The Vaccines Consistency Approach Project: an EPAA initiative

De Mattia Fabrizio[1], Hendriksen Coenraad[2], Buchheit Karl-Heinz[3], Chapsal Jean-Michel[4], Halder Marlies[5], Lambrigts Denis[6], Redhead Keith[7], Rommel Eddy[8], Scharton-Kersten Tanya[9], Sesardic Thea[10], Viviani Laura[11], Ragan Ian[12]

ABSTRACT

The consistency approach for 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. By using highly sensitive non-animal methods, the consistency approach has the potential to improve the quality of testing and to foster the 3Rs (replacement, refinement and reduction of animal use) for quality control of established vaccines. This concept offers an alternative to the current quality control strategy which often requires large numbers of laboratory animals. In order to facilitate the introduction of the consistency approach for established human and veterinary vaccine quality control, the European Partnership for Alternatives to Animal Testing (EPAA) initiated a project, the "Vaccines Consistency Approach Project", aiming at developing and validating the consistency approach with stakeholders from academia, regulators, OMCLs, EDQM, European Commission and industry. This report summarises progress since the project's inception.

KEYWORDS

Vaccine quality control, 3Rs, consistency testing, EPAA.

ACKNOWLEDGEMENT

The authors wish to thank EPAA for their encouragement and financial support of the project and of the writing of this publication.

1 De Mattia Fabrizio: MSD Animal Health, PO Box 31, 5830 AA Boxmeer, The Netherlands.
2 Hendriksen Coenraad: Utrecht University, Department of Animals in Science and Society, Faculty of Veterinary Medicine, P.O.Box 80.166, 3508TD Utrecht, Netherlands.
3 Buchheit Karl-Heinz: European Directorate for the Quality of Medicines & HealthCare, Council of Europe, 67081 Strasbourg, France.
4 Chapsal Jean-Michel: Independent.
5 Halder Marlies*: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Systems Toxicology Unit/EURL ECVAM, 21027 Ispra, Italy.
6 Lambrigts Denis: GSK Vaccines, Wavre, Belgium.
7 Redhead Keith: Vaccine & Assay Consultancy, Watford, WD25 OEX, UK.
8 Rommel Eddy: Rommel Consulting Partners, B-1370 Jodoigne, Belgium.
9 Scharton-Kersten Tanya: Novartis Vaccines and Diagnostics AG, Basel, Switzerland (currently: International AIDS Vaccine Initiative New York, New York, USA).
10 Sesardic Thea: Division of Bacteriology, National Institute for Biological Standards and Control (NIBSC). Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom.
11 Viviani Laura: Novartis Vaccines and Diagnostics AG, Basel, Switzerland.
12 Ragan Ian: EPAA Project Coordinator.

*The views, conclusions and recommendations expressed in this article are those of the author and do not necessarily represent views or policies of the European Commission.

1. INTRODUCTION

Quality testing is an essential element of the process by which medicinal products are released for use. Demonstration of consistency in production is the general basis for quality testing, typically by using analytical methods. However, in the case of vaccines, which are complex immunobiological products containing antigens, adjuvants, excipients and preservatives, a different paradigm is used and individual batches (lots) are regarded as unique products; therefore, regulators require that extensive quality testing is performed on each batch of a licensed vaccine, generally by using animal tests for potency and safety, before its release onto the market. The consistency approach offers an alternative to this uniqueness paradigm. It proposes shifting the focus of quality testing from the product to the production process by using intensive in-process testing and modern quality systems (Good Manufacturing Practices, GMP). These tools ensure a consistent production of vaccine and allow the monitoring of the quality of vaccine products by testing and comparing crucial parameters relevant for the efficacy and safety to a reference batch shown to be efficacious and safe in the intended recipient species [1,2]. These crucial parameters are defined in this paper for each vaccine included the project. The consistency approach is not therefore the one-to-one replacement of an *in vivo* method by a non-animal based method designed to bring the same type of information as provided by the *in vivo* method.

The consistency approach is currently used for routine batch release testing of new generation vaccines. These are well-defined immunobiologicals for which the consistency approach is part of their development, and *in vitro* and/or analytical tools have been developed which are suitable for their characterization. In contrast, established vaccines are less well-defined products and more difficult to characterise (*e.g.* diphtheria-, tetanus- acellular pertussis vaccine (DTaP), rabies and clostridial vaccines). Despite extensive availability of such vaccines for several decades and the use of huge numbers of doses in the field, the consistency approach for the release of established older vaccines still has to be implemented for routine use and the required non-animal based tests remain to be developed in some instances. However, some progress has been made recently. New provisions for additional systems monitoring the consistency of production have been incorporated in the General Notices of the European Pharmacopoeia [3] and have been in force since July 2014.

Launched in 2005, EPAA is a joint initiative between the European Commission (DG Enterprise and Industry, DG Research and Innovation, DG Health and Consumer Protection, DG Environment, DG Joint Research Centre), trade federations and companies promoting the development and implementation of 3Rs methods. In 2010, EPAA organised with the European Centre for the Validation of Alternative Methods[13] (ECVAM, European Commission Joint Research Centre) a workshop on the consistency approach for the quality control of established human and veterinary vaccines. The published meeting report [4] indicated how this approach could be further developed and made recommendations for its implementation. An essential recommendation was as follows: "*A technical platform, set up by EPAA, should be created to deal with general strategies and policies to introduce the consistency approach, to define minimal acceptance criteria for the consistency tests (in vitro and analytical) and to set up specific technical task forces to address specific vaccines, tests, and their validation*".

2. ESTABLISHING THE PROJECT

Based on the report's recommendation, EPAA initiated a project to develop the consistency approach for established human and veterinary vaccines. Originally titled the 'Application of the 3Rs and the Consistency Approach for Improved Vaccine Quality Control', the project is currently known as the 'Vaccines Consistency Approach Project'. The EPAA set up a Project

13 Since 2011, European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM).

Committee (whose members are listed in this article's authorship) in charge of managing the project. The kick-off meeting of the project was held in Brussels on 7th April 2011 over two days and brought together nearly 60 participants from academic institutions, regulatory bodies (EMA, USDA, FDA, CCVB, CBGTD), national control authorities (OMCLs), EDQM, the European Commission, and vaccine manufacturers.

2.1. Scope and Objectives

The objective of the project was defined at the first meeting as addressing, for specific tests and products, the proof-of-concept and preliminary validation stages of the development of alternative approaches and not the formal validation, demonstration of transferability, large-scale collaborative validation studies and transfer to a regulatory context, which are in practice under the responsibility of the EDQM Biological Standardisation Programme (BSP). That being said, one of the priority vaccines had a test at a sufficiently advanced stage of development that the project has taken it to the stage of a formal EDQM collaborative study under the BSP. Longer term, global acceptance and harmonization are obvious, but perhaps unrealistic goals in the time scale of this project. Therefore, aiming towards local (European) acceptance and introduction in the EU regulation via EDQM's groups of experts 15 and 15V, was seen as a more realistic ambition. The original definition of the scope was important to give clarity to the aims of the project but opportunities to take advantage of further progress will not be ignored.

Different key factors for success were identified. The best vaccine candidates for the project should be those that are produced by multiple manufacturers, that have extensive historical quality control data and that have an anticipated high benefit from application of the 3Rs (i.e. use of large numbers of animals and/or severe procedures). Relevant consistency parameters should be identified and be predictive of efficacy and safety but not necessarily correlated directly to the current *in vivo* tests. Finally, a broad consensus between regulators and manufacturers on the selected products, protocols, parameters and acceptance criteria would be necessary to ensure that the developed methods can be implemented in guidelines and harmonised between authorities.

The workshop proposed that the projects should be a mixture of quick wins, where 3Rs benefits could be achieved with existing techniques and within the existing regulatory framework, and hard wins that would require research and preliminary validation. Delegates provided their views on suitable topics at the workshop and subsequently these were collated and used to help the Technical Committee (see below) in its subsequent consideration of priorities. The meeting also discussed and agreed on the practical aspects of the EPAA vaccine project as described in the following sections.

2.2. Organization of the Project

The project is composed of two collaborative arms, a Project Committee (PC) chaired by the project coordinator, Ian Ragan, and a Technical Committee (TC) chaired by Coenraad Hendriksen, a member of the PC. As mentioned above, the PC organises and coordinates project activities and also monitors and reports project progress to EPAA. For practical reasons, the establishment of a single TC for human and veterinary products was favoured, at least initially. The main role of the TC was to identify priorities for the application of the consistency approach and to establish expert working groups for these vaccines as described in detail below.

2.3. Composition of the Technical Committee

Based on the roles attributed to the TC and expectations from it, the presence of representatives from EDQM, EURL ECVAM, OMCLs, and regulatory authorities for both human

and veterinary vaccines was recommended. In order to avoid the creation of a too large group in which active participation of all members would be compromised, representation of individual companies and national control authorities via their sector associations was encouraged (Table 1). Nevertheless, members were considered to act as individual experts rather than just representatives of their parent organizations. Due to the primary European focus of the project, TC members were selected from within European associations and organizations. Non-European organizations and regulatory bodies were offered the status of observers and participated mainly via teleconference.

2.4. Generic data packages

One of the first tasks of the Technical Committee was to discuss and agree generic data packages for the application of the consistency approach to human and veterinary vaccine products, a process helped by sharing perspectives from the two sectors and identifying common needs.

A fundamental point is that replacement or deletion of *in vivo* tests requires the acceptance that such changes might result in a loss of information that is not entirely compensated for in a one-to-one manner by alternatives. Consequently, the data package must contain adequate evidence of the consistency of the production process: raw material control, quality systems (QA and cGMP), in-process controls and quality control testing. In the discussion on the generic data package, a number of points were raised:

- Uptake of alternative methods is slow because of the lack of global acceptance and the cost, despite the fact that once established the alternative method would be cheaper.
- Manufacturers need guidance on product-specific validation and how to establish correlation between an alternative approach to quality control and reference or gold standard methods and processes.
- A large amount of historical data on quality exists for the older vaccines, yet these continue to be those that use the most animals despite the availability of many approved alternatives. These historical data could be used to analyze retrospectively the consistency of production of specific vaccines.
- Consistency data from an agreed number of commercial batches are better for validation purposes than data from a period of manufacturing (i.e. one year). The proposal of ten recent batches was thought to be reasonable although it was pointed out that for some new vaccines; data on only three batches were required for licensing. This might be the consequence of a better quality control during manufacture, i.e. the application of the consistency approach for these new vaccines.
- Statistical analyses of consistency and trend analysis are already in use by manufacturers. Outcome of these analyses should be used in a consistency approach to determine the specification limits and alerts limits in consistency testing and should be part of the generic data package.
- What is required from the analytical tools is the demonstration that the product batch is within the characteristics (to be specified) of the batch(es) that have been shown to be safe and effective in the target species and that the consistency approach is able to detect a batch which is deviating from consistency in production. For validation, batches that have been produced in an inconsistent way might be used.
- Stability of the vaccine is an important parameter that also needs to be taken into account as part of consistency testing.
- It is clear that the data package supporting change from terminal *in vivo* testing to the consistency approach should provide measurements of specific and relevant characteristics, allowing assessment of product quality, ability to monitor stability and identification of

batches that have not been produced consistently according to predefined criteria. It is also important that the entire process generating the data should be robust and adapted to national regulatory requirements.

2.5. Project Priorities

The task of establishing priorities was aided by the creation of an inventory of existing efforts to develop alternatives. This was intended not only to facilitate the identification of potential target areas for the project but also to provide information on the development of alternatives for all stakeholders in the field and to encourage collaboration between those involved.

Three main product areas for veterinary and/or human use were selected as focal points for the project, namely, vaccines for rabies, clostridia, and diphtheria/tetanus/pertussis (DTaP) as described below.

2.5.1 Rabies vaccine

A proposal, common to both the human and veterinary fields, was a project on inactivated rabies vaccine and this was seen therefore as an appropriate priority that could take advantage of the synergy between the human and the veterinary aspects. The current immunization-challenge test for batch release (the NIH test) has poor reliability, has biosafety issues associated with the manipulation of live virus, is ethically worrying due to the high numbers of animals used and its severity and is expensive because of animal costs and the biosafety requirements [5,6]. Its elimination and application of the consistency approach involving *in vitro* methods would reduce the release cost by 80-90% and shorten lead times, thereby providing scientific, ethical and economic benefits. A serological test for batch release (single dose assay; immunization followed by *in vitro* quantification of antibodies) of veterinary vaccines has been developed, validated [7] and accepted [8] but there are already available adequate tools for *in vitro* tests based on G-glycoprotein antigen quantification by ELISA that should permit the complete replacement of *in vivo* testing [9]. For non-adjuvanted vaccines (e.g. those for human use) the scientific challenges are few, but the use of adjuvants by manufacturers of veterinary vaccines presents greater problems [6].

2.5.2 Clostridial vaccines

The second proposal for veterinary products was clostridial vaccines. MSD Animal Health (MSD-AH) has developed cell-line based assays for the in-process control testing of several clostridial strains, a work supported by the NC3Rs (UK) [10]. These tests, which measure the toxicity of the toxin, residual toxicity of the toxoid and the antigenicity of the toxoid, show excellent correlation with the *in vivo* tests (the minimum lethal dose test (MLD), the antigenicity of the toxin (L+ test) and the total combining power test (TCP)) and could in principle replace the use of tens of thousands of animals per year. Such tests are not yet available for all important clostridial strains such as *tetani* and *chauvoei*. Moreover, implementation of a full *in vitro* consistency approach will require monitoring of the antigen, of the adjuvant and of the interaction between the two. The latter is difficult as many different adjuvants are used, some of which interfere with antigen determination. The application of the consistency approach is certainly feasible for clostridial vaccines but *in vitro* tests remain to be developed for some important strains e.g. *chauvoei*. It also needs to be shown that the currently developed tests are also suitable with other products and for different manufacturers and national control authorities.

2.5.3 DTaP vaccine

For human vaccines, DTaP was selected as the second project in view of the numbers of animals that are used for safety and potency evaluation. The project needed to address the removal of *in vivo* potency testing for all components as well as animal tests of residual toxicity/reversal testing required for tetanus (T) and pertussis (aP) components. The task is not trivial - single dilution serology tests are struggling for acceptance [11] and the shift to *in vitro* tests will remain a challenge as these vaccines are most often combined in current formulations. This then makes the test validations quite complex and raises ethical and risk/benefit questions for the scope of validation activities which would be required. The key issues are to identify the relevant process parameters and to ensure method development and validation for antigen characterization, content and residual toxicity.

2.6. Expert Working Groups and Workshops

With the project priorities list clearly defined, four working groups composed of experts for each of the priorities were established: one each for human rabies, veterinary rabies, DTaP and clostridial vaccines. The involvement of experts in each working group is an important feature in order to bring forward these four project priorities to the next step of addressing the technical gaps in the implementation of the consistency approach. Most of the members of the Project Committee and the Technical Committee are also members of one or more of the working groups thereby ensuring continuity and communication between the various layers of organisation.

The work on generic data packages, selection of priority vaccines and establishment of expert working groups was carried out at the first meeting of the Technical Committee on 30th September 2011. Since then, there have been three further Technical Committee meetings, on 6th February 2012, 18th March 2013 and 21st March 2014. During this period, the expert working groups have held their own meetings: the DTaP vaccine group in August 2012; the human rabies group in October 2012; the veterinary rabies group in November 2012; and the clostridial group in March and September 2013. In the sections that follow, the achievements of each working group to date have been summarised.

3. ACHIEVEMENTS OF THE EXPERT WORKING GROUPS

3.1 Human Rabies Vaccine Working Group

In-process controls are already routinely use by manufacturers who have extensive experience and historical data on their performance, but it must be understood that manufacturers use different viral strains and have developed their own in-house tools (e.g. with different monoclonal antibodies). The working group therefore proposed that the way forward for product characterization would require agreement on the most suitable monoclonal antibodies for G-glycoprotein quantitation [12,13] and the demonstration of the test's ability to differentiate potent from sub-potent batches. It is of the essence for consistency tests to reliably identify batches that do not meet the predefined consistency criteria. However, direct correlation between the current challenge NIH test and an *in vitro* test is neither necessary nor possible considering the high variability of the NIH test. The term 'concordance' has therefore been adopted to define the desired relationship between the NIH test and an *in vitro* alternative and thought is needed on the definition of sub-potent batches in the context of consistency testing. In summary, it was concluded that suitable in-process testing tools (ELISAs) are in place [14] and that while continuous monitoring of the rabies production process already takes place, the specification and alert limits still need to be defined in order to correctly assess the product quality.

As follow-up to these discussions, a workshop was organised to discuss the practical aspects of *in vitro* potency testing for human rabies vaccines. It involved 22 participants comprising manufacturers from EU and non-EU countries, OMCLs, academia, validation bodies, and regulatory bodies. The purpose of the workshop was to focus on gaps in technical knowledge and validation of *in vitro* antigen quantification methods and to propose solutions for the replacement of the NIH test. The participants were therefore invited to review the available methods (e.g. ELISA formats, reagents and reference standards), and they concluded that the best way forward was to identify a single test format (a sandwich ELISA for the trimeric form of the G-glycoprotein) to be validated through a future EDQM collaborative study aimed at facilitating global acceptance by regulators and industry. As a matter of principle, the participants agreed that following approval of such an assay, it should not be permissible to use the challenge test to investigate batches which failed the ELISA. However, *in vivo* testing (serological in preference to challenge testing) might be needed to re-establish consistent production following a major change in the production process.

Since various monoclonal antibodies have already been generated and are being used for in-process controls, it was agreed that a preliminary pre-validation study was necessary to select the most suitable ELISA format and reagents for the future collaborative study. The planning and design of this has been developed under the joint leadership of one of the authors (Jean-Michel Chapsal and Noël Tordo (Institut Pasteur)). Products provided by manufacturers under Material Transfer Agreements were to be tested in several participating laboratories, using well-characterised monoclonal antibodies. The participants, products and antibodies are summarised in Table 2. The sub-potent batches were produced by heat treatment, an approach that follows the requirements given in the Ph. Eur. (*e.g.* for inactivated polio vaccines) or by the WHO (*e.g.* for hepatitis B vaccines) and accepted by authorities for other viral vaccines. The study design was to run the ELISA and NIH tests in parallel on the potent batches, sub-potent batches and a 50/50 mixture of the two. The testing was undertaken in 2014 and data analysis carried out by a contracted statistician. The study results will be discussed at a workshop held jointly between the human and veterinary rabies working groups in 2015 in order to evaluate the potential for harmonisation of rabies testing using the non-animal method and to assess the suitability of the proposed test for veterinary adjuvanted products.

3.2. Veterinary Rabies Vaccine Working Group

An internal survey among European manufacturers of veterinary rabies vaccine revealed that in-process controls, usually ELISAs, are already being implemented by most manufacturers, but *in vivo* tests remain in use by all for batch release. The essential difference between human and veterinary rabies vaccine is the use of adjuvants in the latter which makes antigen (i.e. glycoprotein) quantitation of the final product more difficult [6, 10]. Therefore, antigen quantitation has been done up to now only as an in-process control, as there is no available validated method for doing this in the final adjuvanted product [9]. However with so much historical data and in the absence of failed final batches, indicating that process quality is already adequate, it is valid to ask why more tests would be required and what more they would add. Manufacturers could use their historical data to establish alerts and set specifications on parameters for antigen and adjuvant quality. These limits would of necessity be manufacturer and product-specific and would need to guarantee that a batch that does not meet the consistency criteria would be detected. It is true to say that historical data on the consistency of antigen production encourage greater faith in the control of the process than in characterization of the final adjuvanted product which would, in any case, be difficult for veterinary rabies vaccines. However, despite this, antigen characterization in the presence of adjuvant, adjuvant-antigen interaction and the link to potency are still seen as gaps to implementation of a consistency approach for veterinary rabies vaccines [6, 9].

Stability testing is also important as all the current data come from the use of the NIH test and not from an *in vitro* test. However, as long as the production process is not modified, product stability should be predictable and the *in vivo* test should only be used in exceptional cases (as was proposed above for human rabies vaccine).

To develop these ideas further, a workshop was organised to discuss implementation strategies for a consistency approach for veterinary inactivated rabies vaccines. It involved 24 participants from European vaccine manufacturers, OMCLs, regulatory bodies and academia. The aims were to present data from in-house testing that could support the waiving of the current test by *in vitro* methods; to exchange information on reagents and methods that can be used for the *in vitro* quantification of rabies G-glycoprotein for in-process control and for control of the final product; to discuss the implementation strategies and potential gaps in consistency testing of veterinary rabies vaccines; and to discuss the way forward to implement globally a consistency approach as an alternative to rabies challenge testing of the final product.

There was broad agreement that a package that encompassed monitoring parameters in raw materials, production, formulation and the finished product would be sufficient to assure the quality of veterinary rabies vaccines. The trimeric form of rabies G-glycoprotein is considered as the target of choice to monitor antigen quality both in-process and in the final product. However, it is important to evaluate these data in combination with other process parameters and in that case the total G-glycoprotein content could also be acceptable. Several manufacturers are working on *in vitro* alternatives to measure rabies antigenic mass during the process and in the final product. In addition, USDA has developed new hybridomas which they will make available and they are currently working on an ELISA assay to measure trimeric G-glycoprotein. The workshop concluded that during the production stage, ELISA assays can reliably measure the G-glycoprotein content of in-process material and demonstrate the consistency of production in manufacture. These in-process data are typical of the information that could be compiled and presented to the authorities as part of a consistency approach package. Furthermore, results of *in vitro* tests for the final product were also presented by several participants. A number of manufacturers and academic organizations (*e.g.* Justus Liebig University, Giessen, Germany) have their own tests either ready or currently in development. These tests are able to quantify the rabies antigenic content in vaccines and are able to discriminate between potent and sub-potent batches (personal communication). However, no agreement was obtained on the release criteria, alert limits and the amount of data needed to compile a dossier for a consistency approach for batch release.

The participants indicated that a single test format is desirable for the sake of harmonization. However, this is difficult to achieve due to the different production and formulation processes used by veterinary vaccine manufacturers. Therefore, it was agreed that the work of the human rabies vaccine group should be followed and the suitability of *in vitro* methods developed for human rabies vaccines be evaluated for veterinary vaccines. At least some of the monoclonal antibodies used for the ELISAs for human vaccines should also be applicable to veterinary vaccines since they recognise many strains.

3.3. Clostridial Vaccine Working Group

In-process testing for Clostridial vaccines relies on a variety of analytical techniques and animal tests for measuring the toxicity of the toxin (MLD), the antigenicity of the toxin (L+ test), the residual toxicity of the toxoid (MLD test) and the antigenicity of the toxoid (TCP). However, these are manufacturer-and product-specific and based on what is contained in the marketing authorization, hence complete harmonization of in-process controls is unlikely.

MSD-AH's alternative to current in-process animal tests is to replace these which use mice only as indicators of toxicity, with in-house developed and validated cell-line based assays. The

cell-based tests are more sensitive, more accurate and quicker as well as avoiding the use of animals [10]. MLD and L+ replacement tests for *Clostridium perfringens* type D vaccine have already been submitted to the regulators and accepted as variations in the product registration file. Similar tests for other Clostridial species are at various stages of development and validation.

For release testing of potency, a partial replacement of the animal test could be achieved by using cell-based assays or ELISAs [15] instead of mice for the toxin neutralisation test (TNT) of antitoxin in rabbit sera. Furthermore, although serological potency testing does not fulfill the overarching goal of eliminating animal tests completely, it certainly has welfare advantages over challenge tests, and the project to replace the guinea pig challenge test for *Clostridium chauvoei* with a serological assay is a positive step in terms of advancing the 3Rs [16,17].

As with rabies veterinary vaccines, a full replacement of animal testing is complicated by the presence of adjuvants and mixtures of toxoids in some vaccines. However, ELISAs are in use for certain Clostridial strains that produce a single major toxin. Furthermore, a cell-based replacement for the TNT test could work for multiple toxins. The avoidance of use of both rabbits and mice will need direct quantitation of antigen in combination with the consistency approach. This would have the potential to replace not only the TNT test but also the TCP method. Finally, strict control over the vaccine blending process is vital in order to guarantee the release of safe and efficacious batches.

The expert working group decided to focus on the validation of *in vitro* replacements for the MLD and TCP tests for *Clostridium septicum*. This was selected since the cell-line based assays are already in use in MSD-AH, and because manufacturers and authorities in the group involved are familiar with the organism. The programme of work for formal validation of these tests was developed in two workshops held in 2013. The meetings brought together other clostridial vaccine manufacturers from France, New Zealand, Spain and the USA and national testing laboratories from France, Germany, Hungary, Spain, Switzerland and the USA (see Table 3) to work out the details of a collaborative study to determine the transferability of the two tests and the concordance between the *in vitro* and *in vivo* versions. The study was overseen and analysed by EDQM. This is a truly international effort and the active participation of the USDA increases the chances that the new tests will eventually be validated by authorities outside Europe. The results will be presented and discussed at a workshop in September 2015.

This project is merely the first stage of a very large and necessary project to develop similar tests for other Clostridial species and external research funding will be essential for the work to progress. Furthermore, the successful development of replacements for these in-process controls still leaves the more difficult task of applying the consistency approach to the whole process in order to avoid final product testing in animals. A summary of current tools and gaps is given in Table 4.

3.4. DTaP Vaccines Working Group

For release of the final product and for stability testing, WHO and Ph. Eur. require testing of each component for potency/immunogenicity, but specific toxicity and reversal tests may be waived for D and T final bulk and final product, provided that the production method is validated to ensure that the final product would comply. Some of the various *in vivo* tests for safety testing of purified toxoid (freedom from toxin and reversion to toxin) have been replaced by cell based assays for D but have not as yet been developed or introduced for T and aP. After confirming their applicability, *in vitro* biochemical binding/enzymatic activity tests developed for aP [18,19,20] and T [21,22] might be useful measures for designing consistency parameters for final products and for in-process controls.

Potency tests are needed if the product is too complex for physicochemical analysis or if the process is not precise enough to predict with confidence the biological activity. Protection challenge models can be replaced by serological potency for D & T vaccines. Although this does not fulfill the overarching goal of eliminating animal tests completely, it certainly has welfare advantages over toxin challenge tests, and represents a positive step to advancing the 3Rs and as part of consistency evaluation [11]. However, new technologies have become available which are able to predict with confidence the quality of D and T toxoid vaccines even in complex combinations.

Consideration could be given to removing potency testing for certain products where potency is less challenging (such as combined vaccines with reduced antigen content intended for booster immunizations of adults and/or adolescents) and this could certainly be supported by application of the consistency approach to the production process. *In vitro* antigen assays to monitor antigen content and degree of adsorption to adjuvant as in-process controls [23,24] are now included in the new revision of WHO requirements [25,26]. Capture ELISAs for D and T toxoid show excellent specificity and transferability and may also be useful for stability testing, as loss of D antigen with age or temperature correlates very well with loss of immunogenicity.

The development of similar assays for pertussis toxoid and cell-based assays as replacements for animal tests will require additional studies. Manufacturers' willingness to undertake this would be greatly helped if regulators were able to provide scientific advice of a generally strategic, rather than product-specific nature, preferably at reduced cost compared with the current cost of product-specific advice. Although this possibility has been discussed by the EMA JEG3Rs committee (European Medicines Agency - Joint Expert Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products), the EMA appears currently unable to do this. For new products, the current cost of scientific advice is small compared with the total development cost.

The overall conclusion of these general considerations is that for each of the components, D, T and aP, there are various alternative non-animal tests that are or could be applied for in-process and final product testing. They are at various stages of development and validation and there is much remaining to be done to apply the consistency approach to this vaccine. These aspects were discussed in much more detail at a workshop held late in 2012 which attracted 25 participants from the regulatory, industry and academic sectors. The aim of the meeting was to provide an overview of the current practice and ongoing research studies on manufacturing consistency and batch release of DTaP vaccines; to establish a list of required methods/technologies for in-process control and the characterisation of intermediate and final products; and finally to identify potential gaps in order to plan the way forward.

The conclusions of the workshop and subsequent updates from the expert working group are summarised in Tables 5 and 6 which map current tools and gaps in establishing the consistency approach for D/T and aP components of the vaccine respectively.

In general, for both D and T (Table 5), very few new tests are needed but for those already in use, consistency limits need to be set, for which manufacturers no doubt already have a great deal of relevant data. In stage 3 of the production process, the detoxification step should be validated to confirm that detoxification of the crude or purified toxin is complete and irreversible through characterizing the kinetics of inactivation and identification of key parameters such as temperature, time, toxin concentration, and concentration of detoxifying reagent. In stage 4, a number of methods are already in use by manufacturers to monitor toxoid purity and quality, including iso-electric focusing, fluorescence spectroscopy, SDS-PAGE, SE-HPLC and antigenic fingerprinting [20,21]. In addition to these methods, cell-based tests of D toxin/toxoid toxicity are in use but need to be standardised and validated across different manufacturers and products. Cell based methods for T and aP are still in early stages of development or validation. At the

blending/final bulk stage 5, toxoid quality and integrity can be monitored in the presence of adjuvant using DAFIA (Direct Alhydrogel Formulation Immunoassay). It is not clear whether particle size analysis is necessary or even relevant at this stage and at the final batch stage of production. Finally, in the consistency approach, *in vivo* quality control should not be needed either at final bulk or final batch stages.

The aP production process varies widely and approaches include either co-purification to yield preparations enriched in protective antigens (pertussis toxin, filamentous haemagglutinin, and pertactin) but depleted in endotoxin (lipo-oligosaccharide) or individual purification of each antigen using combinations of several separation methods. As with D and T, the detoxification step should be validated with appropriate attention to the critical parameters to confirm complete inactivation, and lead to the elimination of the histamine sensitization test (HIST). Toxoid quality and integrity may be monitored using a range of physicochemical and immunochemical methods (see also Table 6). Application of the consistency approach, as with D and T, should not require *in vivo* quality control at the final bulk or batch stages.

4. CONCLUSIONS AND NEXT STEPS

4.1. The following summarises briefly the status of each of the four priority projects:

4.1.1 Human rabies vaccines

Tools for monitoring the production process are available. However, a selection of suitable materials (e.g. monoclonal antibodies) has to be tested and the best candidate has to be identified. These gaps in technical knowledge have to be addressed and solutions proposed, including an EDQM collaborative study, focused on in-process control and replacement of the NIH test. After the workshop of the human rabies vaccines group a number of antigen quantification ELISAs have been selected to enter the pre-validation phase. The results and conclusions will be presented at a workshop in May 2015.

4.1.2 Veterinary rabies vaccines

Testing of adjuvanted vaccines remains an important issue for this type of product, as well as manufacturer-specific processes which render a single format for the consistency approach unlikely. The aim therefore is not a collaborative study but the definition of a common framework for application of the consistency approach which lays out the stages, methodologies, required information and evaluation strategies, and which can be adapted for each manufacturer. The veterinary manufacturers will take advantage of the work going on in the parallel human rabies vaccine project to evaluate the suitability for their own products of *in vitro* tests(s) developed for human rabies vaccines.

4.1.3 Clostridial vaccines

The cell-line based assay for *Clostridium septicum* MLD and TCP tests developed at MSD AH UK has entered a collaborative study led by EDQM that could result in the inclusion of the new *in vitro* assays as validated alternatives in the European Pharmacopoeia. The results of the collaborative study will be presented at a workshop in September 2015.

4.1.4 DTaP vaccines

Funding may be needed to support research work in order to develop biochemical and cell-based assays as replacements for animal safety tests and to devise coherent consistency

approaches. It is likely that a common approach may not be feasible due to manufacturer-specific in-process controls and production processes. However, these manufacturers' specificities are not incompatible with application of a consistency approach, as it is intrinsic to the consistency approach to demonstrate consistency in production within the manufacturer's production process. A fruitful area for future work is the detoxification process that can be approached through the validation of non-animal alternatives and through waiving of animal based tests, on the basis of consistency of manufacture and historical data.

4.2. General conclusions

Evolution of the project to date illustrates that there is no single path to implementing the consistency approach and that pragmatism must be favored. For example, validating a precise and reproducible *in vitro* test by reference to an imprecise and highly variable *in vivo* test raises many issues. The proposal that correlation should be replaced by concordance means that the alternative test must be capable of detecting sub-potent final batches. The extensive discussions that have taken place in the expert working groups about how to create sub-potent batches artificially are a consequence of the fact that, for many vaccines, current manufacturing quality systems do not produce such batches. In these cases, the logical conclusion is that there is no need for testing of the final batch as long as consistency parameters and acceptance limits are appropriately set for the in-process controls. Nevertheless, the project is in general adopting a strategy of pursuing both options: new non-animal tests plus increased attention to manufacturing quality control. After all, the goal of EPAA is advancing the 3Rs, and both options lead to the same result, even if we may have to consider replacing challenge tests with serological assays as a stage on the way to implementing processes that avoid use of animals completely.

It is also clear from the four chosen priorities that the state of readiness for implementing alternatives is highly variable and has to be approached in different ways that take account of business considerations as much as scientific gaps. The different business models for veterinary and human rabies vaccine manufacture require for example different strategies for the elimination of the NIH test and different strategies for acceptance of new tests by regulatory bodies and their uptake by the manufacturers. The initial proposal to maintain a single project team for veterinary and human vaccines has proved to be a good decision in retrospect and there is no suggestion to change this in the future.

The EPAA has limited ability to address gaps that need filling through research. For the projects in the portfolio, gaps are currently being filled through collaborative efforts between manufacturers and EDQM/OMCLs on validation studies (as it is the case for *Clostridium septicum* and human rabies vaccines) or, for more basic R&D, by the manufacturers and OMCLs individually. All of this work costs money and for manufacturers, any such activity has to be seen to be cost-effective and long term benefit has to be balanced against short term outlay. The piecemeal nature of current research activities runs the risk of duplication of effort and EPAA has a role to play in encouraging communication and data sharing as well as facilitating the validation studies. EPAA itself, being part funded by the European Commission, is ineligible to participate in European funding schemes such as the Framework Programmes, but is able to bring suitable consortia and collaborations together and aid in communication and dissemination activities. Therefore it can be a significant partner for other research funders such as the National Centre for the 3Rs in the UK and the Swiss 3R Research Foundation, where the EPAA's close links with national, regional and global regulatory and standards bodies (OMCLs, EMA, EDQM, EURL ECVAM, FDA etc) are important for progressing newly developed tests into validation studies.

What for the future? The current portfolio of projects merely scratches the surface of the work that is needed to develop the consistency approach for all established vaccines and to ensure

its global acceptance and use. The EPAA project has brought together the relevant bodies and through its pilot projects laid the foundation for a major effort to achieve this goal. EPAA itself cannot do this and consequently the industrial representatives of the PC and TC have worked to develop a project of much wider scope which has been accepted into the Innovative Medicines Initiative 2 portfolio and launched in its 3rd Call in 2014 [27]. This will have the potential to address the research gaps referred to earlier and, being industry-led, to provide a faster route to validation and uptake.

5. ABBREVIATIONS

3Rs, Replacement, Refinement and Reduction of experiments on animals; ANSM, Agence Nationale de Sécurité du Médicament et des Produits de Santé; BSP, Biological Standardisation Programme; CBGTD, Canadian Biologics and Gene Therapies Directorate; CBER, Centre for Biologics Evaluation and Research; CCVB, Canadian Center for Veterinary Biologics; cGMP, Current Good Manufacturing Practice; DAFIA, Direct Alhydrogel Formulation Immunoassay; DG, Directorate General; DTaP, for Diphteria, Tetanus and acellular Pertussis vaccine; EDQM, European Directorate for the Quality of Medicines and HealthCare; ELISA, Enzyme-linked immunoassay; EMA, European Medicines Agency; EPAA, European Partnership for Alternative Approaches to Animal Testing; EURL ECVAM, European Union Reference Laboratory for Alternatives to Animal Testing; FDA, Food and Drug Administration; GMP, Good Manufacturing Practice; HIST, Histamine Sensitisation Test; IFAH, International Federation for Animal Health ; IMI 2, Innovative Medicines Initiative 2; INCQS-FIOCRUZ, Instituto Nacional de Controle de Qualidade em Saúde - Fiocruz; ISP, Institut scientifique de Santé Publique; JEG3Rs, Joint Ad-hoc Expert Group on the Application of the 3Rs in the Regulatory Testing of Medicinal Products; LAL, Limulus Amoebocyte Lysate; MLD, Minimum Lethal Dose; NC3Rs, National Centre for the 3Rs; NICPBP, National Institute for the Control of Pharmaceutical and Biological Products; NIBSC, National Institute for Biological Standards and Control; NIH, National Institutes of Health; OMCL, Official Medicines Control Laboratory; PC, Project Committee; Ph. Eur., European Pharmacopoeia; QA, Quality Assurance; RIVM, Rijksinstituut voor Volksgezondheid en Milieu; SDS-PAGE, SDS Polyacrylamide Gel Electrophoresis; SE-HPLC, Size Exclusion HPLC; TC, Technical Committee; TCP, Total Combining Power; TNT, Toxin Neutralisation Test; USDA-CVB, US Department of Agriculture - Centre for Veterinary Biologics; WHO, World Health Organisation.

6. REFERENCES

[1] Hendriksen C, Arciniega JL, Bruckner L et al. The consistency approach for the quality control of vaccines. *Biologicals* 2008; 36(1): 73-77.

[2] Hendriksen CF. Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. *Expert Rev. Vaccines* 2009; 8:313-22.

[3] General Notices, Ph.Eur Suppl. 8.2. Strasbourg, France: Council of Europe; 2014.

[4] De Mattia F, Chapsal JM, Descamps J et al. The consistency approach for quality control of vaccines - a strategy to improve quality control and implement 3Rs. *Biologicals* 2011; 39(1):59-65.

[5] Daas A, Milne C. Establishment of batch 4 of the Biological Reference Preparation (BRP) for rabies vaccine (inactivated) for veterinary use. *Pharmeuropa Bio Sci Notes* 2004(1):17-22.

[6] Bruckner L, Cussler K, Halder M et al. Three Rs approaches in the quality control of inactivated rabies vaccines. The report and recommendations of ECVAM workshop 48. *Altern Lab Anim* 2003;31(4):429-54.

[7] Krämer B, Schildger H, Behrensdorf-Nicol HA et al. The rapid fluorescent focus inhibition test is a suitable method for batch potency testing of inactivated rabies vaccines. *Biologicals* 2009;37(2):119-26.

[8] Rabies vaccine (inactivated) for veterinary use, monograph 0451. Ph. Eur. 8th ed. Strasbourg, France: Council of Europe; 2014.

[9] Stokes W, McFarland R, Kulpa-Eddy J et al. Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: state of the science and planning the way forward. *Biologicals* 2012;40(5):369-81.

[10] Redhead K, Wood K and Jackson K. Testing of veterinary clostridial vaccines: From mouse to microtitre plate. *Dev. Biol. (Basel)* 2011;134:45-50.

[11] Stickings P, Rigsby P, Coombes L et al. Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. *Procedia in Vaccinology*, 2011;5:200-12.

[12] Bunschoten H, Gore M, Classen IJT et al. ADME characterization of a new virus-neutralizing epitope that denotes a sequential determinant on the rabies glycoprotein. *J. Gen. Virol.* 1989;70:291-98.

[13] Wiktor TJ, Gyorgi E, Schlumberger HD et al. Antigenic properties of rabies virus components. *J. Immunol.* 1973;110(1):269-76.

[14] Gibert R, Jallet C, Poirier B, Tordo N et al. A relevant in vitro ELISA test in alternative to the in vivo NIH test for human rabies vaccine batch release. *Vaccine* 2013;31(50):6022-29.

[15] Wood KR. An alternative to the toxin neutralization assay in mice for the potency testing of the Clostridium tetani, Clostridium septicum, Clostridium novyi type B and Clostridium perfringens type D epsilon components of multivalent sheep vaccines. *Biologicals* 1991;19(4):281-86.

[16] http://www.forschung3r.ch/en/projects/pr_136_13.html.

[17] Frey J, Johansson A, Burki S, et al. Cytotoxin CctA, a major virulence factor of *Clostridium Chauvoei* conferring protective immunity against myonecrosis. Vaccine 2012; 30(37):5500-05.

[18] Yuen CT, Horiuchi Y, Asokanathan C et al. An in vitro assay system as a potential replacement for the histamine sensitisation test for acellular pertussis based combination vaccine. Vaccine 2010;28(5):3714-21.

[19] Isbrucker R, Arciniega J, McFarland R et al. Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. *Biologicals* 2014;42(2):114-22.

[20] Hoonakker ME, Ruiterkamp N, Hendriksen CFM. The cAMP assay: a functional in vitro alternative to the in vivo Histamine Sensitization test. *Vaccine* 2010;28(5):1347-52.

[21] Behrensdorf-Nicol HA, Bonifas U, Hanschmann KM. Binding and cleavage (BINACLE) assay for the functional in vitro detection of tetanus toxin: Applicability as alternative

method for the safety testing of tetanus toxoids during vaccine production. *Vaccine* 2013; 31(52):6247-53.

[22] Behrensdorf-Nicol HA, Bonifas U, Isbrucker R et al. Results of an international transferability study of the BINACLE (binding and cleavage) assay for in vitro detection of tetanus toxicity. *Biologicals* 2014; 42(4):199-204.

[23] Coombes L, Tierney R, Sesardic D et al. *In vitro* antigen ELISA for quality control of tetanus vaccines. *Biologicals* 2012; 40(6):466-72.

[24] Coombes L, Stickings P, Tierney R et al, Development and use of a novel in vitro assay for testing of diphtheria toxoid in combination vaccines. *J Immunol Methods* 2009; 350(1-2):142-49.

[25] Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed). *WHO Technical Report Series* 2012;980:271-334. Available at : http://www.who.int/biologicals/WHO_TRS_980_WEB.pdf?ua=1.

[26] Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed). *WHO Technical Report Series* 2012;980:211-70. http://www.who.int/biologicals/WHO_TRS_980_WEB.pdf?ua=1.

[27] http://www.imi.europa.eu/sites/default/files/uploads/documents/IMI2Call3/IMI2Call3_TopicTextWebFINAL.pdf.

Table 1 – Composition of the Technical Committee

Group Member	Affiliation
Svein Rune Andersen	Norwegian Medicines Agency, EMA representative
Lukas Bruckner	Institute of Virology and Immunology, Switzerland
Karl-Heinz Buchheit	EDQM representative
Jean-Michel Chapsal	Independent, PC member (ex-Sanofi Pasteur and Vaccines Europe representative) Leader of human rabies working group
Fabrizio De Mattia	MSD Animal Health, PC member and IFAH representative Leader of vet rabies working group
Marlies Halder	EURL ECVAM, PC member
Coenraad Hendriksen	University of Utrecht, TC Chair and PC member
Gideon Kersten	RIVM, Netherlands
Beate Krämer	Paul Ehrlich Institute, Germany
Denis Lambrigts or Johan Descamps	GSK Vaccines, PC members and Vaccines Europe representatives
Sylvie Morgeaux	ANSM, France
Ian Ragan	EPAA, Project Coordinator
Keith Redhead	Vaccine & Assay Consultancy, UK (ex-Merck AH).
Fabrice Ribaucour	Leader of clostridials working group.
Fabrice Ribaucour	ISP, Belgium
Eddy Rommel	Rommel Consulting Partners, Belgium. Technical Expert
Tanya Scharton-Kersten or Laura Viviani	International AIDS Vaccine Initiative, New York (ex-Novartis)/Novartis
Thea Sesardic	NIBSC, UK. Leader of DTaP working group.
Catrina Stirling	Zoetis, IFAH representative
Ellen-Margrethe Vestergaard	Danish Medicines Agency, EMA representative

Observer	Affiliation
Oksana Yarosh	CCVB, Canada
Theresa Finn	FDA, USA
Richard Isbrucker	Health Canada
David Dusek	USDA-CVB, USA
Bill Stokes	Independent
Suresh Jadhav	Serum Institute of India

Table 2 – Human rabies vaccine Working Group members

Group Member	Affiliation
C. Milne	EDQM
E. Terao	EDQM
E. Meyer	Paul Ehrlich Institute, Germany
S. Morgeaux	ANSM, France
R. Gibert	ANSM, France
D. Wilkinson	NIBSC, UK
M. Page	NIBSC, UK
J. Shin	WHO, Geneva
G. Pulle	BGTD, Canada
W. Chizhikov	CBER, USA
D. Volokhov	CBER, USA
R. Levis	CBER, USA
L. Yuhua	NICPBP, China
C. Shouchun	NICPBP, China
S. Shajhahan	Novartis
L. Viviani	Novartis
JM. Chapsal	Independent (ex-Sanofi Pasteur)
F. Guinet-Morlot	Sanofi Pasteur
M. Halder	EURL ECVAM
W. Correa de Moura	INCQS-FIOCRUZ, Brazil
B. Poirier	Bpstat Consulting
N. Tordo	Institut Pasteur, France
C. Rupprecht	Ross University, USA
Yashpal Kaushik	Bahrat Biotech, India
Sunil Gairola	Serum Institute of India
Chunlai Jiang	Jilin University, China

Table 3 – Clostridial vaccines working group members

Group Member	Affiliation
Behr-Gross, Marie-Emmanuelle	EDQM
Daas, Arnold	EDQM
Jorajuria, Sylvie	EDQM
Balks, Elisabeth	PEI
Kegel, Birgit	PEI
Bruckner, Lukas	IVI
Englebert, Laurence	Zoetis
Fernandez, Conchita	CZV
Kadra, Benaouda	Ceva
Siklodi, Botond	Ceva
Martinez Arias, Oscar	Syva
Ragan, Ian	EPAA
Redhead, Keith (chair)	MSD AH
Srinivas, Geetha	USDA
Bryant, Sharon	MSD AH
Callus, Marion	MSD AH
Fabian, Katalin	NEBIH
Icin, Sabahattin	Bornova Institute
Stirling, Catrina	Zoetis

Table 4 – Methods that assure the quality, safety and efficacy of clostridial toxoid components in veterinary vaccines

This list of test methods are those performed by and required of only one manufacturer. Other manufacturers may have a different selection of test methods that they are required to perform under their Marketing Authorisations.

1. Full characterization of seeds and fermentation media			
/			
2. Toxin production			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Culture purity	Microbiological	No	No
Growth rate	Microbiological	No	No
pH	Chemical	Yes	No
Yield of toxin production	*In vivo*	No	Yes, cell assays could be developed for most species
Toxin antigenicity	*In vivo*	No	Yes, cell assays could be developed for most species
3. Detoxification			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Confirm detoxification	*In vivo* or Vero cell assay	No	Vero cell assay may require dialysis of test material
Toxoid antigenicity	*In vivo*	Yes	Yes, cell assays could be developed for most species
Residual formaldehyde	Chemical	Yes	No
4. Bulk toxoid			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Sterility	Microbiological	No	No
Specific toxicity, tetanus only*	*In vivo*	No	Yes, cell or biochemical assay

5. Blending/final bulk			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Adjuvant characterisation	Chemical	Yes	No
Adjuvant concentration	Chemical	Yes	No
Preservative content (multiple doses only)	Biochemical	Yes	No
Sterility	Microbiological	No	No
Free formaldehyde	Chemical	Yes (depends on Limit of Detection)	No
Specific toxicity, tetanus only*	*In vivo*	No	Yes, cell or biochemical assay
Potency	*In vivo*	Not relevant for consistency approach	Not relevant for consistency approach
pH	Chemical	Yes	No

6. Final batch			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Sterility	Microbiological	No	No
pH	Chemical	Yes	No
Osmolarity	Yes	Yes	No
Appearance	Visual examination	No	No

* Tetanus toxoid is the only clostridial toxoid used in veterinary vaccines that currently requires this test, whether for tetanus-only or combined vaccines.

Table 5 – Methods that assure the quality, safety and efficacy of the diphtheria and tetanus components in vaccines

<table>
<tr><th colspan="4">1. Full characterization of seeds and fermentation media</th></tr>
<tr><td colspan="4">/</td></tr>
<tr><th colspan="4">2. Toxin production</th></tr>
<tr><th>Test</th><th>Existing Method</th><th>Consistency limits needed?</th><th>Additional methods needed?</th></tr>
<tr><td>Culture purity</td><td>Microbiological</td><td>No</td><td>No</td></tr>
<tr><td>Growth rate</td><td>Microbiological</td><td>No</td><td>No</td></tr>
<tr><td>pH</td><td>Chemical</td><td>Yes</td><td>No</td></tr>
<tr><td>Yield of toxin production/ concentration</td><td>Flocculation</td><td>Yes, for Lf and Kf</td><td>No but other suitable immunoassays can be used</td></tr>
<tr><th colspan="4">3. Detoxification and purification</th></tr>
<tr><th>Test</th><th>Existing Method</th><th>Consistency limits needed?</th><th>Additional methods needed?</th></tr>
<tr><td>Free amino groups</td><td>Chemical</td><td>Yes</td><td>No</td></tr>
<tr><td rowspan="2">Confirm detoxification</td><td rowspan="2">In vivo. For D: Vero cell assay</td><td rowspan="2">Yes</td><td>For D: No</td></tr>
<tr><td>For T : Yes</td></tr>
<tr><td>Residual formaldehyde</td><td>Chemical</td><td>Yes</td><td>No</td></tr>
<tr><th colspan="4">4. Bulk purified toxoid</th></tr>
<tr><th>Test</th><th>Existing Method</th><th>Consistency limits needed?</th><th>Additional methods needed?</th></tr>
<tr><td>Sterility</td><td>Microbiological</td><td>No</td><td>No</td></tr>
<tr><td>Toxoid purity quality/ structure/integrity/ aggregation/proteolysis</td><td>None*</td><td>Yes</td><td>Yes
e.g.physicochemical & immunochemical</td></tr>
<tr><td>Antigenic purity</td><td>Flocculation and protein (nondialysable) nitrogen</td><td>Yes</td><td>No but other suitable assays can be used instead of flocculation</td></tr>
</table>

Specific toxicity	*In vivo*. For D: Vero cell assay	No**	For T: Yes. Vero cell assay should be standard for D (Ph Eur)
Reversion to toxicity	*In vivo*. For D: Vero cell assay	No**	For T: Yes. Vero cell assay should be standard for D (Ph Eur)
Stability on storage	All of above	As above	As above
5. Blending/final bulk			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Adjuvant characterisation	Chemical & biochemical	Yes	No
Adjuvant concentration	Biochemical	Yes	No
Antigen content and degree of adsorption	Flocculation test and ELISA	Yes	No
Toxoid quality/structure/ integrity	None	Yes	Yes e.g. DAFIA and ELISA
Preservative content (multiple doses only)	Biochemical	Yes	No
Sterility	Microbiological	No	No
Free formaldehyde	Biochemical	Yes (depends on Limit of Detection)	No
Specific toxicity	*In vivo*	No	Note: This test may be omitted for routine batch release once consistency of production is demonstrated

Potency	*In vivo*	Not relevant for consistency approach	Not relevant for consistency approach***
pH	Chemical	Yes	No
Stability on storage	All of above	As above	As above
6. Final batch			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Identity	Immunochemical	No	No
Antigen content and degree of adsorption	Immunochemical	Yes	No
Preservative content	Biochemical	Yes	No
Adjuvant concentration	Biochemical	Yes	No
Sterility	Microbiological	No	No
pH	Chemical	Yes	No
Osmolarity	Yes	Yes	No

* Many methods providing additional information on antigen characterization exist but they may not be widely used by all manufacturers with consistency limits.

** When the Vero cell test is performed for D, consistency limits may be applicable. Binding and enzyme activity biochemical assays developed for T are good consistency indicating tests. Cell based assay for T under development.

***Serology can be used as a parameter for monitoring consistency, but only for a limited period of time to confirm feasibility of consistency approach.

Table 6 – Methods that assure the quality, safety and efficacy of the acellular pertussis component in vaccines

1. Full characterization of seeds and fermentation media			
/			
2. Toxin production			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Culture purity	Microbiological	No	No
Growth rate	Microbiological	No	No
pH	Chemical	Yes	No
Rate and yield of antigens production	Immunoassays	Yes	No
3. Characterization of antigens prior to detoxification or chemical treatment			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Characterization of antigens	Physiochemical, immunochemical	Yes	No
Purity	Chemical, physiochemical	Yes	No
Impurities	Chemical	Yes	No
Residual endotoxin	LAL test	Yes	No
Sterility	Microbiological	No	No
4. Detoxification			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Confirm detoxification	*In vivo* or CHO cell	No	No. CHO cell should be used
Residual formaldehyde	Chemical	Yes	No
5. Bulk pertussis antigen			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Antigen content	Protein/ Immunoassays	Yes	No
Sterility	Microbiological	No	No

Antigen purity quality/ structure/integrity/ aggregation/proteolysis	None	Yes	Yes e.g. physicochemical & immunochemical
Residual activity of PT	*In vivo* or CHO cells	Yes	No. CHO cell should be used
Residual level of endotoxin	LAL test	Yes	No
Stability on storage	All of above	As above	As above
6. Blending/final bulk			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Adjuvant characterisation	Chemical & biochemical	Yes	No
Adjuvant concentration	Biochemical	Yes	No
Antigen content and degree of adsorption	Biochemical	Yes	Yes
Toxoid quality/ structure/integrity	None	Yes	Yes
Preservative content (multiple doses only)	Biochemical	Yes	No
Sterility	Microbiological	No	No
Free detoxifing agent	Biochemical	Yes (depends on Limit of Detection)	No
Specific toxicity - residual activity of PT*	*In vivo*	Yes	Yes. Various methods under consideration *
Reversion to toxicity	*In vivo*	Yes	Yes. Various methods under consideration*
Potency/immunological activity	*In vivo*	Yes	Yes
pH	Chemical	Yes	No

Stability on storage	All of above	As above	As above
7. Final batch			
Test	**Existing Method**	**Consistency limits needed?**	**Additional methods needed?**
Identity	Immunochemical	No	No
Antigen content and degree of adsorption	None	Yes	Yes
Preservative content	Biochemical	Yes	No
Adjuvant concentration	Biochemical	Yes	No
Sterility	Microbiological	No	No
pH	Chemical	Yes	No
Osmolarity	Yes	Yes	No

* Binding and enzyme activity biochemical assays are good consistency indicating tests. Various cell based assays under consideration. Applicability to final bulk to be determined.