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PERSPECTIVE

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Rational arguments for regulatory acceptance of consistency testing: benefits of non-animal testing over *in vivo* release testing of vaccines

Marcel H.N. Hoefnagel[a], Paul Stickings[b], Dean Smith[c], Carmen Jungbäck[d], Wim Van Molle[e] and Lorenzo Tesolin[e]

[a]Quality Department, CBG-MEB (Medicines Evaluation Board), Utrecht, The Netherlands; [b]Medicines and Healthcare products Regulatory Agency, Hertfordshire, UK; [c]Bacterial and combination vaccines division, Health Canada, Ontario, Canada; [d]International Association for Biological Standardization for Europe (IABS), Lyon, France; [e]Quality of Vaccines and Blood Products, Sciensano, Brussels, Belgium

ABSTRACT

Introduction: There are rational arguments to replace existing *in vivo* potency and safety assays for batch release testing of vaccines with more advanced non-animal techniques to measure critical quality attributes. However, the introduction of *in vitro* alternatives to replace *in vivo* release assays of authorized vaccines is challenging.

Areas covered: This report describes the hurdles encountered in substituting *in vivo* assays and ways to overcome these and provides arguments why more advanced *in vitro* alternatives are superior, not only as a tool to monitor the quality of vaccines but also from a practical, economical, and ethical point of view. The rational arguments provided for regulatory acceptance can support a strategy to replace/substitute any *in vivo* batch release test if an appropriate non-animal testing strategy is available.

Expert opinion: For several vaccines, *in vivo* release assays have been replaced leading to an optimized control strategy. For other vaccines, new assays are being developed that can expect to be introduced within 5–10 years. From a scientific, logistical, and animal welfare perspective, it would be beneficial to substitute all existing in vivo batch release assays for vaccines. Given the challenges related to development, validation, and acceptance of new methods, and considering the relatively low prices of some legacy vaccines, this cannot be done without government incentives and supportive regulatory authorities from all regions.

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3R; assay variability; consistency approach; *in vivo* assays; potency testing; batch release testing; reverse characterization; vaccines

1. Introduction

The increased availability of advanced analytical techniques allows the development of *in vitro* tests to replace/substitute the historically developed *in vivo* tests used in safety and potency batch release testing of vaccines. A vast effort was made in the past years to develop *in vitro* methods for release testing of vaccine batches, and the control strategy for many vaccines is now entirely comprised of non-animal methods. More and more tests are reaching a stage of technological readiness to allow moving from *in vivo* final product testing to a control strategy based on animal-free tests. However, to replace a control strategy based on *in vivo* testing, generally, an extensive data package will have to be presented in variations to the existing marketing authorizations. Because, for legacy vaccines, *in vivo* assays have been the approved methods for a long time (decades in many cases), some regulators may be reluctant to accept the new batch release strategy. Contrary to legacy vaccines, many recently approved vaccines do not include an *in vivo* test (for potency and/or safety) in the batch release specification. These vaccines and their manufacturing processes are much better characterized as modern pharmaceutical development requires a much better understanding of processes and products. For the drug substance/drug product (DS/DP), a quality target product profile (QTPP) and critical quality attributes (CQA) have to be established, and process understanding should be demonstrated [1]. Using the QTPP, a control strategy can be defined based on a consistent manufacturing process and justified release testing specification. This is in line with the consistency approach for the replacement of *in vivo* testing [2,3]. The consistency approach for batch release testing of established vaccines promotes the use of *in vitro*, analytical, non-animal-based systems allowing the monitoring of quality parameters during the whole production process.

2. Hurdles

The replacement of an *in vivo* batch release test with non-animal tests is generally not straightforward, and numerous hurdles that prevent or hamper the introduction of alternatives have been identified [4,5]. Romberg et al. [4] report several psychological and regulatory hurdles that are listed in Table 1. For all these arguments to refrain from replacement, valid counterarguments can be given (Table 1). These counterarguments are further alluded to in this report. Weißer and Hechler [10] and Van den Biggelaar and coworkers [5] further suggested that 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. This is a valid suggestion because replacement of *in vivo*

CONTACT Marcel H.N. Hoefnagel mh.hoefnagel@cbg-meb.nl Quality Department, CBG-MEB (Medicines Evaluation Board), Graadt van Roggenweg 500, Utrecht 3531 AH, The Netherlands

Article highlights

- Replacement of *in vivo* QC (Quality Control) *assays* is rational and driven by better science, not just (valid) ethical concerns, and should be encouraged by regulatory authorities.
- *In vitro* tests are superior to *in vivo* assays for quality control of vaccines. Due to their inherent large variability and poor discriminative power, *in vivo* batch release assays are less suited to monitor process consistency. Furthermore, they are costly, and the lead time for *in vivo* testing is often several months with practical implications.
- For vaccines initially authorized with an *in vivo* potency assay for batch release, there should be a commitment to replace this with an *in vitro* method in due time.
- Due to the challenges and considerable effort involved substitution of long existing *in vivo* assays for legacy vaccines, this cannot be done without government incentives and supportive regulatory authorities.
- Despite the many practical and regulatory hurdles, for several existing vaccines, *in vivo* assays have been successfully substituted with *in vitro* assays, leading to an optimized control strategy.

testing is time-consuming and costly. These costs can be recovered over time through savings achieved with the routine use of a non-animal testing strategy.

The review by Van den Biggelaar et al. [5] mentions several technical hurdles encountered and expected which are listed in Table 2. For several of these, there are potential ways of solving them as briefly indicated in Table 2, and this is further alluded to in this report.

3. Examples of successful replacement or deletion of *in vivo* tests

Despite these hurdles, there are several examples of successful introduction of testing strategies to replace *in vivo* batch release tests, especially in recent years:

- The *in vivo* potency for the *Haemophilus influenzae* type b vaccine has been replaced by a physicochemical test, especially because of its poor performance. The WHO Technical Report on this vaccine states: *in light of the limited value of the mouse immunogenicity test, physicochemical testing of Hib conjugate vaccines is particularly important to ensure the consistency of manufacture of the batches* [15]. This is also a good example of how poorly representative *in vivo* potency tests can be for the human immune system.
- The *in vivo* mouse serology assays for potency testing of hepatitis A and B vaccines have been replaced with Antigen content tests [16,17].
- The histamine sensitivity test for pertussis has been replaced by the *in vitro* CHO cell clustering assay [18,19].
- The diphtheria-specific toxicity test in guinea pigs has been replaced with an *in vitro* Vero cell assay for testing diphtheria toxoid [20,21].
- Replacement of the serological *in vivo* potency test for vaccines containing inactivated Newcastle disease virus (NDV) with an ELISA-based quantification of hemagglutinin-neuraminidase content of the vaccine [22,23].
- Replacement of the *in vivo* potency test for rabies with an ELISA for quantification of the viral G-protein [24–27].
- Replacement of the *in vivo* potency test for poliomyelitis vaccine (inactivated) by an *in vitro* D-antigen ELISA [28], albeit that the *in vivo* potency test is still mentioned in the monograph alongside the D-antigen ELISA as alternative [29].
- Replacement of the monkey neurovirulence test for the three types of the Sabin live poliovirus, by MAPREC (mutant analysis by PCR and restriction enzyme cleavage) [30,31].

Table 1. Psychological and regulatory hurdles for replacement/substitution of *in vivo* methods reported by Romberg et al. [4] and counterarguments as discussed in the current paper.

Hurdle	Counterargument
'Comfort' with the current *in vivo* test, which seems to work well (as vaccine failures are rare).	There is sufficient evidence that *in vivo* assay in fact suffer from poor precision and high variability making them unsuitable for use in a routine control strategy where the main objective is to demonstrate batch-to-batch consistency [6,7] (and references therein). The fact that vaccine failures are rare is more because of the consistent manufacturing process and its control that ensure product quality.
Public health impacts-concerns over consequences that would be associated with release of sub-potent medicinal products. Current conventional products were authorized using the established *in vivo* test.	Quality control with modern analytical (non-animal) tests is likely to be superior to existing animal tests for the purpose of monitoring manufacturing consistency and identifying substandard batches and is the accepted strategy for most newly introduced vaccine.
Conventional wisdom: validation would require that a new assay is directly compared against the existing animal test, which is costly and for veterinary vaccines this includes trials in target species.	The regulatory approach has changed in recent years (as mentioned in European Pharmacopoeia monographs and EMA Guidelines [8,9]. New clinical trials are no longer required when *in vitro* tests shall replace *in vivo* tests for QC purpose and the inherent variability of many *in vivo* assays means that direct comparison with an *in vitro* assay to demonstrate a correlation is not scientifically justified.
Regulators want a single format for all manufacturers. Current industrial and regulatory dogma calls for a single assay to determine product potency.	This approach requires more and more reevaluation. The variety of products, especially so-called modern products, does not allow a single format testing of all products. This is also recognized in the European Pharmacopoeia text on substitution of *in vivo* methods [8].
Unilateral acceptance by one regulatory body will not incentivize the industry and acceptance of a new approach must be global. Manufacturers are reluctant to invest in an alternative test without assurance of regulatory acceptance.	There is strong effort on a better communication and harmonization of decisions involving all competent authorities and organizations (e.g. ICH, EDQM, ICMRA, and WHO)*.

Note: *(ICH= International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, EDQM= European Directorate for the Quality of Medicines, ICMRA= International Coalition of Medicines Regulatory Authorities, WHO = World Health Organization).

Table 2. Technical hurdles encountered and expected as reported by Van den Biggelaar et al. [5] and counterarguments or potential solutions as discussed in the current paper.

Hurdle	Counterargument/Potential Solution
It is difficult to mimic vaccine-induced immune responses using *in vitro* test methods	Animal *in vivo* testing is also a model for the immune response in humans. For sufficiently characterized vaccines, *in vitro* testing of CQA related to potency/safety are state of art for more recently developed and authorized vaccines
Knowledge about critical quality attributes of vaccines and critical process parameters of vaccination is often limited	Only true for legacy vaccines, but reverse characterization will allow to obtain this knowledge [6,7]. Efforts on e.g. diphtheria and tetanus have started in the last decades, and publications demonstrate good progress [11–14]
Measuring vaccine properties of adjuvanted vaccines is complex	Testing can be performed at stages prior to adjuvant addition, there is the option of desorption and not all assays show interference of e.g. aluminum adjuvant
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	This is not always needed, and there are accepted strategies to obtain these when needed e.g. as indication in the European Pharmacopoeia text on substitution of *in vivo* methods [8]

- Replacement of *in vivo* testing for adventitious agents by nucleic acid amplification techniques (NAT) and Next-Generation Sequencing methods (NGS) [32–35].

Efforts to replace other legacy *in vivo* methods are continuing and include:

- The replacement of the tetanus-specific toxicity test by the BINACLE assay for *in vitro* detection of active tetanus neurotoxin in toxoids is currently being validated as part of the European Biological Standardization Programme [36].
- The replacement of the *in vivo* potency assay of human rabies vaccines with an ELISA test (BSP148) [37–39].
- The replacement of the diphtheria toxoid and tetanus toxoid *in vivo* potency assays with an ELISA for antigen quantification [13,14,40,41] and evaluation of a cell-based assay cell-based *in vitro* assay for testing of immunological integrity of tetanus toxoid [42].
- Physicochemical characterization of the tetanus and diphtheria toxoids [43,44]
- The development of a cell-based potency assays for the tick-borne encephalitis vaccine (TBEV) and inactivated infectious bronchitis virus (IBV) and Newcastle disease virus poultry vaccines [45–47].

The above-mentioned studies show the feasibility of 'everse characterization'for legacy vaccines and that based on these characterization studies, *in vivo* potency tests for batch release can be replaced or substituted. Successful examples for the introduction of alternative potency tests mentioned earlier above involved antigens that are less complex than others still to be tackled. Even then, these prior examples of replacing the existing *in vivo* tests required a strategy that involved additional characterization studies, and the ongoing efforts for vaccines such as tetanus and diphtheria further illustrate that these studies are time-consuming because they include both the physicochemical characterization and development of assays to measure content and ensure (antigenic) integrity.

Apart from replacement by a non-animal method, other approaches have also led to the removal of *in vivo* testing from the batch release testing panel from vaccines. For instance, a risk assessment was introduced as a basis of the testing strategy for the test of extraneous agents in viral vaccines for human use instead of the test on adult mice and guinea pigs [33,48].

Moreover, several *in vivo* assays related to safety testing of vaccines have been removed in recent years from the batch release testing panel because there was no added value of the assay. These tests had been introduced at a time when scientific knowledge was limited. However, over time, it had become apparent that there was no scientific justification for these tests in ensuring the safety of the vaccines as they did not measure what they were expected to do. A good example is the Abnormal Toxicity Test (ATT) aka General Safety Test; the Expert Committee on Biological Standardization (ECBS) recommended the discontinuation of this test in routine testing in all future WHO Recommendations, Guidelines, and manuals for biological products published in the Technical Report Series and that a clear indication be made in its report that the inclusion of this test in previously published WHO Technical Report Series documents be disregarded [49]. The ATT was also removed from the Ph. Eur. monographs because of lack of scientific relevance [50]. Also, the pertussis toxoid irreversibility test and the requirement to test the final lot for residual toxin were removed from the monograph of the European Pharmacopoeia [51,52].

The first incentive for the replacement and deletion of the *in vivo* test from the panel of batch release tests for vaccines may initially have been 3 R considerations. However, currently, it is more and more recognized that in a state-of-the-art control strategy, the analytical and *in vitro* methods are superior to *in vivo* methods, as discussed in the next section.

4. Superiority of *in vitro* techniques/disadvantages of *in vivo* assays

At the time many legacy vaccines (e.g. diphtheria, tetanus, and pertussis vaccines) were developed, the analytical capabilities were limited and *in vivo* assays were the methods of choice for safety and potency testing for batch release. However, with currently available physicochemical, immunochemical, and *in vitro* cell-based methods, all CQA of vaccines can generally be identified and sufficiently controlled such that *in vivo* testing is no longer needed to ensure vaccine quality. Occasionally, vaccines are still brought to the market that require *in vivo* potency testing for batch release because the *in vitro* potency testing is not fully developed and/or validated [53], but it is

expected that there is the intent and commitment of implementing an *in vitro* method [54].

There are sound scientific rationales that provided a mandate for the replacement/substitution of existing *in vivo* assays with appropriately designed *in vitro* alternatives because of the disadvantages of *in vivo* tests.

A major disadvantage of the *in vivo* assays, especially for potency, is their inherent variability ([6,7], and references therein) and the resulting poor precision and discriminative power. This variability (with a CV of up to 101%) makes these assays less suitable to monitor production consistency, which is also reflected in the relatively wide acceptance criteria for *in vivo* potency tests for batch release. This also has several practical implications including the need for manufacturers to use a relatively high target antigen content to ensure that the potency test lower limit is met. Despite these high antigen content targets, *in vivo* potency tests still occasionally need to be repeated due to the variability of the assay. Moreover, the lead time for *in vivo* testing is around 2 to 2.5 months. This has led to supply shortage in cases of re-tests or production problems as reported by industry for some products [50]. In addition, the qualification and replacement of the reference vaccine are time-consuming and costly with regard to animal use. Also, the effect of aging of the reference vaccine can be a major problem with *in vivo* testing, as this can result in a drift in potency results over the years [55].

Based on these issues, it can be considered that analytical, immunological, and *in vitro* tests are superior with respect to monitoring manufacturing consistency. A good example is the G-Protein-based ELISA assay for potency testing of Rabies vaccine which has a very high precision (95% confidence interval between 93% and 107%) compared with the NIH method for which the European Pharmacopoeia defined confidence limits of 25–400% [25]. Furthermore, when the product has been appropriately characterized, suitable *in vitro* methods monitor all relevant CQA with sufficient precision that any deviations in manufacturing are more likely to be identified early, before the final potency test, which is more efficient. It is sometimes argued that *in vitro* methods are not a complete immune system [56,57] and therefore *in vivo* testing would be better. However, *in vivo* testing in animals like mice or guinea pigs is only a model and often a poor representative for the human immune system. Good examples include the Hib conjugate vaccine, for which the *in vivo* assay was replaced because this mouse immunogenicity test could not ensure consistency of potency [15] and the Histamine sensitization test (HIST) for Pertussis vaccines [18,19,58]. HIST does not directly measure the toxicity of pertussis toxins and has recently been deleted from the Ph. Eur., since it was determined to be of no benefit within the product control strategy. This is another argument in favor of a control strategy measuring CQAs based on understanding of the immune system and the technical knowledge of the antigen.

Another example of superiority of *in vitro* methods is the *in vivo* adventitious virus testing for the presence of adventitious viral agents in biopharmaceuticals produced from animal or human cells which is applied to cell banks and as an in-process and lot release test to detect adventitious viruses. In comparison to the *in vitro* virus test methods, the *in vivo* adventitious virus tests have a higher rate of both false positives and false negatives [59]. These false positives or false negatives can take months to resolve, similar to batches failing in an initial *in vivo* potency batch release test. Furthermore, NGS methods have shown a higher sensitivity for the detection of adventitious agents than the *in vivo* methods [32,60].

There are also several practical considerations to prefer *in vitro* methods for batch release testing of vaccines. First, in general, *in vivo* tests take a long time to complete, several tests take more than a month, where analytical or *in vitro* tests can generally be done within days. This long time needed for testing has implications for the availability of vaccines, especially in situations where shortages exist. This also has economic implications for the manufacturer because the vaccines cannot be released and sold until testing is completed. Another economic consideration is the cost of *in vivo* tests, which are generally more expensive than non-animal methods. As a result of the variability of the *in vivo* tests, large numbers of animals are needed to establish their precision (Coefficient of Variance) [6,7].

In conclusion, based on the arguments provided, there are rational considerations, other than ethical concerns (3 R) to avoid *in vivo* assays in batch release testing of vaccines and to replace existing *in vivo* tests with *in vitro* alternatives.

5. Changes in regulatory guidance in Europe in support of introducing *in vitro* tests as replacement for *in vivo* tests

The value of the replacement of *in vivo* batch release testing by non-animal methods has also been recognized by European and International Regulators, and several regulatory documents were published to support the introduction of non-animal methods [8,9,61,62]. These documents provide guidance on how to deal with specific hurdles encountered for the replacement or substitution of *in vivo* release assays for vaccines.

6. Strategies to overcome hurdles/difficulties

As referred to in the introduction (Tables 1 and 2) several hurdles have to be overcome before an existing *in vivo* assay can be replaced. These hurdles are partly dependent on the strategy of replacement of the *in vivo* assay. In the case of a one-to-one replacement of e.g. a potency assay, it should be demonstrated that the new assay's performance is equal to the existing assay. A good example is the replacement of the *in vivo* Histamine Sensitization Test (HIST) for residual toxicity in acellular pertussis vaccines by the Chinese hamster ovary (CHO) cell clustering assay [18].

The potency assay(s) is expected to measure the integrity of the antigen by targeting epitope(s) relevant to the protection offered by the vaccine, and the epitope(s) should preferably be conformational in order to have a stability indicating assay [8]. In the case of the replacement of the Newcastle disease virus (NDV) *in vivo* test, it was successfully demonstrated that the protective serological response after immunization highly correlates with the hemagglutinin-neuraminidase (HN) and fusion (F) proteins content of the

vaccine [22]. In some vaccines, the antigen has to be in a specific conformation to elicit a protective immune response [63], and the potency assay should capture that conformation. However, for many vaccines, the real challenge is in identifying the protective epitope which is often not (fully) known and the protective mechanism is not (fully) elucidated (e.g. [64]). It should also be noted that not only the protective epitope related to the humoral response is important but also the T-cell response is generally an important aspect of the immune protection [65,66]. The potential limitations of an *in vitro* approach to measure potency must be viewed with the knowledge that *in vivo* potency assays may be a poor representation of the human immune response. Despite the challenges faced when developing an *in vitro* potency assay, almost all recently developed vaccines come to the market with an *in vitro* potency assay for batch release.

It is recognized that an *in vitro* assay will very likely measure a different quality attribute than the *in vivo* assay, and in view of the variability of the *in vivo* assays, it may not be scientifically justified to establish a correlation between the existing *in vivo* method and the proposed *in vitro* assay. This has been recognized by regulators, and the European Pharmacopoeia [8] has published a chapter on a strategy to deal with situations where it is not possible to show agreement between the *in vitro* and *in vivo* methods due to low discriminating power and/or high variability of the *in vivo* method. It is assumed that the product has a well-established safety and efficacy profile with a consistent manufacture. Furthermore, the design of the *in vitro* assay (for potency) has to reflect both antigen content and functionality because *in vivo* potency assays generally cover both content and functionality. If this cannot be achieved by a single assay, the strategy used may be a substitution with multiple assays. Notwithstanding, the *in vitro* method(s) should provide the same level of confidence in the control of safety and efficacy of the product. To establish such confidence, all CQAs related to safety and efficacy have to be known. For legacy vaccines (e.g. diphtheria, tetanus and acellular pertussis (DTaP)) that have been developed at a time when their analytical capabilities were limited, it may be needed to identify the CQAs with extensive characterization. Based on that 'reverse characterization a (panel of) test(s) can be proposed to substitute the *in vivo* potency or safety batch release test.

Clearly, such characterization effort is time-consuming and costly, and manufacturers will only be interested if there is sufficient confidence that the substitution effort will be successful and (globally) accepted by the regulators. In that frame, the IMI VAC2VAC project was established in which a public–private consortium of 22 partners collaborated in the development of *in vitro* assays that will support regulatory acceptance of the substitution of the *in vivo* assays for established vaccines where potency and/or safety batch release testing in animals is currently still required [67]. Only in such consorted approach, it will be feasible to develop a control strategy without *in vivo* batch release testing for these legacy vaccines.

Another hurdle mentioned is that regulators may prefer a single format for all manufacturers of the same type of antigen (e.g. diphtheria toxoid). Indeed, currently all manufacturers are expected to perform the same type of *in vivo* potency test for batch release of e.g. diphtheria toxoid (DT) and tetanus toxoid (TT) compared to a standard reference preparation. However, there are wide variations in the *in vivo* approaches used and currently accepted for testing of a specific antigen in vaccines of different manufacturers (challenge or serology test using Single or Multiple dilutions, performed in mice or guinea pigs, etc.; in any combination)

Although it would be preferable to have a single format for all manufacturers, especially for control laboratories that often test the same antigen from different vaccines, this should not be a requirement for individual products. This is also recognized by the European Pharmacopoeia [8]. This pharmacopoeia general text has more detailed information on substitution of *in vivo* tests for release testing and also on more practical considerations, such as on non-conformant or subpotent samples, to compare the *in vitro* and *in vivo* methods. Such samples may not be available due to the well-maintained production consistency of the established vaccines. Therefore, it can be accepted that the method is validated using samples of different concentrations and samples subjected to different stresses can be used to assess the stability indicating potential of the method.

Another practical hurdle is the presence of aluminum adsorbed to antigens in several adjuvanted vaccines that may interfere with, for example, immunochemical potency assays. This can be overcome be different approaches, e.g. desorption, provided it can be demonstrated that this does not impact the antigen quality/integrity [68,69]. If no appropriate method can be developed to deal with aluminum interfering with the *in vitro* potency test, it may be acceptable to perform potency release testing prior to adsorption, as long as it is demonstrated that the content and quality of the antigen is representative of the antigen in the final drug product. The presence of an adjuvant such as aluminum is likely to have a potentiating effect *in vivo* and will be reflected in the assay response from an *in vivo* potency assay. Such a potentiating effect may not be measured by an *in vitro* potency assay, and it may be necessary to develop non-animal methods that are capable of measuring the potentiating effect of an adjuvant in the vaccine. For example, inflammasome activation may be used to measure adjuvant biological activity as an important quality attribute for control or characterization of aluminum-based adjuvant and a cell-based quantitative *in vitro* assay of NLRP3 inflammasome activation as a readout for vaccine adjuvant (aluminum) biological activity was recently developed [70]. Even if not required for routine batch control, such non-animal methods to measure adjuvant activity can still be valuable as a characterization tool during non-clinical development of a vaccine. For routine batch control testing, the use of physicochemical tests (in addition to an *in vitro* potency test) that measure adjuvant content and the degree of antigen adsorption are likely to be sufficient for monitoring manufacturing consistency.

7. Conclusion & discussion

The information provided in this report can be used in the design and argumentation of a rational strategy for replacement of an individual *in vivo* batch release test for vaccines that should be acceptable for the Regulatory Authorities. Several examples

illustrate that replacement of *in vivo* tests can be achieved, also sometimes using a strategy that is not in line with the conventional approach for a one-to-one replacement. The examples described show that generally, the introduction of *in vitro* batch release testing is an improvement in the control strategy because the introduced *in vitro* tests are much more suited to monitor manufacturing consistency than *in vivo* assays. Despite this, there are still several *in vivo* batch release tests that have not been substituted by an *in vitro* alternative. Even though most successes to date are for relatively less complex antigens/vaccines, considerable effort was needed to achieve the outcome (e.g [16,17]). All of these efforts and the changes we have seen so far help to promote the concept of moving from a control strategy with an *in vivo* potency test to one without any animal test. This, combined with advances in analytical techniques, can be an incentive to tackle the more complex antigens/vaccines that remain to be addressed. For some *in vivo* batch release tests, the *in vitro* alternatives are in mature state of development, but additional work is needed to introduce the *in vitro* alternatives (DT and TT [13,14]), whereas for others the *in vitro* alternatives are close to introduction (e.g. tick-borne encephalitis virus vaccine [71]).

It is noted that some alternatives have only been accepted by a part of the jurisdictions [71]. Therefore, manufacturers may still be reluctant to develop on alternatives for the existing *in vivo* batch release assays due to their limitations. However, given the superiority of *in vitro* methods to control the quality of vaccines, regulators could be expected to give more incentive to manufacturers to substitute the *in vivo* assays. Current incentives may be more motivated from an ethical or 3 R perspective, but it is clear that *in vivo* methods should also be discouraged from a scientific perspective when the purpose is to demonstrate batch-to-batch consistency as part of routine quality control point.

8. Expert opinion

Although *in vivo* models for immunogenicity/potency are likely to remain important for non-clinical development of vaccines, they are not the right tool for ongoing monitoring of production/batch consistency once clinical performance is established. From a quality control perspective, *in vivo* potency assays for vaccine batch release should be avoided where possible because of their limitations. These include the relatively poor precision, the high cost, and long lead time. Still, sometimes, a vaccine is initially authorized with an *in vivo* potency assay for batch release because a suitable *in vitro* method is not yet fully developed or validated. In such cases, there should be a commitment to replace this assay with an *in vitro* method in due time. Equally, it would be beneficial to substitute existing *in vivo* assays for legacy vaccines including the diphtheria, tetanus, pertussis, and polio vaccines. In our view, none of the many hurdles that have been identified to complicate substitution or replacement of *in vivo* assays will prove to be prohibitive. That does not suggest that the introduction of *in vitro* methods can be done lightly. Substitution of *in vivo* assays still requires appropriate characterization studies which may be challenging and cumbersome especially for complex antigens such as toxoids, or whole cells as in the case of whole-cell pertussis vaccine. However, this extensive characterization will also be the basis for the superior control strategy and its justification. After all, the introduction of the *in vitro* methods will also include some uncertainty because there will not necessarily be a like vs. like replacement.

The replacement of *in vivo* tests by non-animal alternatives is likely to be facilitated by the rapid development of (game-changing) novel analytical technologies, like we have seen for NGS and the replacement of some *in vivo* adventitious agent tests [31–35]. Similar impact may be expected from novel technologies like organ-on-a-chip, advancements in culturing different cell types, novel gene expression assays, single-cell genome analysis. Such advances may allow the development of potency assays that can monitor quality attributes more directly related to the mode of action. In addition, analytical techniques including mass spectrometry and flow field fractionation are advancing rapidly allowing more detailed physicochemical analysis of complex biopharmaceuticals, which can further facilitate the development of analytical tools to monitor specific CQA of vaccines to ultimately replace existing *in vivo* release assays.

Due to the challenges related to replacing existing *in vivo* batch release assay ('reverse characterization' for identification of Critical Quality Attributes including those related to the Mode of Action and the development and validation of assays), individual companies may be reluctant to start on such uncertain and costly endeavor. Therefore, pre-competitive collaboration or public–private partnerships like the IMI VAC2VAC project (www.vac2vac.eu) are needed to further support the development of *in vitro* methods to substitute *in vivo* assays.

However, given the progress that has been made with the development of *in vitro* methods and the characterization of the various legacy vaccines [13,14,36–47], it can be expected that most (if not all) of the *in vivo* potency batch release assays for vaccines will be phased out in the coming 5 to 10 years. This will require the regulatory authorities of all regions to support this and recognize the superiority of *in vitro* potency methods for the routine quality control of vaccines. For globally operating vaccine manufacturers, the acceptance of an *in vitro* approach for routine batch release testing by regulatory authorities in some, but not all, jurisdictions will lead to significant logistical problems because of the need to maintain two different testing programs in parallel. This could slow down the phasing out of *in vivo* tests for routine batch testing, delaying the introduction of a more advanced control strategy.

Abbreviations

ATT Abnormal Toxicity Test; CQA Critical Quality Attributes; DS Drug Substance; DT Diphtheria toxoid; DP Drug Product; HIST Histamine sensitization test; NGS Next-Generation Sequencing; QC Quality Control; QTPP Quality Target Product Profile; TT Tetanus toxoid

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Declaration of interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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