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Replacement, Reduction and Refinement of Animal Testing in the Quality Control of Human Vaccines

Brief description of ongoing projects

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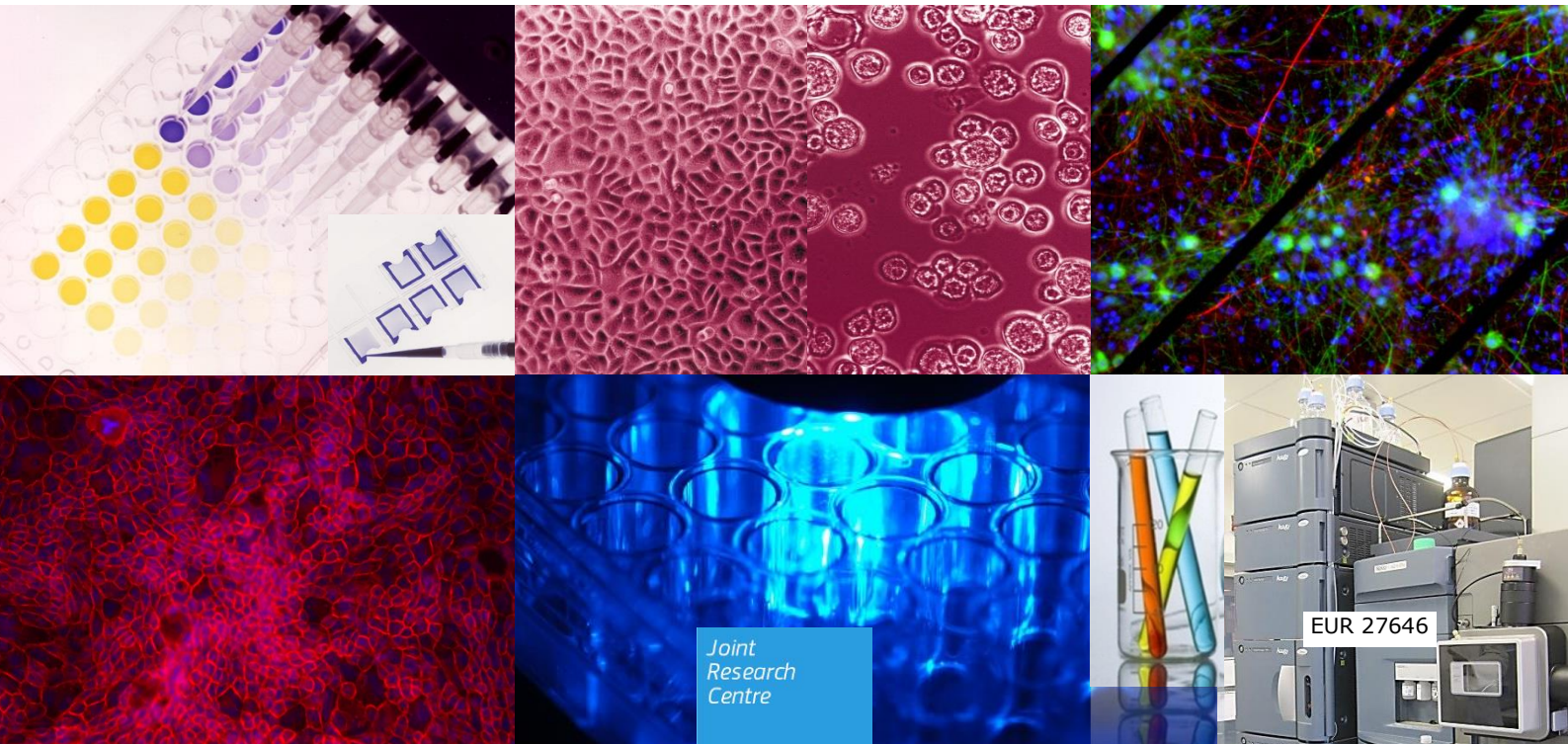

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Replacement, Reduction and Refinement of Animal Testing in the Quality Control of Human Vaccines

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Abstract

Vaccines are recognised as a highly cost effective tool for preventing infectious diseases. They are derived from biological sources and due to the complexity of composition and heterogeneity of products, vaccine lots undergo legally required quality control before they are released. Traditionally, laboratory animals have played an important role in quality control of vaccines and still, many laboratory animals are used in Europe for this purpose. Over the last decades, Replacement, Reduction and Refinement (3Rs) methods to classical animal tests have been developed by control authorities, academia and vaccine manufacturers.

The purpose of this report is to inform the EURL ECVAM stakeholders on ongoing activities in development and validation of 3Rs methods for the quality control of vaccines for human use. The focus of the report is on methods for lot release testing (e.g. safety, pyrogenicity, potency) and projects related to the implementation of the consistency approach to established vaccines such as diphtheria, tetanus, pertussis and rabies vaccines.

1. Introduction

Vaccines are recognised as a highly cost effective tool for preventing infectious diseases. Their importance is likely to increase in the future given the emergence of antibiotic-resistant strains of bacteria and viral infections.

Vaccines are derived from biological sources and due to the complexity of composition and heterogeneity of products, vaccine lots undergo legally required quality control before they are released (EC, 2001). Quality control tests are described in the marketing authorisation for a given product and granted by the competent authorities. Changes to the marketing authorisation, e.g. use of a different method for the lot testing, have to be approved by the competent authorities.

In Europe, lot quality control is performed by the manufacturer and may additionally be performed by an Official Medicines Control Laboratory as laid down in the EU guidelines on Official Control Authority Batch Release [1]. Traditionally, laboratory animals have played an important role in quality control of vaccines and still, many laboratory animals are used in Europe for this purpose.

Over the last decades, Replacement, Reduction and Refinement (3Rs) methods to classical animal tests have been developed by control authorities, academia and vaccine manufacturers. Milne and Buchheit (2012) provide an overview of 3Rs methods which have been incorporated into European Pharmacopoeia (Ph.Eur.) monographs after their formal validation under the umbrella of the Biological Standardisation Programme[2,3,4] run by the European Directorate for the Quality of Medicines & HealthCare (EDQM) and co-sponsored by the Council of Europe and the European Commission.

The purpose of this report is to inform the EURL ECVAM stakeholders on ongoing activities on the development and validation of 3Rs methods for the quality control of vaccines for human use. The focus of the report is on methods for lot release testing (e.g. safety, pyrogenicity, potency) and projects related to the implementation of the consistency approach to established vaccines such as diphtheria, tetanus, pertussis and rabies vaccines.

[1] https://www.edqm.eu/en/Human-OCABR-Guidelines-1530.html
[2] http://www.edqm.eu/en/Biological-Standardisation-Programme-mission-60.html
[3] http://www.edqm.eu/en/BSP-Work-Programme-609.html
[4] https://www.edqm.eu/en/BSP-programme-for-3Rs-1534.html

2. Consistency approach

In order to apply the consistency approach in vaccine production and quality control, it is necessary that the vaccine is thoroughly characterised, the crucial characteristics and associated parameters for quality (e.g. safety and efficacy) known and the tools for monitoring them in place via a quality system (e.g. Good Manufacturing Practice). By this, characteristics of a new lot of a vaccine can be compared to those of a reference lot (clinical lot) which has been shown to be safe and efficacious.

The consistency approach is already in place for the newer, well-defined vaccines, e.g. human papilloma virus vaccine and polysaccharide conjugate vaccines such as meningococcal, Haemophilus type B, and pneumococcal vaccines.

How the consistency approach could be applied to established vaccines (i.e. vaccines which are less well-defined since they are produced by inactivation or attenuation of a virulent microorganism or by detoxification of the toxin thereof) was the topic of several workshops organised by EURL ECVAM (Metz et al., 2007a; Hendriksen et al., 2008) and co-organised with the European Partnership for Alternative Approaches to Animal Testing (EPAA; De Mattia et al., 2011). As a follow-up of the latter, EPAA launched in 2011 a project to develop the consistency approach for established human and veterinary vaccines: *Application of the 3Rs and the Consistency Approach for Improved Vaccine Quality Control*. Within the framework of this project a series of meetings and workshops on specific vaccine groups were held with the aim to discuss the consistency approach and its implementation in more detail with experts from regulatory bodies, manufacturers and academia. De Mattia et al. (2015) provide a general description of the project, summaries of meetings, workshops and discussions on possibilities to implement the consistency approach for the four priority vaccine groups (diphtheria, tetanus and acellular pertussis vaccines; human rabies vaccines; veterinary rabies vaccines and clostridial vaccines). Section 5.4 of this report describes one of the activities launched within this EPAA project aiming at replacement of an *in vivo* test for potency testing of human rabies vaccines.

The potential of the consistency approach in vaccine quality control is underlined by the recent call[5] for proposals within the Innovative Medicines Initiative 2 (IMI 2) programme. The aim is to develop/identify non-animal based techniques for vaccine characterisation and parameters being critical for the vaccine quality. In addition, aspects of validation and promotion to regulatory acceptance should be addressed[6].

The IABS conference on *The consistency approach and alternative methods: towards non-animal based testing in vaccine development and QC* took place on 16-18 September 2015 (Egmond aan Zee, NL). Further information on the conference is available on the conference website[7] and relevant presentations are summarised in the following sections.

The Ph.Eur. states in the Section *General Notices* that a manufacturer needs to demonstrate that a product is in compliance with Pharmacopoeia quality. However, this does not imply that all tests in a monograph need to be performed. A manufacturer could demonstrate that a product is of Pharmacopoeia quality *"…on the basis of its design, together with its control strategy and data derived, for example, from validation studies of the manufacturing process."* By including the statement *"… manufacturers may consider establishing additional systems to monitor consistency of production. With the agreement of the competent authority, the choice of tests performed to assess compliance with the Pharmacopoeia when animal tests are prescribed is established in such a way that animal usage is minimised as much as possible."* Ph.Eur. encourages the

[5] http://www.imi.europa.eu/content/imi-2-call-3-1
[6] Grant negotiations with the VAC2VAC consortium started in December 2015
[7] http://www.consistency-congress.org/

use of 3Rs methods and the implementation of the consistency approach (EDQM, 2015a).

3. Safety tests

In general, there are two types of safety tests, specific safety tests associated with properties of a given vaccine (discussed under 3.2-3.5) and general safety tests to detect non-specific contaminations (also called abnormal toxicity test or test for innocuity).

3.1 Abnormal toxicity test

The abnormal toxicity test (ATT) is a general safety test (using mice and guinea pigs) which is intended to detect non-specific contaminants causing adverse effects (EDQM, 2015b). After introduction of Good Manufacturing Practice and stringent quality criteria for the starting materials, the relevance of this test was questioned (Hendriksen et al., 1994). As a follow-up of the outcome of a retrospective analysis of ATT data carried out by the Paul-Ehrlich-Institut (Germany; Duchow et al., 1994; Krämer et al., 1996) and an enquiry launched by EDQM (EDQM, 1996), the ATT was deleted as lot release test from Ph.Eur. monographs for human vaccines and completely removed from all monographs for sera and immunoglobulins for human use as well as for immunobiologicals for veterinary use (Schwanig et al., 1997). At present (status 2015), 36 European Pharmacopoeia monographs for human vaccines still stipulate under *General Provisions* in the Production section that *"The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9.)"* (EDQM, 2015c).

Despite the deletion of the ATT as a lot release test in Europe, manufacturers producing for the global market may still perform the ATT, since it is stipulated by international requirements (e.g. World Health Organization [WHO]) and national requirements (e.g. Russia, China, Japan, Argentina, Mexico). However, recently revised WHO recommendations (for example, hepatitis B vaccines, WHO, 2013a; diphtheria vaccines, WHO, 2014a; tetanus vaccines, WHO, 2014b) now state that the test for innocuity *"… on the final lot may be omitted from routine lot release once the consistency of production has been demonstrated"* subject to approval of the national regulatory authority. Also, other countries allow waivers of the ATT (e.g. India, personal communication) or are moving towards deletion of the ATT (e.g. Brazil, personal communication). Only recently, US FDA revocated the general safety test (GST), since *"GST requirements are no longer appropriate to help ensure the safety, purity, and potency of licensed biological products"* (US FDA, 2015).

Deletion of the ATT/GST/test for innocuity from regulatory requirements was one of the topics discussed at a recent EPAA workshop (see Section 7.2 of this report).

3.2 Absence of toxin and irreversibility of toxoid for tetanus vaccines

The test for absence of toxin and irreversibility of toxoid is stipulated by Ph.Eur. monograph for tetanus vaccines for human use (EDQM, 2015d) or combined vaccines with a tetanus component to ensure complete and stable inactivation of tetanus toxin after detoxification with formaldehyde. The test is carried out in guinea pigs, which would develop signs of tetanus disease in the presence of active tetanus toxin.

Behrensdorf-Nicol et al. (2013) have developed a promising *in vitro* method, the so-called BINACLE (binding and cleavage) assay. It mimics two important functional properties of intact tetanus toxin molecules: a) their binding via the heavy chain to

specific receptors and b) the proteolytic activity of the light chain, namely the cleavage of synaptobrevin-2. Other proposed *in vitro* methods (Leung et al., 2002; Kegel et al., 2007) only capture the second step and can therefore not discriminate between active and detoxified tetanus toxin (Behrensdorf-Nicol et al., 2008; Behrensdorf-Nicol et al., 2014). After successful transfer to several laboratories and promising results with regard to reproducibility (Behrensdorf-Nicol et al., 2014), validation of the BINACLE assay is ongoing under the umbrella of the EDQM BSP.

Rajagopal et al. (2015) are developing a functional cell-based assay covering important stages of *in vivo* tetanus toxin action: toxin binding to the cell surface, endocytosis, translocation of the toxin light chain, and enzymatic cleavage of the intracellular target: vesicular associated membrane protein-2. Preliminary results show that neuronal cells differentiated from pluripotent mouse embryonic stem cells can be used to detect active tetanus toxin and their sensitivity is comparable to the current *in vivo* assay.

3.3 Residual pertussis toxin and irreversibility of pertussis toxoid for acellular pertussis vaccines

The test for residual pertussis toxin and irreversibility of pertussis toxoid (EDQM, 2015e) is stipulated by the Ph.Eur. monograph for acellular pertussis vaccines as in-process test and for lot release to detect active pertussis toxin (EDQM, 2015f). For in-process testing, manufacturers use the *in vitro* Chinese Hamster Ovary (CHO) cell assay, which is based on the observation that in the presence of active pertussis toxin CHO cells grow within 48 h in clusters (Gillenius et al., 1985). The CHO assay cannot however be used for the testing of adjuvanted acellular pertussis vaccines due to the inherent cytotoxicity of adjuvants. The only currently accepted assay for residual pertussis activity testing of adjuvanted acellular pertussis is the histamine sensitisation (HIST) assay carried out in mice (EDQM, 2015e; WHO, 2013b). It is based on the fact that mice get more sensitive to the effect of histamine when exposed to active pertussis toxin. Sensitised mice die when challenged with a normally non-lethal histamine dose (Corbel and Xing, 2004). There are several variations of the HIST protocols in use and regulatory requirements differ. In general, the assay is considered to have a high intra- and inter-laboratory variability and in order to meet the statistical requirements for a valid assay, several repetitions are often necessary (Bache et al., 2012; Isbrucker, 2012).

Over the last 15 years, an increasing number of possible alternatives to the HIST have been developed, which can be divided into three groups: a) assays that measure a single biochemical function of pertussis toxin, e.g. enzymatic activities or binding (Cyr et al., 2001; Gomez et al., 2006; Gomez et al., 2007; Isbrucker et al., 2010; Xing et al., 2012); b) in addition to the CHO assay, other cell-based assays that measure the whole pertussis toxin function (Bache et al., 2012; Hoonakker et al., 2010, 2015; Isbrucker, 2012) and c) assays that measure biomarkers, i.e. effects on the transcription profile of human cells after pertussis toxin exposure (Vaessen et al., 2013; Vaessen et al., 2014).

As a follow-up of a workshop organised at the Paul-Ehrlich-Institut in 2011, the international "Working Group for Alternatives to HIST" was established bringing together manufacturers, regulatory bodies and academia (Bache et al., 2012). This working group organised a collaborative study (under the umbrella of the BSP114 project) involving 12 laboratories. It aimed at comparing the sensitivity of several *in vitro* methods under development or in use as in process controls by manufacturers (Isbrucker et al., 2014). Participants received seven vaccines, pertussis toxin reference preparation and agreed protocols for sample preparation (e.g. spiking of vaccines with pertussis toxin, test concentrations, desorption of pertussis toxin) and were asked to test at least three of the vaccines with their assay(s). The results of the study were presented and discussed at the International Workshop on Alternatives to the Murine Histamine Sensitization Test (HIST) for Acellular Pertussis Vaccines: State of the Science and the Path Forward (28-29 November 2012) organised by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the Interagency

Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and their International Cooperation on Alternative Test Methods (ICATM) partners (Isbrucker et al., 2014). There was agreement that biochemical assays are useful to monitor pertussis toxin activity; however, since they only capture a single function of the pertussis toxin, preference was given to the further development of the cell-based assays capturing the full function of the pertussis toxin. At the time of the workshop, the most promising cell-culture based assays were: the cAMP assay (Hoonakker et al., 2010) and the modified CHO clustering assay using a porous cell culture insert to prevent contact of the cells with the adjuvant (Isbrucker et al., 2014).

As a follow-up of the workshop, the "Working Group for Alternatives to HIST" organised a second collaborative study under the umbrella of the BSP114 project, which evaluated two CHO cell-based methods with acellular pertussis vaccines representative for the market. As in the first study, pertussis reference preparation has been used for the spiking of vaccine samples. The study took place during 2014. The results have been reviewed and discussed by regulators and manufacturers at the international workshop *In search of acceptable alternatives to the murine histamine sensitization test (HIST): What is possible and practical?* hosted by the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs; London, 4-5 March 2015). Participants concluded that the indirect CHO-cell based assay (i.e. the above mentioned modified CHO clustering assay) is a suitable alternative for replacement of HIST and ready for product-specific validation at manufacturer level. The results of the collaborative study will be published in *Pharmeuropa Bio & Scientific Notes*. Incorporation of the method into the Ph.Eur. monograph and other national and international regulatory requirements was recommended. The recently revised WHO recommendations for acellular pertussis vaccines (WHO, 2013b) already foresee the possible use of an alternative method to the HIST and state that if an alternative assay is used, it should be at least as sensitive and specific as a validated HIST assay and should be approved by the national regulatory authority.

3.4 Specific toxicity test for whole-cell pertussis vaccines

The test for specific toxicity (mouse weight gain test [MWGT]) is stipulated by the Ph.Eur. monograph for whole-cell pertussis vaccines for lot release to detect active pertussis toxin (EDQM, 2015g). The MWGT has been criticised for its lack of specificity, since not only active pertussis toxin but also other toxins (e.g. endotoxin) typically present in whole-cell pertussis vaccines could decrease the weight gain of mice.

Van Straaten-van de Kapelle et al. (1997) compared in a collaborative study the performance of several *in vitro* and *in vivo* assays designed to detect endotoxins (*in vitro* Limulus Amoebocyte Lysate assay) or pertussis toxin (*in vivo* MWGT, leukocytosis promotion test, HIST; CHO assay). None of the tests performed well with regard to inter-laboratory reproducibility, most likely due to the variety of protocols used by the participating laboratories. The accuracy of the MWGT was lower than that of the other assays. Van Straaten et al. (2002) proposed to combine the mouse toxicity and immunogenicity test in one animal model. Specific toxicity is determined by measuring endotoxin levels (weight reduction 16 h post vaccination) and pertussis toxin levels (increase in leukocytes after 7 days), whereas serum antibody levels after 28 days are used as a measure of immunogenicity.

Since the introduction of acellular pertussis vaccines in 1990s, whole-cell pertussis vaccines have lost their importance in Europe and other regions. However, due to the low production costs and since the relative protective efficacy of the best whole-cell pertussis and acellular pertussis vaccines are comparable, whole-cell pertussis vaccines remain the vaccine of choice in many developing countries. In the interest of the 3Rs, it might be worth to explore whether any of the methods mentioned above or those described in Section 3.3 is applicable for specific toxicity testing of pertussis vaccines.

3.5 Neurovirulence testing for lot release of live polio vaccines

Neurovirulence testing is required for live, attenuated vaccines deriving from neurotropic wild-type viruses and ensures the absence of residual neurotoxicity or reversion to neurovirulence. In most cases, e.g. for mumps, tick-borne encephalitis, yellow fever vaccines, neurovirulence testing is performed during development of the vaccine; however, for live polio vaccine it is required also for the lot release at the bulk stage. Historically, non-human primates are used for neurovirulence testing (see review by Levenbook, 2011).

Following the validation in a WHO-collaborative study (Dragunsky et al., 2003) *in vivo* tests based on transgenic mice carrying the human poliovirus receptor (TgPVR21 mice) can be used instead of non-human primates for neurovirulence testing of poliovirus serotypes 1, 2 and 3 (WHO, 2002; 2014c).

Moreover, *in vitro* methods (mutation analysis by PCR and restriction enzyme cleavage; MAPREC) are available for monitoring individual mutations in each of the three poliovirus serotypes, which are associated with reversion to neurovirulence (see review Levenbook, 2011). Since the MAPREC for poliovirus serotype 3 correlates well with *in vivo* neurovirulence, it is used as a screening method and only bulks passing should be tested *in vivo* (EDQM, 2015h; WHO, 2014c).

Neverov and Chumakov (2010) propose massively parallel sequencing (MPS) for identifying and quantifying the mutation profiles of oral polio vaccines. As reported by Rubin (2011), MPS based methods may facilitate the monitoring of the genetic consistency of live viral vaccines, and in the case of oral polio vaccine have the potential to replace the *in vivo* neurovirulence test. The WHO announced in 2013 an international collaborative study that will assess the utility of massively parallel sequencing for monitoring molecular consistency of oral polio vaccine. The study involves national control authorities and vaccine manufacturers and will also develop common approaches, standards, and acceptance criteria needed for introduction of the new method to regulatory decision-making (WHO, 2013c). Preparation of the study is ongoing and testing will start in early 2016.

4. Pyrogenicity

The Ph.Eur. includes three methods for detection of fever inducing contaminants (i.e. endotoxin from gram-negative bacteria or non-endotoxin pyrogens) in vaccines: the *in vivo* pyrogen test carried out in rabbits (EDQM, 2015i), and two *in vitro* methods, namely, the bacterial endotoxin test (BET; EDQM, 2015j) and the monocyte activation test (MAT; EDQM, 2015k).

The BET (or Limulus Amebocyte Lysate assay) is the appropriate test for detection of endotoxins from gram-negative bacteria and over the last 30 years, it replaced the rabbit pyrogen test in most Ph.Eur. monographs, WHO guidelines, FDA requirements for human vaccines. However, the BET may not be applicable to all vaccine products, since vaccine components as the adjuvant aluminium hydroxide may interfere with clotting reaction, which occurs in the presence of endotoxins.

In contrast to the rabbit pyrogen test and the BET, the MAT is based on human cells. As described in Ph.Eur. 2.6.30 (EDQM, 2015k), the MAT is used to detect substances that activate human monocytes or monocytic cells and induce the release of cytokines. Since these cytokines are playing an important role in fever pathogenesis, the MAT is suitable to detect contamination with pyrogens. Revision of Ph.Eur. 2.6.30 is ongoing and the proposed new text was recently published in *Pharmeuropa* (EDQM, 2015l) Improvements to the text are based on the results of an EDQM survey carried out in 2013. It is underlined that the MAT is suitable, after a product-specific validation, as a replacement

for the rabbit pyrogen test. With regard to coverage of pyrogens, it is noted that *"The MAT detects pyrogenic and pro-inflammatory contaminants, including endotoxins from gram-negative bacteria and 'non-endotoxin' contaminants, including pathogen-associated molecular patterns (PAMPs), derived from gram-positive and gram-negative bacteria, viruses and fungi, and product-related and process-related biological or chemical entities."*

Several individual vaccine Ph.Eur. monographs (e.g. monographs on hepatitis B, meningococcal polysaccharide, pneumococcal polysaccharide, rabies, tick-borne encephalitis vaccines) are still listing the *in vivo* pyrogen test and do not refer to the MAT, nevertheless manufacturers and official control laboratories are encouraged to validate the MAT for the relevant products.

Koryakina et al. (2014) describe the validation of the MAT using cryopreserved peripheral blood mononuclear cells for a vaccine *"… consisting of three recombinant proteins and outer membrane vesicles from a gram-negative bacterium and included aluminum hydroxide as adjuvant."*

5. Potency tests

The purpose of potency testing is to demonstrate with a suitable method that a given vaccine lot would induce a protective immune response comparable to that shown to be efficacious in humans. Depending on the type of vaccine, this may involve animal tests, e.g. immunisation-challenge or immunisation-serology assays for some inactivated vaccines, or may be carried out *in vitro* (antigen quantification assays for live vaccines or well-defined inactivated vaccines). For the immunisation-challenge assays, animals are immunised and after a given period infected with the virulent agent to measure protection against the disease. These assays are often multi-dilution assays with the option of using a single-dilution version. Immunisation-challenge assays may use high numbers of animals and involve severe pain and distress, since insufficiently protected animals (i.e. those which received a vaccine dilution) will develop the disease. More humane animal-based models for measuring potency are immunisation-serology assays, where animals are immunised and after a certain period the antibody levels induced by the vaccine are measured with an immunochemical *in vitro* method.

The use of physico- and immunochemical techniques to characterise antigens and to apply them for the vaccine quality control has been discussed since several years in the light of the consistency approach (Metz et al., 2007; Hendriksen et al., 2008; De Mattia et al., 2011). Some of the vaccines described in the following are adjuvanted and possible interference with the adjuvants must be considered when developing new methods.

5.1 Diphtheria vaccines

The Ph.Eur. lists three possible assays for potency testing of diphtheria vaccines or vaccines containing a diphtheria component. It is clearly stated that the immunisation-serology method should be preferred over the two (intra-dermal or lethal; multi- or single-dilution) immunisation-challenge methods (EDQM, 2015m).

Metz et al. (2003; 2007) showed that physico- and immunochemical techniques as SDS-PAGE, primary amino group determination, fluorescence spectroscopy, circular dichroism and biosensor analysis can be used to characterise diphtheria antigen and detect differences in experimentally produced diphtheria toxoid. The results obtained correlated well with the *in vivo* potency test. The Institute for Translational Vaccinology (Intravacc, NL) and Bilthoven Biologicals are currently testing (in close collaboration with the Serum Institute of India) 20 routinely produced lots of diphtheria toxoid at the bulk and final product stage with the currently regulatory required tests and a suite of additional

physico- and immunochemical assays (Hendriksen 2014, personal communication) as described by Metz et al. (2003; 2007).

Researchers at NIBSC have developed an ELISA for diphtheria antigen quantification (bulk stage or after desorption of the adjuvant on the final product) and propose its use to demonstrate consistency of production (Coombes et al., 2009; 2012; 2015). Zhu et al. (2009) developed a methodology, the Direct Alum Formulation Immunoassay (DAFIA), which can directly demonstrate aspects of antigenic quality without prior desorption and used at the final product stage. This technique is part of the suite of assays used by Intravacc (see above).

Validation of antigen quantification assays for diphtheria vaccines is listed in the EDQM BSP workprogramme for 2015 and beyond[8].

5.2 Tetanus vaccines

The Ph.Eur. monograph includes two assays for potency testing of tetanus vaccines or vaccines containing a tetanus component. It is clearly stated that the immunisation-serology method should be preferred over the immunisation-challenge method (EDQM, 2015n).

A recent publication of Metz et al. (2013) reports on the suitability of physico- and immunochemical methods to detect differences in the quality of ten experimentally produced tetanus toxoids. The authors underline that the methods still need to undergo validation using routinely produced vaccine lots.

Several ELISAs have been described for quantification of tetanus antigen (Prieur et al., 2002; Coombes et al., 2012 & 2015; Metz et al., 2013).

Validation of antigen quantification assays for tetanus vaccines is listed in the EDQM BSP workprogramme for 2015 and beyond[9].

5.3 Whole-cell pertussis vaccines

The potency of whole-cell pertussis vaccines is measured with an immunisation-challenge assay known as mouse protection or Kendrick test (EDQM, 2015o; WHO, 2007) which is highly variable, requires large number of animals and induces severe pain and suffering (i.e. intracerebral challenge, non-protected animals develop pertussis). The validation of an immunisation-serology assay is ongoing within the EDQM BSP104. In principle, guinea pigs are immunised with whole-cell pertussis vaccine and the level of induced antibodies is measured with an ELISA using whole-cell pertussis bacteria for coating. This assay has been developed and prevalidated in a small-scale study funded by the Joint Research Centre (von Hunolstein et al., 2008) and is based on the work carried out by van der Ark et al. (1994; 1996; 1998; 2000).

5.4 Rabies

Potency testing of rabies vaccines is carried out with a multi-dilution immunisation-challenge assay in mice (EDQM, 2015p). The assay (also known as NIH test) has been criticised for many years due to its high variability, the large numbers of mice used and the associated severe suffering and distress. Over the last 15 years, several workshops have been dedicated to the use of 3Rs in the quality control of rabies vaccines e.g. Bruckner et al. (2003); Stokes et al. (2012). Some of the workshop recommendations as the use of humane endpoints in the NIH test (Bruckner et al., 2003) have been

[8] http://www.edqm.eu/en/BSP-Work-Programme-609.html
[9] http://www.edqm.eu/en/BSP-Work-Programme-609.html

implemented into the Ph.Eur. rabies monograph. In addition, the use of alternative methods to the NIH test e.g. an immunisation-serology assay or antigen quantification with an immunochemical assay are mentioned in the recently revised monograph (EDQM, 2015o), provided that they are validated against the NIH test.

Human rabies vaccines are one of the priorities identified by the EPAA vaccines consistency project. In order to address the specific needs for use of the consistency approach and replacement of the NIH test, a workshop was organised in 2012 (see flash report[10] on EPAA website; De Mattia et al., 2015). One critical step in the production of rabies vaccines is the definition of the antigen content in the final product. Manufacturers use *in vitro* antigen quantification assays for this purpose, however, due to regulatory requirements they need to carry out the NIH test on the final lot. There was agreement that an appropriate antigen quantification assay should be able to detect the native trimeric form of glycoprotein-G, a surface protein of the rabies virus, inducing the production of protective antibodies. In order to select the most suitable method for further validation, it was proposed to organise a collaborative study testing vaccines from several manufacturers and of different qualities with the methods currently in use by manufacturers and control laboratories.

This study was organised by an international working group formed after the workshop and chaired by Jean-Michel Chapsal (formerly Sanofi Pasteur) and Noel Tordo (Institute Pasteur). One out of the three ELISA methods correctly estimated the antigen content of all vaccine samples (incl degraded samples). The results of the study and possible follow up have been discussed at a workshop in May 2015 (scientific paper in preparation). It was agreed that additional work would be needed in order to present a proposal to the EDQM Biological Standardisation Programme for full validation of the ELISA.

6. General aspects

6.1 Facilitate and promote product-specific validation

Before a new method can be used for the quality control of a vaccine, it has to be demonstrated that the method is valid in the given laboratory for the given product. This process is generally known as product-specific validation and is a prerequisite for the acceptance of a new method, also for those included in Ph.Eur. monographs after validation within the BSP of EDQM. Product-specific validation is perceived as a hurdle to the swift implementation of 3Rs methods. In order to facilitate and promote product-specific validation of 3Rs methods, expert groups of Ph.Eur. and the European Medicines Agency (EMA) are working on guidelines covering various aspects of product-specific validation. Whereas the Ph.Eur. group of experts 15 is focusing on guidance on data requirements for introducing a new method, the EMA JEG 3Rs[11] group suggests in a recently published concept paper[12] the development of guidance on the use of data generated in large-scale collaborative studies (e.g. those run under EDQM BSP) for product-specific validation. Both documents should become available for public commenting in 2016.

In this context, attention is drawn to Article 13 "Choice of Methods" of Directive 2010/63/EU on protection of animals used for scientific purposes (EU, 2010), which states that that *"a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union."* DG Environment states in *Questions & Answers on the legal*

[10] https://circabc.europa.eu/sd/a/6cfc6e14-4c81-4981-a424-5b51b982da1d/flash-report-vaccines-workshop-october-2012_en.pdf
[11] Joint Committee for Medicinal Products for Veterinary Use/Committee for Medicinal Products for Human Use Ad-hoc Expert Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (JEG 3Rs)
[12] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/07/WC50016 9977.pdf

understanding[13] of Directive 2010/63/EU that *The methods specified in the European Pharmacopoeia can be considered as "recognised by EU legislation" within the meaning of Article 13 of the Directive through a number of EU pieces of legislation such as Directives 2001/82/EC, 2001/83/EC, and 2003/63/EC, as amended, on medicines for human and veterinary use. If a product specific validation is a prerequisite for the use of such a method, the Directive does not foresee a waving from the requirement on the basis of time or cost. … Since a Directive gives generally the Member States some room for manoeuvre concerning its aims, the Competent Authority* [for implementation of 2010/63/EU] *should apply the proportionality principle in their actions. The Competent Authority has to be satisfied that all efforts to complete the validation are made within a reasonable time to comply with the requirements of this Directive. Should the validation fail due to scientific reasons and thus the method proved not valid for the product in question, the use of the animal method could in those cases continue to be allowed. Once a successful validation is completed, or the manufacturer fails to undertake the validation in a reasonable time, the animal method could no longer be authorised.*

6.2 International harmonisation

Due to differences in regional regulatory requirements, manufacturers may need to carry out different tests for lot release when marketing their products outside of Europe. One example is the general safety test (or ATT or test for innocuity; see Section 3.1), which was deleted more than 15 years ago from the Ph.Eur. monographs for lot release, but is still required in many countries outside of Europe.

Several of the leading human pharmaceutical manufacturers call in their review paper (Garbe et al, 2014) for the removal of the ATT from pharmacopoeias and regulatory requirements on a global level. The ATT was introduced in the early 1900s as a test during production of antiserum preparations to detect the level of the preservative phenol. It remained in the regulatory requirements as "safety" test, however, as Garbe et al (2014) state, *the test lacks scientific merit and is neither specific, reproducible, reliable, nor suitable for the intended purpose*.

The EPAA has launched a project aiming at international harmonisation of lot release methods (information available on the EPAA website[14]). Within the framework of this project, the EPAA convened the international workshop *Modern science for better quality control of medicinal products: Towards global harmonisation of 3Rs in biologicals*. It was organised by Katrin Schütte (EU Commission, Directorate General for the Environment, Belgium) and Anna Szczepanska (European Federation of Pharmaceutical Industries and Associations, Belgium) as joint leaders of the EPAA Biologicals project team and took place in September 2015 (report in preparation).

Two case studies presented were related to human vaccines, the deletion of the ATT from national and international requirements and the use of *in vitro* potency assays for diphtheria and tetanus vaccines and the way forward to possible global acceptance of their use. The report of the workshop is in preparation and will become available in early 2016.

Conclusion

The quality control of established vaccines such as diphtheria, tetanus, pertussis and rabies vaccines is focused on the final product control and often involves animal tests. As the report shows progress has been achieved and new approaches to the quality control as the consistency approach have the potential of further reducing animal use.

[13] http://ec.europa.eu/environment/chemicals/lab_animals/pdf/qa.pdf
[14] http://ec.europa.eu/enterprise/epaa/platform-regulation/biologicals/biologicals-project_en.htm

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List of abbreviations and definitions

3Rs	Replacement, reduction, and refinement
ATT	Abnormal toxicity test
BINACLE assay	Binding and cleavage assay
BET	Bacterial endotoxin test
BSP	Biological Standardisation Programme
cAMP	Cyclic adenosine monophosphate
CHO cells	Chinese hamster ovary cells
DAFIA	Direct alhydrogel formulation immunoassay
EDQM	European Directorate for the Quality of Medicines & HealthCare
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EPAA	European Partnership for Alternative Approaches to Animal Testing
EU	European Union
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
GST	General safety test
HIST	Histamine sensitisation assay
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IMI 2	Innovative Medicines Initiative 2
intravacc	Institute for Translational Vaccinology
JEG 3Rs	The Joint Committee for Medicinal Products for Veterinary Use/Committee for Medicinal Products for Human Use Ad-hoc Expert Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products
MAPREC	Mutation analysis by PCR and restriction enzyme cleavage
MAT	Monocyte activation test
MPS	Massively parallel sequencing
MWGT	Mouse weight gain test
NIBSC	The National Institute for Biological Standards and Control
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NC3Rs	UK National Centre for the Replacement, Refinement and Reduction of Animals in Research
NIH	National Institute for Health
PCR	Polymerase chain reaction
Ph.Eur.	European Pharmacopoeia

QC	Quality control
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TgPVR21	Transgenic mice carrying the human poliovirus receptor
US FDA	United States Food and Drug Administration
WHO	World Health Organization

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