on lethal challenge end-points.

In-vivo models of Serological Analyses to Refine Animal Use

A slight modification of the traditional potency assay has been achieved by developing serological assay that measures antibody level or some other aspects of the adaptive immune response instead of direct challenge or *in-vivo* toxin neutralization. In serological methods the amount of protective antibody produced is measured which serves as an indicator of vaccine potency. The procedure is significantly less severe than challenge methods. Nowadays, most of the vaccines licensed today depend on the induction of serum antibodies for their efficacy. For various pathogens particular levels of antibodies have been identified or suggested that confer protection (see the 'Clinical development' section) [43]. Moreover, antibody responses are easy to measure in ELISA or in newly developed multiplex assays analyzing titers for several antigens simultaneously in very small serum volumes [44-47].

Serological methods for several models like clostridial species, diphtheria, pertussis, rabies and leptospiral are available and even validated in large scale through inter laboratory studies organized by the European Directorate for the Quality of Medicines and HealthCare (EDQM) and, in the case of the tetanus vaccine, in collaboration with the EU European Centre for the Validation of Alternative Methods [48,49]. Ph.Eur. has revised their monographs based on the output of these products and now include serology as an alternative to using the challenge procedure. Examples of serological potency assays as 3R alternatives have been shown in the Table 3 below:

Table 3. *Examples of serological potency assays as 3R alternative*.

S No	Vaccines	Animal Model	Traditional Potency test	Serological ELISA Assay as 3 R alternative
1	Tetanus toxoid vaccine and tetanus component in combined vaccines (*Clostridium tetani*)	Mouse/guinea pig	Lethal/paralytic challenge test	Single-dilution immunization and serology[b,c]-*in-vitro* toxin-binding inhibition (ToBIb,[c]), indirect ELISA[b]
2	Tetanus and diphtheria	Guinea pig	Serological test	Single dose

3	Diphtheria toxoid vaccine and diphtheria component in combined vaccines *Corynebacteriumdiphtheriae*)	Lethal/intradermal challenge	Guinea pig	Single-dilution immunization and Serology-ELISA or Vero Cell Assay[b,c]
4	Acellular pertussis component in combined vaccines Whooping cough (*Bordetella pertussis*)	Mouse	Multiple-dilution mouse serologyc	Immunization (mice) and serology[a,b,c] ELISA
5	Rabies (cell culture, human use)	Mouse	Lethal challenge test	Serology
6	Erysipelas	Mouse	Lethal challenge test	Serology
7	Clostridialnovyi (type B)/perfringens/septicum	Rabbit/mouse	Toxin-neutralization test	Serology

[a]Accepted by U.S.regulatory authorities.

[b]Published in the European Pharmacopoeia.

[c]WHO Technical Report Series number and year of publication.

Further, a single-dilution assay can be a valid procedure to demonstrate that a product exceeds the minimal requirement given for potency provided that consistency in production and testing has been proven. Information is presented justifying the use of a single dilution assay based upon quantitative responses for establishing the potency of diphtheria toxoid vaccines. Data of 27 multi-dilution assays on the diphtheria toxoid component of DPT-polio vaccines were retrospectively analysed for consistency in production and testing. Criteria for analysis are given and a protocol for quality assurance of a single dilution assay based upon serology is discussed [50]. However, the use of single dilution assay for the purpose of the batch release is already allowed by WHO. Though, WHO Guidelines should clearly indicate in which circumstances the simplified assay could be used and in which circumstances full assay is required [51].

Besides, Safety, animal welfare (Refine/ reduce), efficiency and test monitoring is very much ensured by ELISA based serological tests if compared to conventional challenge test. In ELISA there is no direct exposure with virulent micro-organisms or toxin in the animal facility as well as with experimental animals, therefore the toxin, pain and distress levels in animals have been reduced from severe to minor. Additionally, the performance time is reduced by omitting the observation period and using a quantitative endpoint (antibody titre) which further allow a substantial reduction in the number of animals required per vaccine dose. Moreover, the serology also offers the storage of test samples i.e. serum material that can be reused for questions coming from post-marketing surveillance or for retesting of samples from vaccine manufacturers by regulatory authorities [52].

Nowadays, combination vaccines are receiving importance because of their continued demand for the protection against multiple diseases, especially in children. However, their potency estimates for each of active compound present a unique challenge. For example, potency testing for the DTaP combination vaccine typically requires separate tests for each of the major components (diphtheria, tetanus, and acellular pertussis). Therefore, combining the individual potency procedures into one serological test for multivalent vaccines would have an immediate and significant impact on reducing animal use.

In-vitro Immunological Tests

Immune response in vertebrate organism is most complex and interactive in nature, involving various cells of the immune system in a cascade of reactions after contact with the antigen. The main determinants in the cascade of reactions are antigen presenting cells (APCs), such as monocytes and dendritic cells, antibody-producing B lymphocytes, helper T cells, killer T cells and memory B and T cells. These cells are further supported by so-called accessory cells (fibroblast, endothelial cells) as well as cell products such as cytokines and chemokines that play an important role in intercellular communications [52].

Today there are several *in-vitro* immunogenicity models are available which range from very simple peripheral blood mononuclear cell (PBMC) cultures to complex co-culture systems including various types of immune cells as well as accessory cells. General conceptions about these models are that they hardly mimic the complex immune response, particularly when correlates of protection for specific immune parameters have not yet been established. However, some have shown good correlation between vaccine quality and cytokine profiles such as stimulation of PBMCs with tetanus toxoid [52].

The major limitations of these models are that they represent only a particular phase during the development of the immune response. Moreover, *in-vitro* conditions (antigen dose, cell density and cell–cell interaction) differ substantially from *in-vivo* situation. In these models, primary responses are difficult to measure due to low number of potentially responsive cells. Therefore, these models at present have been of little help for mandatory required quality control of traditional vaccines. However, the importance is continuously increasing in vaccine development studies that focus on studying a particular aspect of the immune response [52].

Antigen Quantification

Nowadays, some human vaccines do not require the use of animals because *in-vitro* methods have been developed that quantify the presence of the protective antigen. *In-vitro* antigen quantification tests are performed either mainly by determining the number of live particles in case of live attenuated bacterial

vaccines (e.g., Bacillus Calmette- Guérin, typhoid and cholera) or, in case of live viral vaccines, by virus titration in cell cultures using end points such as plaque formation, cytopathology and indirectly by virus neutralization using virus-specific serological reagents.

These tests are considered as state-of-the-art technique for lot-release potency testing of live attenuated vaccines or genetically modified live vaccines based on binding of key protective antigens to specific antibodies in an *in-vitro* immunoassay. At present, these methods represent the most promising *in-vitro* approaches for the replacement of animals used in vaccine potency testing. But these tests have been successfully implemented (regulatory acceptance) for only a few products. These include hepatitis A/B vaccines, inactivated polio vaccine, polysaccharide and polysaccharide conjugate vaccines, and human papillomavirus vaccine as shown in the Table 4 below:

Table 4. *Examples of human vaccine potency tests that replace the use of animals.*

S No	Vaccine	Traditional test procedure	*In-vitro* methods	References for alternative methods
1	Hepatitis A vaccine (hepatitis A virus)	Mouse serology	Antigen quantification,	[53]
2	Hepatitis B vaccine (hepatitis B virus)	Mouse serology	Antigen quantification,	[54]
3	Inactivated polio vaccine (poliovirus)	Mouse serology	Antigen quantification,	[55,56]
4	Human papillomavirus vaccine	Mouse serology	Antigen quantification,	[4]
5	Erysipelothrixrhusiopathiaea (inactivated) (swine Erysipelas)	Immunization challenge test in mice	Antigen quantification-*in-vitro* ELISA	[57]
6	Bovine respiratory viruses (BRV, BVD, PI3, BRSV)a (cattle respiratory disease)		Antigen quantification-*in-vitro* ELISA	[58]
7	Newcastle disease virus (chicken respiratory disease)	Immunization challenge in chickens; serology	Antigen quantification-*in-vitro* ELISA or serology	[59,60]

a-Published in the European Pharmacopoeia.

b-WHO Technical Report Series It.

c-No mouse test in European Pharmacopoeia.

d-Not for routine lot release (Ph Eur).

e-Traditional method is antigen quantification.

f-Accepted by U.S. regulatory authorities.

Currently, *in-vitro* antigen quantification methods represent the most promising approaches for the replacement of animals used in vaccine potency testing. However, there are few limitations in antigen quantification test that it only measures antigen quantity which do not necessarily reflect biological activity. Another limitation for potency testing of inactivated vaccines is that most products include an adjuvant product. These adjuvants cause direct interference with assay (e.g., high background, non-parallelism). Therefore, the adjuvant has to be removed before antigen quantification and also need to ensure that the process used for removal of adjuvant does not affect recovery and/or integrity of the antigen, which could interfere with its detection in the assay [4].

High-Performance Liquid Chromatography (HPLC)/Polymerase Chain Reaction (PCR)

Nowadays, a majority of industries are inclining toward the use of *in-vitro* assays. Therefore, regulators in certain regions have been concerned with assessing the impact of the adjuvant on serial potency. This can be achieved through a variety of physical and chemical assays, including particle sizing, aluminium concentration, HPLC and through following tightly controlled Good Manufacturing Practices (GMP) in production systems and component specifications. Moreover, HPLC as an additional potential assay method has shown outstanding results for the quantification of the new purified peptide or subunit vaccines. But still there is need for further development and validation of this method.

Another method is quantitative polymerase chain reaction (qPCR) which has been proposed as an alternative to potency assay due to its ability to determine the number of organisms based on the nucleic acid copy number. However, since the qPCR does not measure either the expression or conformation of the target antigen, its use is limited to a potential supportive assay in combination with another assay. However, cell cultures based assays are widely accepted. Therefore, the use of cell culture toxicity as a replacement for mouse in clostridium antitoxin determination has also been proposed [61].

Acheviements of 3Rs methods in Vaccinology

In general, the development of an alternative method requires a clear understanding of the purpose for which the assay is to be used. Accordingly, it is important to discriminate between testing procedures for development of novel vaccine, the batch quality control of licensed vaccines and fully established products. Generally, it is possible to use tests which are

different from those used to determine the efficacy of novel formulations for the release of established products. However, these batch release tests should be a subset of those used to characterize the product going into clinical trials. To develop effective and credible alternative methods, it is essential to understand the mechanism of action of vaccine [11].

In vaccines, the relevant and robust assay development generally requires the knowledge of both the mechanism of induction of a protective immune response and the mode of action of the pathogenic entity in causing disease. Moreover, the development of mechanism based assays would also require an understanding of how virulence factors exert their pathogenic effects. Assays developed by following these criteria will lead to the complete replacement of animal models. However, lack of scientific knowledge at present limits the development of such mechanism based assays.

Table 5. *Examples of 3Rs achievements for human vaccine potency tests*.

SNo	Vaccine	Traditional Method	Alternative Method	3R alternates	Reference
1	Japanese encephalitis	mouse immunogenicity assay followed by a Plaque Reduction Neutralization (PRN) Test (PRN Test)	ELISA to determine the E antigen content of the Japanese encephalitis virus	Replacement	[62]
2	Tetanus toxoid vaccines	Lethal/paralytic challenge test in the batch potency testing	ELISA procedure for Batch Potency Testing of Tetanus Vaccines	Reduction (Single-dilution test instead of multi-dilution), Refinement (Endpoint is not death)	[63,64]
			Toxin Binding Inhibition (ToBI) Test for Batch Potency Testing of Tetanus Vaccines	Reduction (Single-dilution test instead of multi-dilution), Refinement (Endpoint is not death)	[65,66]
3	Diphtheria Vaccine	Lethal/Intradermal Challenge Test in the Batch Potency Testing of Diphtheria Vaccine	ELISA Procedure for Potency testing of Diphtheria Vaccines	Reduction & Refinement	[67]
			Vero Cell Assay for Potency testing of Diphtheria Vaccines	Reduction & Refinement	[68,69]
4	Diphtheria component in combined vaccines (*Corynebacterium diphtheriae*)	Guinea pig lethal challenge test	Erythema score following intradermal challenge in guinea pigs	Refinement (Endpoint is not death)	[70,71]
5	Tetanus and Diphtheria vaccines	Combination Tetanus and Diphtheria vaccines-separate serology tests for each vaccine	Tetanus and Diphtheria Serology test-single test for combination vaccine	Reduction	[67]
6	Hepatitis B Vaccine	Potency Test of Hepatitis B Vaccine (Mouse)	Serological Antigen Quantification	Reduction & Refinement	[67]
7	Inactivated Poliomyelitis Vaccine	Serological Potency Test of Poliomyelitis (inactivated) Vaccine (Rat)	Antigen quantification	Replacement	[67]
8	Rabies Vaccine	Lethal challenge test for Rabies Vaccine	Single dilution assay	Reduction	[67]
9	Inactivated rabies virus vaccine	Mouse multiple-dilution lethal challenge test	Convulsions, paralysis, paresis		
			Humane Endpoints for Rabies Potency Testing	Refinement	[67,72]
		NIH mouse protection test	Multi-dose serological assay, based on vaccination of mice and subsequent determination of neutralizing antibodies in vitro.	Reduction, Refinement & Replacement	[73]
		In-vivo rabies vaccine potency test (NIH test	Time Resolved Fluoroimmunoassay (TRFIA) for the assay of rabies virus glycoprotein	Replacement	[74]
10	Oral Polio Vaccine	Oral Polio Neurovirulence Test (Monkey intra-cerebral)	Mutant Analysis by PCR and Restriction Enzyme	Reduction	[75,76]
			Cleavage (MAPREC test) TgPVR21 Mouse Neurovirulence Test	Replacement	[77]
11	Diptheria Vaccine	Residual Toxicity in Diptheria Vaccine (Guinea pig)	Vero Cell Test of Diphtheria Toxoid Vaccines	Replacement	[78]
12	HBsAg Vaccine	*In-vivo* method for HBsAg potency	*In-vitro* method for HBsAg content using Auszyme EIA kit	Replacement	[79]

			In-vitro method for HBsAg content using an in-house-developed method based upon an inhibition ELISA	Replacement	[80-82]
13	Anthrax Vaccine Adsorbed	*In-vivo* mouse immunogenicity test	Toxin neutralization assay (TNA) to measure antibodies to anthrax Protective Antigen (PA)	Replacement	[83]
14	Whole cell pertussis (wP) vaccine	Lethal Challenge potency testing	Two Phase Studies: Evaluation phase and Validation phase with Humane endpoints	Reduction and Refine ment	[84]
15	Acellular pertussis component in combined vaccines Whooping cough (*Bordetella pertussis*)	Multiple-dilution mouse serology	Immunization (mice) and Serology ELISA	Replacement	[85-87]
16	Hepatitis A vaccine (hepatitis A virus)	Mouse serology	Antigen quantification	Replacement	[88,89]
17	Inactivated polio vaccine (poliovirus)	Rat serology	Antigen quantification	Replacement	[88,90,91]
18	Human papillomavirus vaccine	Mouse serology	Antigen quantification	Replacement	[92]
19	Rotavirus vaccine	*In-Vivo* potency assays	Cell-based viral replication followed by quantitative reverse-transcription polymerase chain reaction (RT-QPCR) analysis	Replacement	[93]
20	Live Rubella Virus Vaccine	*In-Vivo* potency assays	*In-vitro* cytopathic effect (CPE) with rabbit kidney epithelial (RK-13) cell culture	Replacement	[94]
21	Smallpox virus vaccine	Titration onto chorioallantoic membranes of fertilized hen eggs (CAM assay).	Vero cell culture titration assay.	Replacement	[95]
22	Trivalent, live, measles, mumps, rubella vaccines (MMR).	CCID50 and plaque assays	Quantitative PCR after cell culture	Replacement	[96]
23	Whole cell pertussis vaccines	Safety: mouse weight gain test. Potency: Kendrick test-large numbers of animals, severe distress, poor precision and reliability	Respiratory challenge assays, nitric oxide induction assay and serological assays.	Replacement & Refinement	[97]
24	Acellular pertussis vaccines (ACPVs)	The histamine sensitization test (HIST)	Combination of enzyme coupled-HPLC (E-HPLC) and carbohydrate binding assays to examine both the functional domains of PT.	Replacement & Refinement	[98]
25	Cholera	Potency: multidilution vaccination +serology	Enzyme-linked immunosorbent assay (ELISA) titers of the antibody secreted in the cell supernatant.	Replacement	[99]
26	Haemophilus type B conjugate	Multidilution vaccination +serology .	High throughput SBA for anti-Hib antibodies that would be useful for evaluating various Hib vaccines	Replacement & Refinement	[100]

At the moment, there is a paradigm shift in the concept of vaccine quality control from classical to consistency approach. Main emphasis is being put on ensuring the consistency in production of a vaccine and not regarding each batch produced as a unique product. Therefore, the main focus should remain in monitoring of consistency rather than to establish the true effectiveness of a vaccine. The characteristics of a new batch of vaccine should be similar to those of a batch which has been shown to be safe and efficacious [2].

For classical animal tests, many alternative methods for the quality control of vaccines have been developed in last two decades and even they have been implemented successfully. But in some vaccine products, *in-vitro* assays will not be able to completely replace animal models but in near future, the potency testing in animals is likely to disappear when current vaccines are replaced by highly purified and well-characterised genetically engineered products. As these new generation vaccines are better defined and allow the use of *in-vitro* and physico-chemical methods for their quality control, thus using less or no animals for the quality control of batches. The 3Rs achievement in quality control of vaccine has is shown in Table 5 for human vaccines and Table 6 for veterinary vaccines below:

Table 6. *Examples of 3Rs achievements for veterinary vaccine potency tests*.

S No	Vaccine	Traditional Method	Alternative Method	3R alternates	Reference

1	Inactivated swine erysipelas vaccines	Lethal challenge test in the batch potency testing of inactivated swine erysipelas vaccines	Serological Method (ELISA) for the Batch Potency Testing of Inactivated Swine Erysipelas Vaccines	Reduction & Refinement (Endpoint is not death)	[101,102]
2	Safety Test	Target Animal Safety Test	Deletion of test	Replacement	[103]
3	Inactivated rabies virus vaccine (*Lyssavirus rabies*)	Study endpoints used were Moribund condition or death	Convulsions, paralysis, paresis	Refinement	[104-106]
4	Inactivated swine erysipelas vaccine (*Erysipelothrix rhusiopathiae*)	Study endpoints used were Moribund condition or death	Pathognomonic, diamond shaped erythematous skin lesions, elevated temperature, arthritis	Reduction & Refinement (Endpoint is not death)	[107-109]
5	Fowlpox virus vaccine	Study endpoints used were Moribund condition or death	Pox lesions, warty eruptions/scabs on combs and wattles	Reduction & Refinement (Endpoint is not death)	[107,110]
6	Inactivated rabies vaccine (*Lyssavirus rabies*)	Immunization challenge in mice (intracerebral)	Immunization (mice) and serology: *In-vitro* RFFIT (rapid fluorescent focus inhibition test)	Replacement	[111-113]
7	Inactivated swine erysipelas vaccine (*Erysipelothrix rhusiopathiae*)	Mouse lethal challenge test	Immunization (mice) and serology: *In-vitro* antibody quantification-ELISA	Replacement	[114,115]
8	*Clostridium novyi* (Type B); Bovine (Black disease)	Rabbit immunization/mouse toxin neutralization test	Immunization (rabbits) and Serology: *In-vitro* immunochemical method or neutralization in cell cultures	Refinement and Replacement	[116,117]
9	*Clostridium septicum*; Bovine (malignant edema)	Rabbit immunization/mouse toxin neutralization test	Immunization (rabbits) and serology: *In-vitro* immunochemical method or neutralization in cell cultures	Refinement and Replacement	[117,118]
10	*Clostridium perfringens* C/D; Bovine (Enterotoxemia)	Rabbit immunization/mouse TNT	Immunization (rabbits) and serology: *In-vitro* immunochemical method or neutralization in cell cultures	Refinement and Replacement	[116,117,119]
11	Tetanus Antitoxin Products (equine); (*Clostridium tetani*)	Guinea pig immunization/ guinea pig toxin–antitoxin neutralization test	Immunization (guinea pigs) and serology: *In-vitro* toxin binding inhibition (TOBI), indirect ELISA	Refinement and Replacement	[120,121]
12	*Leptospirainterrogans Serovarcanicola* Bacterin Canine leptospiral (inactivated, adjuvanted and non adjuvanted)	Immunization challenge test in hamsters	Immunization (hamsters) and serology: *in-vitro* method to determine antibodies	Refinement and Replacement	[122]
			USDA Supplemental Assay Methods (SAM) 625		[123]
	Leptospirakirshnerii Serovargryppotyphosa		USDA Supplemental Assay Methods (SAM) 626	Replacement	[124]
	Leptospirainterrogans Serovarpomona		USDA Supplemental Assay Methods (SAM) 624	Replacement	[125]
	Leptospirainterrogans Serovaricterohaemorrhagiae		USDA Supplemental Assay Methods (SAM) 627	Replacement	[126]
13	Leptospirainterrogans Serovarhardjobacterin E Bovine	Cattle immunization challenge: Immunization challenge test in hamsters	Immunization (guinea pigs) and serology: microagglutination test	Refinement and Replacement	[127]
	Leptospira hardjo		USDA Supplemental Assay Methods (SAM) 624		[125]
14	Rabies vaccine (*Lyssavirus rabies*)	Multiple-dilution assays	Single-dilution assay	Reduction	[128-130]
			NIH potency test (Center for Veterinary Biologics (CVB) intends to eliminate the upper limit LD50 for a valid challenge when conducting the Rabies Virus)	Reduction	[131]
15	*Brucellaabortus* (cattle brucellosis)	Invivo Potency Assay	*In-vitro* titration method determining colony-forming units (tryptose agar)	Replacement	[132]
16	*Erysipelothrix rhusiopathiae* (swine erysipelas)	Vaccination challenge test in swine	*In-vitro* titration method determining colony-forming units (5% bovine blood agar)	Replacement	[133]
17	*Mannheimiahaemolytica* (*Pasteurellahaemolytica*) (cattle respiratory disease)	Vaccination challenge test in cattle	*In-vitro* titration method determining colony-forming units (trypticase soy agar)	Replacement	[134]

18	*Chlamydophilafelis* (feline respiratory disease)	*In-vivo* Potency Assay	Cell culture-*in-vitro* titration method utilizing indirect fluorescent antibody staining (mouse fibroblasts; MEM)	Replacement	[135]
19	Feline calicivirus (feline respiratory disease)	*In-vivo* Potency Assay	Cell culture-*in-vitro* titration method utilizing plaque-forming units (Crandall feline kidney cells; MEM)	Replacement	[136]
20	Feline Rhinotracheitis Virus (feline respiratory disease)	*In-vivo* Potency Assay	Cell culture-*in-vitro* titration method utilizing plaque-forming units (Crandall feline kidney cells; MEM)	Replacement	[137]
21	Mareks disease virus (poultry neoplastic disease)	Vaccination challenge test in chickens	Cell culture-*in-vitro* titration method (primary chick embryo fibroblasts; M199)	Replacement	[138]
22	Porcine transmissible gastroenteritis caused by coronavirus TGEV (swine infectious diarrhea)	*In-vivo* Potency Assay	Cell culture-*in-vitro* titration method utilizing cytopathic effect (swine testicular cells; MEM)	Replacement	[139]
23	Porcine rotavirus (swine infectious diarrhea)	*In-vivo* Potency Assay	Cell culture—*in-vitro* method utilizing cytopathic effect or indirect fluorescent antibody technique (Rhesus monkey kidney cells; MEM)	Replacement	[140]
24	Infectious canine hepatitis caused by canine adenovirus Type 1 (canine hepatitis)	*In-vivo* Potency Assay	Cell culture-*in-vitro* method utilizing cytopathic effect (primary dog kidney cells; MEM)	Replacement	[141]
25	Canine distemper virus (canine viral disease)	*In-vivo* Potency Assay	Cell culture-*in-vitro* method utilizing cytopathic effect (Vero cells; MEM)	Replacement	[142]
26	Infectious bursal disease virus (IBDV) (poultry immunosuppressive disease)	Immunization challenge test in chickens	Cell culture-*in-vitro* titration method of tissue culture adapted IBDV (primary chick embryo FB; M199/F10)	Replacement	[143]
27	Feline panleukopenia caused by feline parvovirus (feline viral disease)	*In-vivo* Potency Assay	Cell culture *in-vitro* titration method utilizing indirect fluorescent antibody straining (Crandall feline kidney cells; MEM)	Replacement	[144]
28	Mink distemper virus (mink viral disease)	Immunization challenge test in mink	Embryonated chicken eggs-titration of viral plaques on chorioallantoic membrane (CAM)	Replacement	[145]
29	Erysipelothrix rhusiopathiae (inactivated) (swine Erysipelas)	Immunization challenge test in mice	Antigen quantification-*in-vitro* ELISA	Replacement	[146]
30	Bovine respiratory viruses (BRV, BVD, PI, BRSV) (cattle respiratory disease)	*In-vivo* Potency Assay	Antigen quantification-*in-vitro* ELISA	Replacement	[147]
31	Newcastle disease virus (chicken respiratory disease)	Immunization challenge in chickens; serology	Antigen quantification-*in-vitro* ELISA or serology	Replacement	[148,149]
32	Tuberculin, PPD Bovis, Intra-dermic	43 guinea pigs are needed: 20 sensitized to M. avium, 20 sensitized to M. bovis, and 3 non-sensitized to be used as controls.	The alternate testing protocol uses only 15 guinea pigs, eliminates the M. avium sensitized guinea pigs, reduces the number of PPD dilutions for the test, and uses 6 injections per guinea pig.	Reduction	[150]
33	Botulism neurotoxin products	Mouse LD50 Assay for Botulinum Toxin Testing	Acceptance of cell based assay for Botulism neurotoxin products	Refine, Reduce and Replace	[151]
34	Inactivated influenza vaccines	Single radial immunodiffusion (SRID) assay	Monoclonal antibody (mAb)-based potency assays	Refinement	[152]
			New VaxArray® potency reagent kit	Refinement	[153]

Conclusion

In conclusion, there is a need for rigorous in-process quality control monitoring than the end batch testing. Remarkable advancements have been made in the development, maintenance, and upgradation of *in-vitro* potency assays like ELISA, Cell culture, SDS-PAGE, 2-D gel electrophoresis, Serology, etc. which minimize the animal use and suffering. However, many hindrances are still encountered in the implementation of 3Rs principles for vaccine potency assays due to their unique compositions, multivalency, and long life cycles. Therefore, relentless research work is required by both health industries and regulatory agencies for the development and validation of robust 3Rs methods at the global scenario.

Other prevailing challenges in the implementation of 3Rs principles are the lack of comprehensive harmonization of regulatory requirements, cooperation by health authorities to

accept deviations from established guidelines and strong incentive to develop and implement alternatives of animal testings valid for global regulations. All the alternative methods data should be validated with the *in-vivo* methods for comparison and thereafter should be included in the guidelines for quality control. Many countries are still using the traditional methods due to lack of mutual acceptance of data (MAD) between the regulatory agencies. If these advanced validated methods are strictly applied at the global level within the time-bound framework by the regulatory agencies, this might be helpful in reducing the numbers as well as the unwanted suffering inflicted to animals during the potency assays.

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