Substitution of *in-vivo* Method(s) by *in-vitro* Method(s) for the Quality Control of Vaccines

Purpose

The purpose of this general chapter is to provide guidance to facilitate the implementation of *in vitro* methods as substitutes for existing *in vivo* methods, in cases where a typical one-to-one assay comparison is not appropriate for reasons unrelated to the suitability of one or more *in vitro* methods. This general chapter will not discuss the details of assay validation as such, since those principles are described elsewhere.

The general chapter applies primarily to vaccines for human or veterinary use; however the principles described may also apply to other biologicals such as Antisera/Immunosera.

Context

The test methods used for routine quality control of vaccines are intended to monitor production consistency and to ensure comparability of the quality attributes between commercial batches and those batches originally found to be safe and efficacious in clinical studies or, for veterinary vaccines, in the target species.

While the *in vivo* potency and safety assays described within Indian Pharmacopoeia (IP) vaccine monographs have historically played a central role in safeguarding the quality of vaccines, the inherent variability of *in-vivo* assays can make them less suitable than appropriately designed *in vitro* assays for monitoring consistency of production and for assessing the potential impact of manufacturing changes. As a result, it is essential continually to challenge the scientific value and relevance of these *in vivo* test methods. When *in vivo* tests are found to be of limited or no value, it is imperative to eliminate them, given the ethical considerations and the obligations under the relevant conventions. In addition, there is a substantial effort to develop *in vitro* methods (including immunological, molecular and physico-chemical tests) to replace the animal tests. In several cases this has led to the successful introduction of new *in-vitro* methods in vaccine monographs. The use of appropriate *in-vitro* methods not only reduces the use of animals while maintaining or improving the scientific relevance of the assays involved, but also substantially reduces assay variability and the time and resources required, and enhances the predictability of the release of safe and effective vaccine lots for use.

In addition to the benefits resulting from the substitution of appropriate *in-vitro* methods for existing *in-vivo* methods, under the ‘Prevention of Cruelty to Animals Act, 1960' and `CPCSEA Guidelines' so as to prevent the infliction of unnecessary pain, suffering and prevention of cruelty to animals, the IP Commission has committed to the reduction of animal usage wherever possible in pharmacopoeial testing. Alternative methods of analysis may be used for control purposes, provided that the methods used are shown to give results of equivalent accuracy and enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used.

General Considerations

One of the consequences associated with the inherent variability of *in-vivo* assays is the problem this poses with their replacement by the more-consistent *in-vitro* methods, which typically requires one-to-one assay comparison. This can be a challenge in some cases as repeated efforts through multicentre international collaborative studies can fail due to the variability inherent in the *in-vivo* methods. Another consideration is that many *in-vivo* safety and potency assays for vaccines were generally shown to be fit for purpose and have historically proven their value in ensuring the efficacy and safety of vaccines. However, this was in an era when validation requirements and guidelines were not in place, making a formal one-to-one comparison challenging or even impossible in some cases. Since precision, reproducibility, limits of detection and quantification were not established for the *in-vivo* method, the comparability of one method to another becomes difficult to evaluate.

When considering the transition from an *in-vivo*-based to an *in-vitro*-based quality control assay system, it is important to understand what *in vivo* assays can and cannot offer. Although properly established *in-vivo* potency assays in laboratory animals have the potential to measure complex functional responses for demonstrating proof of concept, these do not necessarily predict the actual responses in the target population. In addition, *in-vitro* bioassays have the potential to mimic specific elements of complex *in-vivo* responses with generally lower variability and higher sensitivity.

Another key consideration is that when an *in-vivo* test for a given product is to be replaced with an *in-vitro* test, the quality attribute(s) of the product will likely be assessed differently. Examples include: the determination of antigen content or a functional response (*e.g.* virus or toxin neutralisation) in an *in-vitro* bioassay instead of *in-vivo* potency; molecular consistency instead of *in-vivo* neurovirulence or attenuated phenotype; absence of the extraneous agent genomes using molecular methods instead of absence of micro-organisms through *in-vivo* testing; minimum titer of virus or bacterial count per dose instead of *in-vivo* potency test, Endotoxin testing instead of Pyrogen testing and demonstration of toxin binding and enzyme activity instead of *in vivo* specific toxicity. As a consequence, a demonstration of agreement between the two methods is generally not scientifically justified and should not always be expected. Even where pass/fail results from the two test procedures are in agreement, the correlation between two quantitative methods across the assay range may still be low regardless, the *in-vitro* method(s) or testing strategy must provide at least the same confidence that the key quality attributes, which are necessary to ensure the consistency of a product's safety and effectiveness, are adequately controlled. ~~However, wherever possible correlation between *in vivo* and *in vitro* methods shall be established but not limited to.based on scientific justification.~~

~~While t~~The focus of this general chapter is to provide guidance on the development and replacement of existing methods for approved products, it is important to consider the use of *in-vitro* methods for quality control during product development. ~~and to understand that the use of *in vivo* assays is not mandatory.~~

Alternative approaches for the substitution of *in-vivo* methods

The primary focus for the implementation of any proposed *in-vitro* methods within a quality control system should be of the scientific relevance of *in-vitro* assays for control of the relevant quality attributes. Additionally, any *in-vitro* methods will have to meet the current validation requirements.

In the IP, replacement of *in-vivo* assays or alternate approach for in-vivo methods were done by analysing the data submitted or in reference to suitable international guidelines~~for vaccines are typically replaced by *in-vitro* assays following multicentre collaborative studies~~, but this should not be a prerequisite for *in-vivo* assay replacement initiatives for individual products. Additionally, while it may be desirable to have assays that are widely applicable to a class of products, this should not be a requirement.

As explained in the guidance below, in some cases an existing method may need to be substituted by more than one *in-vitro* test, in order to characterize the key qualitative and quantitative attributes measured by the existing test.

In veterinary vaccine, ~~most~~few of the *in-vivo* safety and efficacy testing procedures are performed in target species. Wherever required, laboratory animals can be used instead of target species, if an appropriate *in-vitro* model is not found (eg. Formulation safety).

Potency tests

When 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* assay, the following approach can be used. It is assumed that the product under consideration has a well-established safety and efficacy profile, with consistent manufacturing.

The *in-vitro* test(s) should be able to detect differences that are relevant to the control of the production process as justified scientifically. This should be supported by data demonstrating the capability of the proposed assay(s) to control key quality attributes of the vaccine and maintain the link between the quality of the batches to be released and those batches found to be safe and efficacious through clinical studies or routine use. With the setting of appropriate specifications, the consistency of manufacturing with the *in-vitro* method(s) will be maintained.

The design of an assay/assay system for vaccine quality control needs to reflect both antigen content and functionality. If a single method is used, it should preferably measure the content and integrity of the antigen by targeting epitope(s) relevant to the protection offered by the vaccine. An example of this would be a monoclonal antibody or monoclonal antibodies against an epitope or epitopes as the main target for generating neutralising antibodies. The epitope or epitopes should preferably be conformational in order to have a stability-indicating assay (as is the case for rabies vaccine). In some cases, a single *in-vitro* method may not adequately reflect the content and functionality. This can be remedied through the use of

multiple assays, as is the case with conjugate polysaccharide vaccines, where molecular size, conjugate integrity, and total and free polysaccharides are examples of relevant measures.

To establish quantitative measurements with an *in-vitro* method, samples that differ in the magnitude of the response will be needed. In most cases, samples that are below the minimum approved potency specification with the *in-vivo* method will not be available because production consistency is generally well maintained, and potency between batches does not differ significantly and/or the precision of the *in-vivo* assay is such that it cannot discriminate between batches unless the difference is very large. Therefore, initial assay evaluation should be performed with samples at different concentrations, which could be followed by testing of samples subjected to different types of stress conditions to assess further the stability-indicating potential of the new method. The inability to demonstrate agreement between an *in-vitro* and an *in-vivo* method does not necessarily mean that the *in-vitro* method is not suitable/relevant. In many cases, an *in-vitro* method will detect changes in the product profile that would not be detected by the *in-vivo* method. In such cases, the *in-vitro* method may be considered superior for monitoring the consistency of production and may be more relevant to assess the impact of manufacturing changes.
In case of live vaccines, sufficient data can be generated during developmental stage and clinical trial stage to establish correlation between potency/sero-conversion and virus titer/ bacterial count. In such circumstances, the bacterial count or virus titer can be used as potency test during the lot release of live vaccines.

For approved veterinary vaccines, *in-vitro* methods for potency to be standardised ~~for approved products~~ in correlation with ~~of~~ test results in laboratory condition and field conditions ~~should~~ needs to be established and compared for effective protection in field situation. For new ~~products~~veterinary vaccines, these comparative studies can be undertaken during the field trials of the product under development.
~~-~~In case of human vaccines , in vitro tests were performed to understand the antigenic and immunogenic profile for release a batch under development. ~~For new products these comparative studies can be undertaken during the field trials of the product under development.~~
Various manufacturers have specific formulations and *in-vitro* test development shall be based on product formulation considering the hinderances caused by components in the product. So these *in-vitro* tests developed by different manufacturer will be specific to their product which may cause different playing field for each product / manufacturer. There are various attempts to harmonize the *in-vitro* test~~s~~ing approaches, standardize methods and the variation observed in *in-vitro* test and *in-vivo* test ~~.~~ Hence, studies on larger perspective to **set up** standards of in-vitro tests are required. ~~have golden standards of *in-vitro* tests are required~~.

Safety tests

Specific toxicity
An *in vitro* method for detection of residual toxic components should be specific and at least as sensitive as the existing *in-vivo* method. Where possible, a fully functional *in-vitro* system

should be used (*e.g.* toxin-sensitive cell line). Where no functional *in-vitro* system is available, an *in-vitro* testing strategy could be based on the detection/measurement of more than 1 parameter, sequentially where relevant, that together reflect the mode of action for the toxic components in question. Examples include the use of assays with immunological and biochemical steps to detect receptor binding and enzyme activity. In most cases, where an *in-vivo* assay is to be replaced there will be data available on the sensitivity of that model for detection of the toxin in question. Therefore, new *in-vitro* methods can be characterised to demonstrate that they are sufficiently sensitive using spiking experiments and referring to historic data for the *in-vivo* assay. Such assays, in conjunction with the appropriate time and temperature conditions, could also be used to demonstrate the absence of reversion of a specific toxoid.

For Veterinary Vaccines

Attenuation or avirulence status of established live vaccine strains can be ascertained by the genomic characterisation. Genome sequence analysis of whole genome or g specific genome regions (if attenuation markers are known) can be used to characterize the vaccine strains genotypically. In this case, target animal testing to determine'non-reversal to virulence'and 'general safety in target species' can be avoided for the established attenuated vaccine strains (*e.g.*Canine distemper virus vaccine strain Onderstepoort, PPRV vaccine strain Sungari/96). Established attenuated strain can be a vaccine strain, which was used in the field for minimum of four years without any major safety issues.

For an inactivated vaccine, effective and complete inactivation of virus or bacteria can easily be established by *in-vitro* passaging of virus in susceptible cell line and by absence of bacterial growth in specific media. Laboratory or target animal testing can be avoided in these cases.

Development of validated *in-vitro* methods is encouraged for evaluating the formulation safety. Until that time, the formulation safety can be evaluated in laboratory animals instead of using target ~~animals,wherever~~animals, wherever possible.

Molecular consistency by deep sequencing versus the neurovirulence test

An *in-vitro* genotypic method to assess the molecular consistency of a viral vaccine has the potential to replace an existing *in-vivo* neurovirulence test and other safety tests such as extraneous virus agents in cell substrate or viral vaccine. . A prerequisite for any *in-vitro* genotypic method is an in-depth knowledge of the molecular markers responsible for the attenuation of the live viral vaccine (as is the the case for oral poliovirus vaccine, for example). In such a case, monitoring the consistency of the vaccine lots would be achieved by confirming the presence of the required molecular attenuation markers and percentage of mutants with methods such as deep sequencing.Wherever the molecular markers of attenuation is not known, whole genome sequencing by NGS can be used to establish sequence similarity of the strain at seed level and end of production stage.

Detection of viral extraneous agents by novel molecular methods

Detection of viral extraneous agents in cell banks, seed lots and cell culture harvests are currently conducted using a panel of *in-vivo* and *in-vitro* methods at different stages of the manufacturing process. Novel, sensitive molecular techniques with broad detection capabilities are available, including deep sequencing or high-throughput sequencing methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods(associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. The use of these new molecular methods has highlighted gaps in the existing testing strategy by identifying previously undetected viral contaminants in final product, the cell banks from which it was produced and intermediate manufacturing stages. These new molecular methods (e.g. deep sequencing or high-throughput sequencing) detect genomes while the existing *in-vivo* methods are based on observations of the effects viruses have on experimental animals. The implementation of such new molecular methods as substitutes for *in-vivo* methods requires a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods. For this purpose, an appropriate panel of representative, well-characterised model viruses should be used to assess the ability of the new method to detect viruses that are (or are not) detected by the *in-vivo* methods, and to determine if the sensitivity is at least equivalent to the sensitivity of the *in vivo* methods. This last element is particularly complex since these new molecular methods do not detect the same characteristic of the viral contaminant (genome for molecular methods versus infectious virus for *in-vivo* methods) and also since no or limited validation data exist for the *in-vivo* methods. It should also be emphasised that the outcome of the new molecular methods is not the final result since the detection of a genome or fragments of a genome does not necessarily indicate the presence of an infectious virus.

Extraneous virus testing by cell culture:

Another effective *in-vitro* method for identifying extraneous agents is using cell culture systems. The cell seeds, neutralised virus seeds and neutralised vaccine lots can be checked in cell culture systems *in-vitro*. Haem-adsorbing viruses, Haem-agglutinating viruses and CPE inducing viruses can be detected using cell culture system. Highly permissive cell lines such as Vero, Bovine turbinate cells (for checking extraneousbovine viruses) and IBRS cells (for checking extraneous porcine virus)can be used. Manufacturers are encouraged to use recombinant trypsin instead of porcine trypsin during manufacturing. Wherever recombinant trypsin is used, testing extraneous porcine viruses is not necessary, if the suspected source of extraneous porcine viruses is trypsin.

General Comments from Sanofi-

This text is very welcome in order to help set guidance on substituting in vivo methods by alternative non animal-based methods which allow to introduce more reliable assays to demonstrate product quality, efficacy and safety.

It is much inspired from Ph Eur chapters 5.27 Comparability of alternative analytical procedures and 5.2.14 Substitution of in vivo method(s) by in vitro method(s) for the quality

control of vaccines. Some text additions performed introduce however some confusion or are contradictory to what is actually meant by the reference texts