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Overcoming scientific barriers in the transition from *in vivo* to non-animal batch testing of human and veterinary vaccines

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ABSTRACT

Introduction: Before release, vaccine batches are assessed for quality to evaluate whether they meet the product specifications. Vaccine batch tests, in particular of inactivated and toxoid vaccines, still largely rely on *in vivo* methods. Improved vaccine production processes, ethical concerns, and sub-optimal performance of some *in vivo* tests have led to the development of *in vitro* alternatives.

Areas covered: This review describes the scientific constraints that need to be overcome for replacement of *in vivo* batch tests, as well as potential solutions. Topics include the critical quality attributes of vaccines that require testing, the use of cell-based assays to mimic aspects of *in vivo* vaccine-induced immune responses, how difficulties with testing adjuvanted vaccines *in vitro* can be overcome, the use of altered batches to validate new *in vitro* test methods, and how cooperation between different stakeholders is key to moving the transition forward.

Expert opinion: For safety testing, many *in vitro* alternatives are already available or at an advanced level of development. For potency testing, *in vitro* alternatives largely comprise immunochemical methods that assess several, but not all critical vaccine properties. One-to-one replacement by *in vitro* alternatives is not always possible and a combination of methods may be required.

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3Rs; *in vitro*; vaccines; quality control; animal model; alternatives; potency; safety


1. Background

Vaccination is a cost-effective strategy to prevent infectious diseases among humans and livestock. Vaccines are biologicals that are used to immunize large groups of healthy individuals and they may be subject to inherent batch-to-batch variation, which is why newly produced vaccine batches require quality assessment before being released to the market. Vaccines have traditionally been generated using trial and error approaches involving *in vivo* experiments [1]. Subsequently produced batches of such established vaccines are often still tested using *in vivo* methods to confirm that the quality of the new vaccine batch meets the specifications as defined in the marketing authorization. These specifications concern purity (i.e. freedom from extraneous matter), potency (i.e. the capacity of a vaccine batch to exert its effect), safety (i.e. relative freedom of harmful effects) and efficacy (i.e. effect of vaccination on the target species/population under ideal circumstances) of the vaccine [2]. Over one million animals were used for batch potency and safety testing of medicinal products in the EU in 2017, which corresponds to approximately 12% of total animal use for scientific purposes in the EU [3]. Nowadays, there are several reasons to move away

from the use of these *in vivo* potency and safety tests to assess the quality of vaccine batches. Firstly, significant improvements in and standardization of the vaccine production process, adherence to good manufacturing practice (GMP) standards and in-process controls have resulted in less batch-to-batch variation and a lower risk of producing unsafe or ineffective products [4]. Secondly, the use of large numbers of animals in experiments that may inflict pain and distress is not in line with the ethics of contemporary research and the 3Rs principles of Replacement, Reduction and Refinement [5–7]. Thirdly, the relevance of some *in vivo* tests is disputed because the test results show high variability and poor reproducibility [7–12]. Fourthly, the use of animal models is expensive, time consuming and risky for personnel when models involve exposure to viable pathogenic organisms [7].

The consistency approach was proposed as a strategy to enable the transition of *in vivo* to *in vitro* batch testing of vaccines [13,14]. This approach is based on the principle that quality is the consequence of consistent production of subsequent batches monitored by a GMP quality system [15]. The evaluation of a number of pre-defined vaccine parameters using *in vitro* physicochemical, immunochemical and cell-

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Article Highlights

- There is a global intent to develop *in vitro* alternatives for animal-based batch potency and safety tests of vaccines. This review describes scientific barriers that have been overcome or still need to be overcome to move the transition from *in vivo* to *in vitro* batch testing forward.
- For safety testing, many *in vitro* cell-based or immunochemical toxicity tests are already available.
- Potency tests are often still performed *in vivo*, especially for vaccines based on inactivated pathogens, toxoids or pathogen subunits. An increasing number of immunochemical alternatives are being developed. These tests assess vaccines for antigen identity, quantity and integrity characteristics. However, some vaccines will require additional testing. For instance, cell-based assays can provide functional information about the interaction between a vaccine and the immune system.
- Adjuvanted vaccines are notably hard to test for quality using *in vitro* alternatives. Fortunately, several desorption and extraction methods of adjuvants have been described.
- Due to quality systems that are in place at the manufacturer there may be a lack of non-compliant vaccine batches, which are needed to validate new *in vitro* test methods. Altered batches, which are purposely made non-compliant batches, may provide a solution.
- Cooperation between scientists in academia, industry and regulatory institutes is key to move the transition to *in vitro* vaccine testing forward by sharing of products, knowledge and technologies.

based test methods should demonstrate that final batches are of consistent quality, making the use of *in vivo* tests unnecessary.

Major steps have been taken to promote the use of *in vitro* alternatives for vaccine batch testing, including the creation of a legal and logistic framework. For instance, Directive 2010/63/EU on the protection of animals used for scientific purposes, which includes regulatory testing of vaccines, states 'The use of animals for scientific or educational purposes should only be considered where a non-animal alternative is unavailable' and thus promotes the use of novel *in vitro* test methods [16]. In addition, a general chapter on the 'Substitution of *in vivo* methods by *in vitro* methods for the quality control of vaccines' has been incorporated in the European Pharmacopoeia to provide guidance on the substitution of an *in vivo* test method with non-animal alternatives in cases where one-to-one test replacement cannot be achieved (5.2.14) [17]. Bodies like the Biological Standardization Programme of the European Directorate for the Quality of Medicines & Healthcare (EDQM), which facilitates multi-center validation studies, and the European Center for Validation of Alternative Methods (ECVAM), which acts as a reference laboratory of the European Union, provide the logistic framework needed to validate novel *in vitro* methods [16,18]. Meanwhile, some *in vivo* tests of vaccines have been replaced by *in vitro* alternatives, whereas others are no longer used as will be discussed further below.

In September 2015, industrial, regulatory and scientific experts gathered for a workshop in the Netherlands to identify drivers and barriers for the implementation of the consistency

approach, of which the results were published [15]. Newer generation vaccines, including recombinant subunit vaccines (e.g. human papillomavirus and hepatitis B) or conjugate vaccines (e.g. *Haemophilus influenzae* type b, meningococcus and pneumococcus) are well defined and can be evaluated for quality using *in vitro* methods, which is why the workshop focused on established live attenuated, inactivated and toxoid vaccines that were in part still tested for quality using *in vivo* methods [17,19,20]. Identification and development of *in vitro* methods that provide alternatives to currently used *in vivo* batch tests of vaccines is hampered by several scientific and other constraints including the following points [15]:

- It is difficult to mimic vaccine-induced immune responses using *in vitro* test methods.
- Measuring vaccine properties of adjuvanted vaccines is complex.
- There is a need to create subpotent formulations for method validation due to a lack of appropriate non-compliant batches to test (and validate) *in vitro* methods.
- Research into *in vitro* test methods needs to be prioritized and financed based on number of test animals, level of severity, and performance of the *in vivo* test.
- Knowledge about the critical quality attributes of vaccines and critical process parameters of vaccine production is often limited.

In the present review we will discuss the scientific barriers that have been overcome since the workshop or still need to be dealt with to enable a complete transition from *in vivo* to *in vitro* vaccine quality testing.

2. Current repertoire of *in vitro* vaccine quality tests

Despite the constraints described above, several human and veterinary vaccines already underwent the transition from *in vivo* to *in vitro* batch testing (Figure 1) [15]. These vaccines could provide a roadmap to guide the transition to *in vitro* for the vaccines that are currently still tested for potency and safety using *in vivo* methods.

For live attenuated vaccines, comprising bacteria or viruses, the use of *in vivo* batch tests is in general not demanded in the European Pharmacopoeia (Supplementary Table 1) [17]. Due to their replicative nature, live attenuated vaccines can be tested for potency by viral or bacterial titration. Some live attenuated viral vaccines (e.g. influenza virus and viral poultry vaccines) are propagated and titrated on embryonated chicken eggs when suitable cell lines are not available. These chicken embryos are not regarded as laboratory animals in view of the European act on the protection of animals used for scientific purposes (Directive 2010/63/EU), which is not applicable to fetal stages of birds [16]. However, the embryos may experience pain and cell-based alternatives to propagate and titrate these viruses are therefore desirable whenever available [21]. Moreover, virus propagation through cell lines allows faster upscaling during epidemics and is better standardized than propagation through eggs [22]. Finally, the transportation of chicken eggs may be prohibited by governments during avian influenza outbreaks.

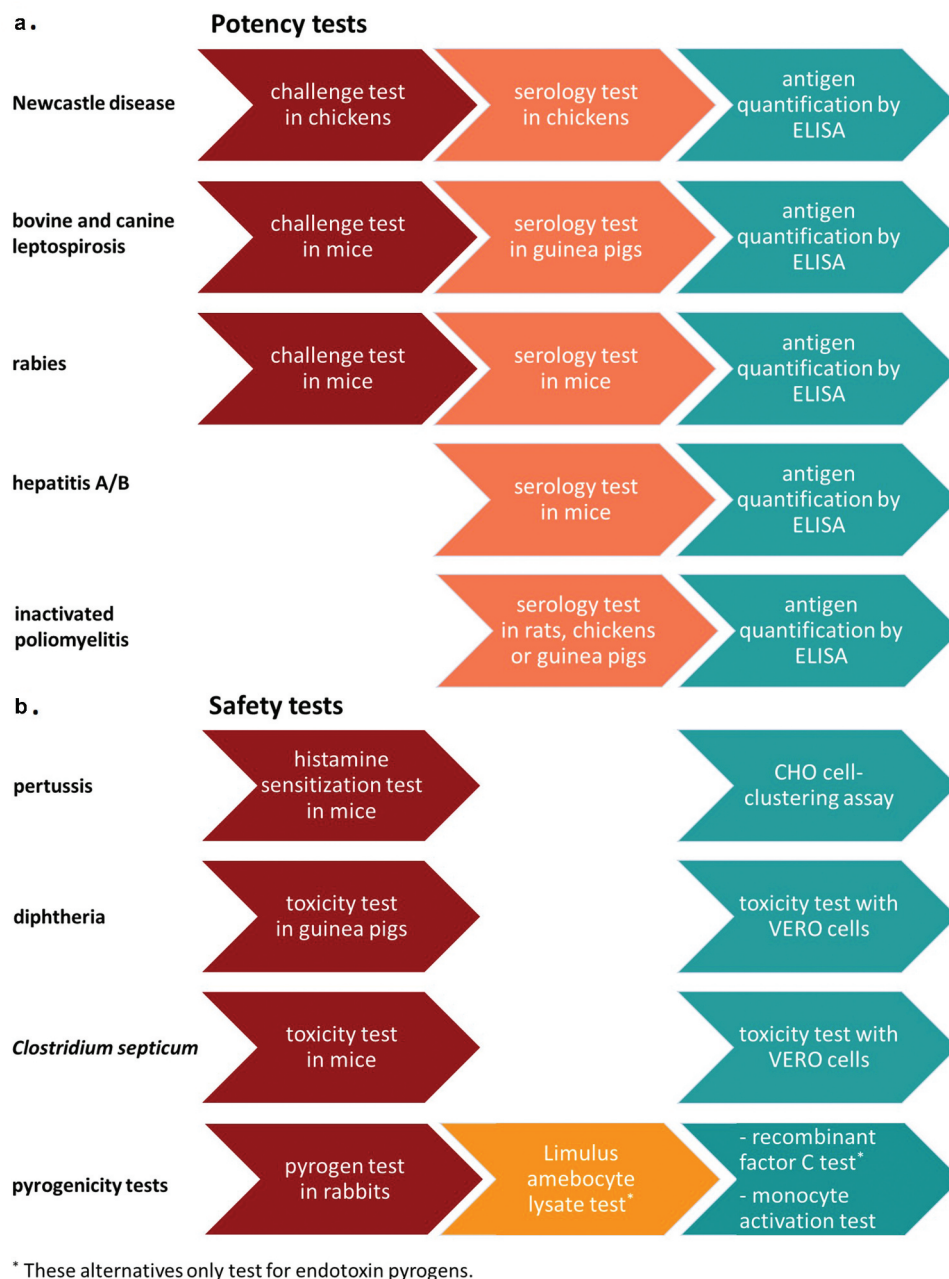


Figure 1. For several established inactivated and toxoid vaccines for human and veterinary use there has already been a transition from *in vivo* to *in vitro* batch testing in Europe [5]. Advancements have been made for both potency (a.) as well as safety tests (b.). Conventional *in vivo* tests are shown in red. Alternative *in vivo* tests with reduced and refined use (i.e. less animals and less discomfort) of animals are shown in orange. Alternative *in vitro* tests are shown in green.

Unlike live attenuated vaccines, inactivated vaccines and toxoid vaccines mostly rely on vaccination-challenge or vaccination-serology potency tests, unless antigen quantification methods are available as *in vitro* alternatives (Supplementary Table 1). These antigen quantification methods may be physiochemical or immunochemical assays that are specific for one or more dominant antigens of the vaccines. In particular, the development of enzyme-linked immunosorbent assays (ELISAs) for antigen quantification has been successful for the replacement of *in vivo* potency tests of several vaccines in Europe, including those against Newcastle disease in poultry, foot-and-mouth disease in

cattle, leptospirosis in cattle and dogs (monographs 0447 and 1939), rabies in both animals and humans, and hepatitis A and B in humans (Figure 1a) [17,20,26–29]. Other ELISAs for antigen quantification have shown to be successful in determining the potency of some but not all inactivated vaccines and toxoid vaccines, but these are not yet fully validated and/or approved by the regulatory authorities in Europe. These include tests for infectious bronchitis virus and infectious bursal disease virus vaccines for poultry, furunculosis vaccines for salmonids, tetanus vaccines for human and veterinary use, and diphtheria vaccines for human use [30–35]. In addition to the ELISA, another

important antigen quantification method is the immunodiffusion test, which is used to determine the content of hemagglutinin antigens of inactivated or subunit influenza vaccines [36]. For the whole-cell and acellular pertussis vaccines, there have been efforts to develop *in vitro* ELISA, Luminex, cell-based and proteomic assays as alternatives [37–41].

With respect to safety, alternative *in vitro* methods have also become available in Europe (Figure 1b). Originally, vaccines were tested for pyrogenicity using the Rabbit Pyrogen Test (RPT), in which temperature changes in rabbits are evaluated after injection of a vaccine. To specifically quantify endotoxin pyrogens, comprising bacterial lipopolysaccharides (LPS), the Limulus Amebocyte Lysate (LAL) test is used. Although the LAL test is legally not regarded as an animal test, horseshoe crabs are captured and bled to obtain their blood cells as the main reagent of the LAL test. The horseshoe crabs do not always survive the bleeding procedure, which is estimated to result in an annual mortality rate of 100,000 horseshoe crabs in North America alone and impacts the survival of this threatened species [42,43]. Nowadays, the blood clotting factor C (rFC) from the horseshoe crab is commercially available as a recombinant protein, but the rFC test has been implemented to a limited extent despite its proven efficacy and its adoption in the European Pharmacopoeia as alternative reagent in endotoxin tests (general text 2.6.14, 2.6.32 and 5.1.10) [17,42]. The Monocyte Activation Test (MAT) was developed to detect both endotoxin and non-endotoxin pyrogens [44–46]. In this test human whole blood or (cryopreserved) PBMCs are stimulated with the vaccine of interest after which the production of pro-inflammatory cytokines (TNF, IL-1 β and IL-6) is assessed in ELISAs. The European Pharmacopoeia states that whenever possible and after product-specific validation, the RPT test should be replaced by the MAT (general text 2.6.8) [17]. To justify the use of the LAL test or the rFC test as alternatives for the RPT test, a risk assessment using the MAT is recommended to rule out the presence of any non-endotoxin pyrogens in a vaccine (general text 5.1.10). These statements promote the use of the MAT as a safety test for pyrogenicity or bacterial endotoxins. The MAT test was recently optimized for pyrogenicity testing of the Encepur vaccine against tick-borne encephalitis [44].

To test for residual toxicity of toxoid vaccines *in vitro* alternatives have been developed including the Chinese hamster ovary (CHO) cell clustering assay as an alternative to the *in vivo* Histamine Sensitization Test (HIST) for acellular pertussis vaccines [47]. In addition, a VERO cell toxicity assay has been developed and implemented for specific toxicity testing of diphtheria toxoid vaccines as an alternative to the *in vivo* test in guinea pigs [48]. Furthermore, a VERO cell toxicity assay to be used instead of the mouse toxicity test for veterinary *Clostridium septicum* vaccines is currently being validated [49]. Finally, the binding and cleavage (BINACLE) assay evaluates residual tetanus toxicity of toxoid bulk that is produced for human and veterinary tetanus vaccines, as an alternative for the currently used test in guinea pigs [50]. The BINACLE assay is currently being validated as part of the European Biological Standardization Programme. Unfortunately, the

BINACLE assay cannot be used for vaccine final products that contain tetanus toxoid in an adjuvant-adsorbed state [50].

In summary, although the quality of many inactivated vaccines and toxoid vaccines is still tested with *in vivo* potency tests, an increasing number of *in vitro* alternatives are being developed to achieve their replacement. These alternatives include immunochemical assays to quantify antigen, but also tests for other critical properties of vaccines, as will be discussed in the next section. For safety tests, many *in vitro* alternatives are already available in Europe and implementation of these methods has now become important to move the transition to *in vitro* safety testing forward.

3. Critical quality attributes of vaccines

Critical quality attributes of vaccines are physical, chemical or microbiological properties of vaccines that should be within certain limits to ensure vaccine quality [51]. The *in vitro* methods that are currently in place for potency testing of vaccine batches are mainly used to evaluate antigen identity, quantity and integrity (Figure 1a). Some of these methods are antigen quantification methods that depend on the integrity of a single antigen [52]. For example, the detection antibody that is used for antigen quantification of inactivated Newcastle disease vaccine is a monoclonal antibody raised against a linear epitope of Newcastle disease virus hemagglutinin-neuraminidase covering 20 amino acids [53]. Similarly, monoclonal antibodies have been used for potency testing of non-adjuvanted veterinary rabies vaccines by quantifying G protein antigen [54]. However, at the population level vaccination efficiency hardly ever depends on a single dominant epitope. Furthermore, the ability of immunochemical assays to measure the relevant epitopes of antigens inactivated with agents like formaldehyde and β -propiolactone should be validated. The inactivation may alter or hide specific epitopes of antigens and hence affect potency as measured *in vitro*, even when the potency as measured *in vivo* remains the same [52,55–57]. The use of a pool of multiple monoclonal, i.e. multiclonal, antibodies in ELISAs can result in more consistent and standardized quality testing of vaccines, without depending too much on single epitopes [52]. Multiclonal antibodies are for example used for antigen quantification of hepatitis A vaccines [58]. In addition to antigen identity, quantity and integrity characteristics, the potency of vaccines may depend on critical quality attributes like vaccine composition, susceptibility of the antigen to proteolytic degradation, the spatial organization (i.e. three-dimensional structure) of the antigen, or the presence of additional immunostimulatory molecules. A combination of assays addressing these different critical quality attributes may thus be required to sufficiently demonstrate batch-to-batch consistency for some vaccines.

The vaccine composition depends on production processes, and inconsistencies in these processes may affect the potency of the vaccine. For instance, for whole-cell pertussis vaccines it was shown that disturbances of the bacterial culture conditions may result in the downregulation of important immunogenic virulence proteins [59]. Mass spectrometry-based proteome analysis was proposed as a method to evaluate the whole protein composition of vaccines to detect

these disturbances [41]. Another applicability of mass spectrometry is the assessment of the stability of antigens and their susceptibility to enzymatic degradation as an important part of antigen processing by antigen-presenting cells [60]. Inactivation by formaldehyde and heat exposure were shown to affect the degradation kinetics of a model antigen and tetanus toxoid, respectively [61,62]. The importance of the spatial organization of antigens has been demonstrated for influenza vaccines by showing that during a priming vaccination whole-inactivated influenza vaccines induced higher antibody titers, both in mice and humans, and superior T cell responses in humans as compared to the less reactogenic split vaccines with spatially disrupted antigens [63–67]. The antigens of the split vaccine lack the proper spatial organization to efficiently induce antibody production [68]. Similar results have been found for detergent-treated inactivated vaccines against Newcastle disease virus for use in poultry [69]. Finally, the presence of immunostimulatory molecules, which may include exogenous adjuvants or endogenous pathogen-associated molecular patterns (PAMPs), is important for vaccine potency. The contribution of PAMPs to vaccine potency has been demonstrated for both viral (e.g. influenza) and bacterial (e.g. pertussis) vaccines [39,64,70]. Similar to the antigens, PAMPs can be destroyed or become less accessible by inactivating agents like formaldehyde or β -propiolactone [55,71]. The presence of immunostimulatory molecules can be evaluated using cell-based assays, as discussed in more detail below. The described quality attributes may require additional testing when antigen identity, quantity and integrity characteristics are insufficient to predict the potency of a vaccine.

The previous section demonstrated that many *in vitro* alternatives to test vaccines for pyrogenicity and to test toxoid vaccines for residual toxicity are already available. Furthermore, toxicity tests that were found to be unnecessary based on historical data have recently been deleted from the European Pharmacopoeia, including abnormal toxicity tests (to detect any unexpected hazards), some specific toxicity tests of human vaccines, and some residual toxicity tests of veterinary vaccines [72,73]. In contrast, the porcine actinobacillosis vaccine, porcine progressive atrophic rhinitis vaccine, and tetanus vaccines for human and veterinary use still require animal-based toxicity tests [17,72]. Moreover, safety tests of the Bacillus Calmette-Guérin vaccine, used to protect against tuberculosis, include the virulent mycobacteria test and the excessive dermal reactivity test, both in guinea pigs, to show absence of virulence and excessive reactogenicity, respectively [17]. An alternative safety assay based on the proliferation of lymphocytes from sensitized guinea pigs has been proposed instead of the currently used excessive dermal reactivity test [74]. A vaccine for rabbit hemorrhagic disease still requires a residual live virus safety test in rabbits [17]. Some live attenuated viral vaccines (e.g. smallpox and poliomyelitis) require neurovirulence safety testing in monkeys or transgenic mice, although deep sequencing methods have been proposed as an alternative strategy to test these vaccines for genetic instability and to prevent the occurrence of neurovirulent viral mutants [17,75,76]. Recently, a model based on brain cells in a transwell system, named the BBB-Minibrain culture device, was developed as a next step in search for an

alternative neurovirulence test [77]. Finally, batch release of pertussis vaccines still requires a test for residual dermonecrotic toxin in mice [17]. Recently, the use of liquid chromatography mass spectrometry to quantify dermonecrotic toxin has been proposed as an alternative *in vitro* method [78].

To summarize this section, antigen identity, quantity and integrity can be considered the most important critical quality attributes of inactivated and toxoid vaccines. However, additional quality attributes including vaccine composition, the spatial organization of the antigen, or the presence of additional immunostimulatory molecules may be critical for specific vaccines. Importantly, it is increasingly recognized that one-to-one replacement of an *in vivo* test for an *in vitro* test will be difficult and that a combination of assays to demonstrate batch-to-batch consistency may be needed for some vaccines [11,79].

4. Mimicking *in vivo* vaccine-induced immune responses using *in vitro* cell-based assays

The use of immunochemical and physicochemical methods for potency testing is based on evidence showing that consistency between new batches and batches of proven potency with regard to aspects like antigen identity, antigen quantity, antigen integrity, antigen structure, vaccine composition, and quantity of immunostimulatory molecules will also warrant consistent potency *in vivo*. For vaccines that are well defined (e.g. recombinant and subunit vaccines) the use of these methods may indeed be sufficient to guarantee the potency and safety of a vaccine. However, for less defined inactivated and perhaps even toxoid vaccines, additional information on the immunostimulatory capacity of vaccines may be needed to generate sufficient evidence about vaccine quality when *in vitro* methods are used. Cell-based assays can assess aspects of vaccine-induced immune reactivity and may identify critical quality attributes of vaccines.

An immune response is initiated by innate immune cells (Figure 2; step 1), among which professional antigen-presenting cells, including dendritic cells, macrophages and B cells process and present antigen, and in addition express co-stimulatory molecules and release cytokines to activate T cells (Figure 2; step 2). Several methods have been proposed to evaluate vaccine potency in cell-based assays with immune cells using either primary cells or immortalized cell lines [40,80–84]. Primary dendritic cell-based assays use monocyte-derived dendritic cells (moDCs) or bone marrow-derived dendritic cells (BMDCs) [40,82–84]. Whereas primary cells more closely represent the physiological nature of immune cells, they are collected from animals or human donors, often have a limited lifespan, are available in limited numbers, and may show variable responses due to genetic diversity of the individual donors. Cell lines do not have these disadvantages and are thus being explored for use in a vaccine quality control setting (Table 1), even though they may be less representative for the *in vivo* situation than primary cells. Most cell-based potency assays measure vaccine-induced activation of dendritic cell-, monocyte- and macrophage-like cells by expression of co-stimulatory molecules (e.g. CD40, CD80, CD83, CD86) or pro-inflammatory cytokines. Furthermore,

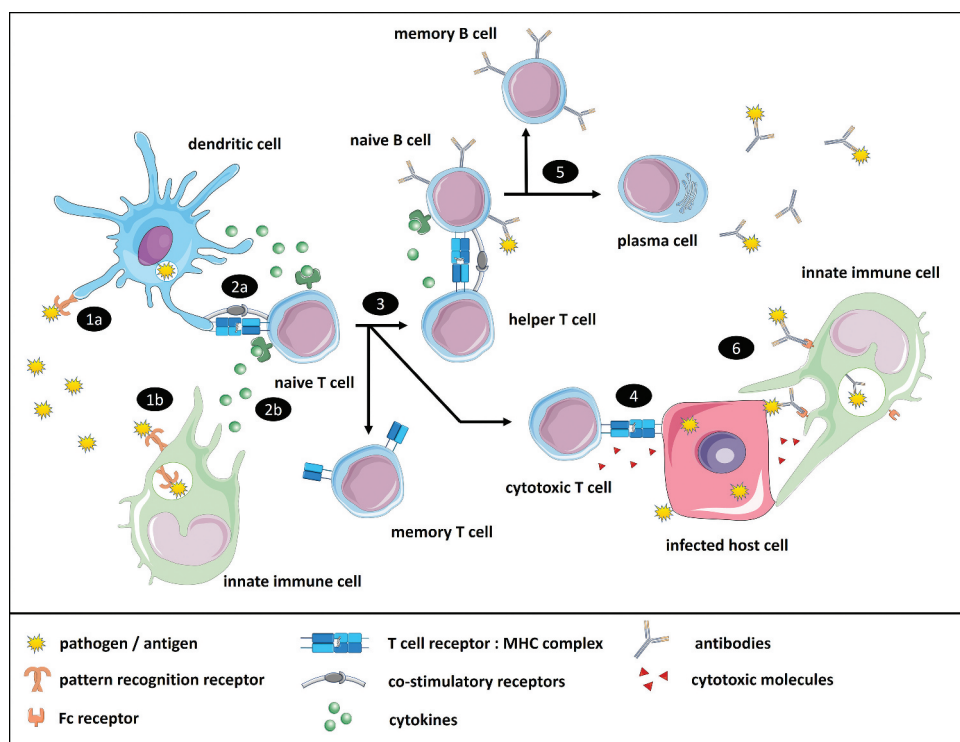


Figure 2. Simplified overview of a vaccine-induced immune response. (1) Innate immune cells recognize pathogen-associated molecular patterns (PAMPs), e.g. bacterial cell wall components or double-stranded RNA that are part of vaccine antigens, through pattern recognition receptors (PRRs). (2) Upon recognition, vaccine antigens are taken up by innate immune cells, and processed for antigen presentation on major histocompatibility complexes (MHC) class I and II to (naive) antigen-specific T cells. Innate immune cells orchestrate the adaptive immune response by releasing cytokines that affect the differentiation of T cells. (3) As a result, CD4+ T cells differentiate into various types of CD4+ helper T cells (Th1, Th2, Th17, Treg etc.) or memory T cells, whereas CD8+ T cells differentiate into CD8+ cytotoxic T cells, or memory T cells. (4) Cytotoxic T cells recognize antigen-derived peptides presented on MHC class I molecules of infected host cells that are subsequently killed. (5) Antigen-specific naive B cells that bind antigens through their B cell receptor will endocytose and process the antigens, and present these on MHC class II molecules to helper T cells. Upon recognition helper T cells will produce cytokines that facilitate B cell proliferation and differentiation into plasma cells or memory B cells. (6) Plasma cells produce antigen-specific antibodies that can neutralize or opsonize pathogens. Opsonized pathogens can be bound by Fc receptors on innate immune cells, resulting in the uptake and destruction of the pathogens. Furthermore, antibodies can bind to antigens exposed on the surface of infected host cells and stimulate antibody-dependent cellular cytotoxicity (ADCC) by innate immune cells. The icons used in this figure are adaptations from icons retrieved from the Servier Medical Art collection, which are licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

Table 1. Myeloid cell lines that have been described for use in potency testing of human and veterinary vaccines.

Representative cell type	Activated by	Activation markers	Does not respond to	Ref
MUTZ-3 human dendritic cells	<i>N. meningitidis</i> outer membrane protein	CD80, IL6, IL-8, TNF	- LPS - <i>H. influenzae</i> type B polyribosyl ribitol phosphate - Whole-cell <i>B. pertussis</i> vaccine - LPS, R848 - whole inactivated / subunit influenza virus	[83] [40] [82]
MM6 human monocyte	LPS, Pam3CSK4 whole-cell <i>B. pertussis</i> vaccine <i>N. meningitidis</i> outer membrane protein	CD80, CD86, IL-1 β , IL-6, IL-12p40, IL-10 IL-1 β , IL-6, IL-12p40 IL-1 β , IL-6, IL-10, CXCL10	Alum, MF59	[40] [40,85] [23]
THP-1 human monocyte	LPS, FSL-1, Pam3CSK4, flagellin, R848 PMA+alum MDP+MPLA+alum	IL-1 β , IL-6, IL-8, PGE ₂ , TNF IL-1 β , CD80 IL-1 β , IL-8, TNF		[88] [24] [25]
HD11 chicken macrophage	Inactivated vaccines for infectious bronchitis virus, Newcastle disease virus Inactivated vaccine for <i>Av. paragallinarum</i>	Phagocytosis Nitric oxide, IL-1 β , CXCL1, CXCL2, IL-10		[117] [107]

dendritic cells have been used in *in vitro* antigen degradation assays, in which the susceptibility of antigens for proteolytic degradation is used as a biomarker for immunogenicity [60]. Reporter cell lines like PRR-expressing human embryonic kidney 293 (HEK)-Blue cells have been used to evaluate the

immunostimulatory properties of clinical isolates of *Bordetella pertussis* and may also be useful to test vaccines for potency [85,86].

Assays with innate immune cells have also been used to evaluate safety aspects of vaccines. Vaccine pyrogenicity can

be evaluated by the MAT using monocytic cell lines like the MM6 cell line [87]. Interestingly, the MM6 cell line has been explored for use in *in vitro* safety tests, as well as potency tests, both using secretion of the pleiotropic cytokines IL-1 β and IL-6 as readouts [40,88]. The use of IL-1 β and IL-6 as readouts for potency is in line with the role of these cytokines in T cell activation and differentiation, whereas their use as readouts for safety is in line with their ability to induce fever and other potential side effects of vaccination [88–90]. Thus, potency and safety limits of pro-inflammatory cytokines need to be identified beforehand to discriminate between potent and potentially dangerous levels of vaccine-induced immune responses [88].

After the innate immune response is initiated by a vaccine, antigen-presenting cells will activate the adaptive immune system (Figure 2; step 3–5). An important aspect of the adaptive immune response for vaccination is the differentiation of B and T cells into effector and memory cells, of which the latter will quickly induce a secondary immune response upon future encounter with the same pathogen. Batches of whole cell pertussis vaccines and rabies vaccines of different potencies were found to differ in their ability to induce T cell responses in splenocytes from vaccinated mice, suggesting that T cell assays can be used to test for vaccine potency when these T cell responses are known correlates-of-protection [91,92]. The activation of naive T cells or the re-activation of memory T cells by vaccines has been mimicked *in vitro* using respectively autologous dendritic cell-T cell co-cultures or PBMCs to evaluate the potency of vaccines against yellow fever and influenza for use in humans, and for vaccines against blue tongue and rabies for use in cattle [93–95]. However, T cells are highly heterogeneous and T cell assays have only been explored for use in pre-clinical development of vaccines. In addition, T cell assays with primary cells require blood collection from vaccinated animals, similar to serological assays. The use of a combination of several epitope-specific T hybridoma cell lines might be more promising for potency testing of vaccine batches, but requires further development [96].

The contribution of assays with innate immune cells to batch testing is product-specific and depends on the presence of PAMPs, adjuvants and other potentially immunostimulatory vaccine components. T cell assays may be useful when T cell responses are known correlates-of-protection. Due to their accessibility, infinite lifespan, high numbers and low variability, (reporter) cell lines are particularly suitable for cell-based assays to test vaccines. Furthermore, cell-based assays can be useful to investigate whether there are any functional synergistic or antagonistic interactions between the adjuvant and other components in the vaccine, as will be discussed further below. Finally, the need for cell-based assays depends on whether the vaccine will be applied for priming or boosting the immune response. For example, booster vaccines may stimulate the reactivation of memory T cells independently of dendritic cells through

memory B cells and are therefore less dependent on stimulating innate immune cells than primer vaccines [97].

5. Adjuvanted vaccines

Many inactivated, toxoid and subunit vaccines require adjuvants to support activation of the immune system. For a long period of time, only aluminum salts and mineral oil emulsions were used as adjuvants. Aluminum salts are used as adjuvants for human vaccines such as diphtheria and tetanus vaccines as well as veterinary vaccines such as the bluetongue vaccine for ruminants, some feline leukemia vaccines and the rabies vaccine for dogs, cats, ruminants and horses [98,99]. The first emulsion adjuvants were Freund's incomplete adjuvant, based on mineral oil emulsified in a water-in-oil formulation, and Freund's complete adjuvant, formulated with tubercle bacteria [100]. Mineral oil-based water-in-oil adjuvants are too reactogenic for use in humans, but are widely used in poultry, cattle and fish [101,102]. The first emulsion adjuvant to be approved in humans was MF59, which is an oil-in-water adjuvant based on squalene [103].

Before batch release, adjuvanted vaccines are tested for identity, concentration, physical properties like viscosity and stability of the adjuvant (monographs 0062 Vaccines for Veterinary Use and 0153 Vaccines for Human Use) [17]. However, emulsion and aluminum salt adjuvants may interfere with *in vitro* methods to evaluate the antigens of final products [104]. The following section describes how this problem can be circumvented by testing intermediary products before blending of antigens and adjuvants, by extracting the antigenic fraction from final products or by using *in vitro* methods that are not affected by adjuvants [105].

Methods to extract antigens from final adjuvanted products have been developed for several vaccines. To enable the quantification of antigen by ELISAs, isopropyl myristate has been used to extract antigens from oil-adjuvanted poultry vaccines for Newcastle disease, infectious bronchitis and infectious bursal disease [30,106]. Similarly, antigens extracted from an oil-adjuvanted poultry vaccine against infectious coryza could be used to evaluate the presence of PAMPs by measuring their effects on nitric oxide production or cytokine expression in cell-based assays [107]. Isopropyl myristate could not be used to extract antigens from vaccines for food-and-mouth disease, which are formulated with Montanide ISA201 oil adjuvant, but an extraction with benzyl alcohol could be used instead for this vaccine, showing that different emulsion adjuvants require different extraction methods [108]. For aluminum salt-adjuvanted vaccines, electrophoretic or chemical competitive desorption methods can be used to retrieve the antigens, although the latter methods may result in denaturation of antigens and affect their integrity [109,110]. Such methods have been used by manufacturers for hepatitis A vaccines in order to enable antigen quantification by ELISA, but for commercial reasons the methods were not disclosed in the resulting publications [58,111].

Not all quantification methods require antigen extraction or desorption from adjuvanted vaccines. With respect to emulsion adjuvants, a multiplexed sandwich immunoassay for influenza vaccines against strains with pandemic potential was shown to be compatible with the squalene-based adjuvant MF59 [112]. An *in situ* method based on the fluorescent nucleic acid-reactive dye SYBR Green II has been developed to determine the stability of an inactivated vaccine for foot-and-mouth disease and was found compatible with aluminum salt, water-in-oil and oil-in-water adjuvanted vaccines [113]. However, to the best of our knowledge there are currently no *in vitro* antigen-specific methods to determine the quality of adjuvanted vaccines formulated as water-in-oil emulsions that do not require extraction of the antigen. Methods that nonspecifically determine protein content are compatible with aluminum salt adjuvants, including fluorescence spectroscopy, based on intrinsic fluorescence of tyrosine and tryptophan amino acids or fluorescent protein-reactive chemicals, and chemiluminescent nitrogen detection [114–116]. These methods are not antigen-specific and therefore not suitable for vaccines that contain antigens in complex media with other proteins, e.g. allantoic fluid present in poultry vaccines [117,118]. Some antigen-specific immunoassays have shown compatibility with aluminum salt-adjuvanted vaccines, which include sandwich and competitive ELISAs, Luminex, and immunofluorescent assays analyzed with a fluorometer or flow cytometer [38,119–123]. Cell-based toxicity assays can be made compatible for aluminum salt-adjuvanted vaccines by using semi-permeable transwell inserts to prevent direct contact between the cells and aluminum salts, as demonstrated by the CHO cell-clustering assay used to test for residual toxicity of pertussis vaccines [47].

In summary, adjuvanted vaccines create additional challenges for the development of *in vitro* potency and safety tests, but adsorption and extraction methods, and the development of adjuvant-compatible methods offer possibilities to overcome these.

6. Altered batches to validate *in vitro* test methods

Non-compliant vaccine batches of substandard quality are needed for the validation of new *in vitro* tests methods by confirming their capacity to discriminate between batches of different qualities [124]. However, modern well-controlled vaccine production processes that adhere to GMP standards rarely result in failed batches anymore and there may be reluctance to share the remaining non-compliant batch material [11,15]. This has led to a paradox in which improved quality of vaccines makes it more difficult to validate new *in vitro* test methods. Another problem is the inherent variability of current *in vivo* release tests that are used as reference tests, which may limit the ability to which they can discriminate between batches of different quality [7–12,125]. In other words, *in vivo* tests may show variation to an extent that some batches fail the test, while they are in fact compliant, i.e. their capacity to induce protective immunity in the target species is sufficient [125]. Such batches can be expected to pass appropriate *in vitro* tests. Likewise, batches may pass *in vivo* tests,

while they are in fact non-compliant, i.e. their capacity to induce protective immunity in the target species is insufficient.

To solve these problems, vaccine batches could be altered intentionally, for example by exposure to stresses that may decrease the stability of a batch during the vaccine production process (e.g. decreased or increased pH, osmolality, temperature), by creating vaccines with reduced antigen content or a different composition, by deviating from the standard inactivation method, or by changing the degree of adsorption to adjuvants [11,34,41,57,124,126,127]. Importantly, altered batches should be representative for non-compliant batches that may realistically be produced as a result of disturbances in vaccine production processes. In the European Pharmacopoeia, heat treatment is given as an example to create a proper altered batch of the inactivated poliomyelitis vaccines, which is used for product-specific validation of the *in vitro* D-antigen assay, in order to replace the former *in vivo* potency test in chickens, guinea pigs or rats (Ph. Eur method 2.7.20) [17]. The altered batches need to be designed and produced already during the development phase of *in vitro* test methods to identify the sensitivity of these assays for detecting non-compliant batches.

7. Cooperation between academia, industry and regulatory institutions is crucial for the transition from *in vivo* to *in vitro* batch testing of vaccines

Two previously identified constraints for the transition to *in vitro* batch testing of vaccines were the lack of priority of research into *in vitro* test methods that are most needed, and the varying knowledge about vaccine production processes among scientists of different disciplines [15]. International, multi-stakeholder (academia, industry and regulators) collaborations were described as key to move the transition to *in vitro* vaccine testing forward. Over the last five years, this necessity was met by the VAC2VAC consortium, funded by the Innovative Medicines Initiative (IMI2) programme [128]. In this consortium, vaccines and their intermediary products (e.g. antigens, adjuvants, excipients and additives), knowledge about production processes, and new technologies for vaccine quality control were shared between the different stakeholders. The aim of the consortium was to develop new tests and approaches that support the use of the consistency approach in batch testing of established vaccines.

Assay development is only the first step of the transition from *in vivo* to *in vitro* batch testing, which has to be complemented by a validation process, first in small-scale feasibility studies to test the transferability of the assay to other labs, later in large-scale multi-center validation studies that are preferably conducted at an international or even global level to standardize novel methods [18,129]. The latter validation studies can be performed as part of the Biological Standardization Programme in Europe, in which Official Medicines Control Laboratories (OMCLs), manufacturers and other stakeholders can participate. Assays that have been successfully validated and are accepted by regulatory authorities can become incorporated into pharmacopoeias [129].

After incorporation, the methods still need to be implemented by individual manufacturers and OMCLs, which have to conduct assay validation before the conventional animal-based test can be fully replaced and eventually eliminated from pharmacopoeias. Engagement between academia, industry and regulators at an early stage of the development of *in vitro* alternatives is essential for getting all stakeholders acquainted with new methods, to facilitate validation studies and to achieve wide implementation of the *in vitro* alternatives [15,18,79].

8. Conclusions

This review describes scientific barriers that hampered the transition from *in vivo* to *in vitro* batch testing of vaccines, especially for those that include inactivated pathogens, toxoids or pathogen subunits, and the opportunities to overcome these. An increasing number of *in vitro* potency and safety tests have been developed to replace *in vivo* tests. Currently implemented *in vitro* potency tests largely comprise ELISAs or other immunochemical methods that assess antigen identity, quantity and integrity characteristics, while other properties may also be critical for vaccine quality. There is an increasing consensus that one-to-one replacement by *in vitro* alternatives is not always possible and that a combination of *in vitro* alternatives may be needed. Cell-based assays can provide functional information about the interaction between a vaccine and the immune system, which is impossible to capture with ELISAs only. Adjuvanted vaccines are notably hard to test for quality using *in vitro* alternatives due to their incompatibility with some of these methods. However, the current scientific literature provides many possibilities to overcome these difficulties, including desorption and extraction methods of adjuvants and strategies to study the potential interactions between different vaccine antigens. The use of purposely altered batches enables the validation of new *in vitro* test methods when there is a lack of non-compliant batches originating from disturbances in the routine vaccine production process due to quality systems and GMP that are in place at the manufacturer. Finally, it is recognized that cooperation between scientists in academia, industry and regulatory institutes is key to move the transition to *in vitro* vaccine testing forward by sharing of products, knowledge and technologies.

9. Expert opinion & five-year view

According to the most recent report on animal use for scientific purposes in the European Union [3], more than a million animals were used in 2017 for regulatory batch testing of medicinal products, predominantly for potency assessment. Furthermore, over 264,000 animals were involved in batch potency tests that were classified as causing severe pain and distress. Replacement of *in vivo* potency and safety batch tests of vaccines by *in vitro* alternatives would reduce the number of animal tests considerably. In particular, batches of inactivated and toxoid vaccines are still assessed for potency and safety using *in vivo* methods (Supplementary Table 1). It is clear that many of these animal models have limitations

including ethical issues, disputed relevance, high variability and high costs, and are therefore in need of replacement.

Improvement of vaccine potency assessment in animal models in terms of both animal welfare and test performance was initially made by introducing humane endpoints and potency tests based on serology rather than challenge. Moreover, some *in vivo* batch potency tests have nowadays been replaced by *in vitro* antigen quantification methods. Nevertheless, replacing *in vivo* batch potency tests for *in vitro* antigen quantification may not be sufficient to ensure all aspects of vaccine quality. With respect to safety testing, many *in vitro* alternatives are already available or at an advanced level of development. Furthermore, some toxicity tests have been or will be waived globally by regulatory authorities since these are no longer considered relevant [72]. Product-specific validation and implementation of existing *in vitro* safety tests will be most important for the coming years to achieve safety testing without the need of animal tests.

As described in this review, increasing evidence shows that properties like vaccine composition, spatial organization of the antigen, and the presence of additional immunostimulatory molecules may be critical for vaccine quality and may thus require testing. One-to-one replacement of an *in vivo* test by an *in vitro* alternative will therefore often not be possible. A combination of physicochemical, immunochemical and cell-based assays may be required to demonstrate batch-to-batch consistency of multiple vaccine properties. In the past few years, studies have explored mass spectrometry to characterize vaccines, and cell-based assays as candidates to evaluate vaccine-induced immune reactivity. In the years ahead, these methods should receive further attention to ensure that replacement of *in vivo* potency tests will evolve beyond the development of suitable antigen quantification methods only. Obstacles for the development of *in vitro* vaccine potency tests, like the presence of emulsion or aluminum salt adjuvants, are being overcome. Several studies have now demonstrated that desorption and extraction methods can be applied to remove adjuvants to enable *in vitro* potency testing. However, a remaining objective is to investigate the extent to which desorption and extraction methods alter vaccine properties that require testing in comparison to the untreated final batches. Finally, regulatory guidelines for the development of altered batches that mimic non-compliant vaccine batches are needed to make sure that new *in vitro* methods are properly validated.

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