Replacement, Reduction, Refinement

Animal welfare progress in European Pharmacopoeia monographs: activities of the European Pharmacopoeia Commission from 2007 to 2017

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ABSTRACT

Since the opening for signature of the European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes in 1986, the European Pharmacopoeia Commission and its experts have carried out a programme of work committed to Replacing, Reducing and Refining (3Rs) the use of animals for test purposes. While updates on achievements in the field of the 3Rs are regularly provided, this article summarises the activities of the Ph. Eur. Commission in this field within the last decade.

KEYWORDS

Replacing, reduction and refining, 3Rs, animal welfare, animal tests, *in vivo* method replacement, European Pharmacopoeia, European Pharmacopoeia Commission.

1. INTRODUCTION

The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) of the Council of Europe was opened for signature on 18 March 1986 [1]. This marked the beginning of an intensification of the activities of the European Pharmacopoeia Commission (Ph. Eur. Commission) to review all animal tests in monographs. Ph. Eur. texts are being continuously reviewed with a view to applying the precepts of the Convention for the '**3Rs**' in the use of animals for test purposes:

- **R**eplacement (animals are no longer used for the test)
- **R**eduction (fewer animals are used to achieve the defined aim of the test)
- **R**efinement (a test that causes less distress to the animals used is carried out).

In addition to the traditional 3Rs, the Ph. Eur. Commission has employed 'Removal', a fourth 'R', as a strategy to end the unnecessary use of animals. This involves the removal of the need for regular performance of an animal test that, after scientific scrutiny, has proved to be no longer relevant and can be deleted without replacement with another test.

A review of the achievements of the Ph. Eur. Commission in the field of 3Rs since the elaboration of the Convention has previously been published [2] and relevant information is also available on the website of the European Directorate for the Quality of Medicines & HealthCare

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(EDQM)[2] and in other publications [3]. This article provides further updates in this field. In addition to legal aspects related to the 3Rs (section 2), it describes the progress made within the last decade in Ph. Eur. texts and the challenges lying ahead (section 3 and tables in the Appendix), and the tools utilised in the implementation of the 3R principles (sections 4-6). It concludes with perspectives for the future (section 7).

2. DIRECTIVE 2010/63/EU

Directive 2010/63/EU on the protection of animals used for scientific purposes [4], which took full effect on 1 January 2013, replaced Directive 86/609/EEC adopted shortly after European Convention ETS No. 123 [1]. It reinforced the 3R principles and introduced tools such as severity classification to estimate the levels of pain, suffering, distress and lasting harm that are inflicted on the animals. Most importantly, Article 13 (Choice of methods) of the directive has a significant impact for users of the Ph. Eur. as it includes the following statement:

> Without prejudice to national legislation prohibiting certain types of methods, Member States shall ensure 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.

Application of the quality requirements of the Ph. Eur. is prescribed in EU legislation [5, 6] and thus can be considered as recognised in the context of Article 13. Further considerations on the potential impact of Directive 2010/63/EU on the implementation of the Ph. Eur. can be found in section 5.2.

3. ACHIEVEMENTS OVER THE LAST DECADE

The Ph. Eur. Commission has taken a multi-layered approach to advance 3Rs improvements. This approach begins with the overarching principles, includes the assessment of strategies applicable across different sectors and is anchored in specific texts in individual monographs which directly reduce, replace, refine or remove the use of animals. The main achievements of the Ph. Eur. Commission in this field over the last decade are described below, while detailed information by class of product on specific texts is provided in the Appendix. Despite the many advances, numerous challenges remain and details of some of those encountered during the last decade are also discussed below.

3.1. Overarching principles

3.1.1. Compliance via validated alternatives

As stated in the General Notices (chapter 1) of the Ph. Eur., the methods described in Ph. Eur. monographs are reference methods, essential in case of dispute. Compliance is requested, but alternative methods may be used as long as they lead to the same pass/fail result. In other cases, a detailed validated procedure may be given as an example of a suitable method, meaning that other methods could be used instead without having to demonstrate their equivalence to the example method.

Moreover, the General Notices state that compliance with Ph. Eur. requirements does not imply that performance of all the tests in a monograph is necessary provided the product would comply if tested. In other words, through its General Notices, the Ph. Eur. already allows flexibility in the application of testing requirements.

3.1.2. Consistency of production

The Ph. Eur. also provides flexibility with respect to test performance in the *Demonstration of compliance with the Pharmacopoeia* section in the General Notices:

2 https://www.edqm.eu/en/alternatives-animal-testing.

> Reduction of animal testing: the European Pharmacopoeia is dedicated to phasing out the use of animals for test purposes, in accordance with the 3Rs (Replacement, Reduction, Refinement) set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. In demonstrating compliance with the Pharmacopoeia as indicated above […], 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.

The concept of waiving tests as part of a strategy for monitoring consistency of production was added to the general monograph *Vaccines for veterinary use (0062)* as well as to three veterinary vaccine monographs, *Canine leptospirosis vaccine (inactivated) (0447)*, *Bovine leptospirosis vaccine (inactivated) (1939)* and *Infectious bovine rhinotracheitis vaccine (inactivated) (2674)* (9th Edition, January 2017). Omission of tests is therefore possible when consistency is demonstrated and in agreement with the competent authority. It is important to note that compliance with the tests described in Ph. Eur. monographs (during production or for the final lot) is usually not sufficient to ensure consistency of production: suitable additional tools such as statistical process control should also be used.

3.1.3. Substitution of in vivo *tests*

Pharmacopoeia monographs are public standards and are intended to provide quality requirements applicable to all products on the market. Application of the 3Rs to animal testing in existing monographs has been seen to require development of an alternative method applicable, without modification, to all existing products. For finished products, notably vaccines, this aim has rarely been achieved in a way that leads to direct application of the 3Rs. The existing products were developed at a time when the animal model was the standard method, despite the associated relatively high variability, and the products were necessarily developed in such a way as to comply with these models. Demonstration of equivalence of an alternative method to the animal model is not simply problematic, in many instances it may also be of limited relevance. This implies that a complete re-evaluation of the aims of the new test needs to be made to define the relevant aspects that must be validated.

With these difficulties in mind, and to facilitate the transition from *in vivo* to *in vitro* methods, the Ph. Eur. Commission developed a new general chapter on the *Substitution of* in vivo *method(s) by* in vitro *method(s) for the quality control of vaccines (5.2.14)* published in Supplement 9.3 (implementation date January 2018). This provides guidance on how to validate alternative *in vitro* methods in scenarios where a direct head-to-head comparison with an existing *in vivo* method is not possible. The general chapter envisages the possibility that the validity of an alternative *in vitro* method can be demonstrated without such a head-to-head comparison (concept of 'substitution') and discusses alternative approaches for *in vivo* method replacement. Specific recommendations on the substitution of *in vivo* potency and safety tests, together with examples, are provided. A cross-reference to Chapter *5.2.14* has been added to the general monographs *Vaccines for human use (0153)* and *Vaccines for veterinary use (0062)* to increase stakeholder awareness of this important text, which provides additional tools for the efforts to reduce animal testing and encourage the use of alternatives.

3.2. Applicability across sectors

3.2.1. Abnormal toxicity

The abnormal toxicity test (ATT) was originally developed in the early 1900s as a safety test intended to detect extraneous contaminants in biological products. The test is based on the injection of the product to be tested into mice/guinea pigs. The product passes the test if no animal shows any sign of illness, relevant body weight changes, or dies within a defined timeframe. The number of animals used in the ATT has been considerable (e.g. 5 mice and 2 guinea pigs are used for each vaccine batch to be tested), making the ATT one of the most controversial animal tests in the Ph. Eur., and therefore a priority target for replacement.

After a review of historical batch data in 1999, references to the ATT were removed from the Tests section of over 80 monographs and replaced by a statement in the Production section, which prescribed that the manufacturing method be validated in such a way as to ensure that the product would comply with the test if it were performed. As a result of this exercise, the ATT was no longer required to be performed routinely on each batch (deletion of the test as a routine batch release test) and had to be performed during product development only.

The relevance of the ATT was reviewed in depth during a European Partnership for Alternative Approaches to Animal Testing (EPAA) International Workshop in September 2015 [7]. The test was considered to be outdated and shown to be 'neither specific, reproducible, reliable nor suitable for the intended purpose'. Additionally, with modern manufacturing and stringent quality measures in place to control and detect contaminants, it has also become unnecessary. It was concluded that the ATT lacked scientific relevance and that its omission would not compromise the safety of biologicals.

Based on this detailed review, the Ph. Eur. Commission deleted the ATT from 49 Ph. Eur. monographs encompassing areas such as vaccines and immunosera for human use, biotherapeutics, allergens, antibiotics/antimycotics and plastic containers. Revised monographs omitting the ATT from the Production section will be included in Supplement 9.6 (January 2019) with simultaneous suppression from the Ph. Eur. of general chapter *Abnormal toxicity (2.6.9)*, as it will no longer be referred to in any monograph and will thus be obsolete. With the publication of Supplement 9.6, the complete suppression of the ATT from the Ph. Eur. will have been achieved.

3.2.2. Rabbit pyrogen test (RPT)

About 60 Ph. Eur. texts still refer to the pyrogen test (*Pyrogens 2.6.8*). Among these are monographs on vaccines for human use, blood products, antibiotics, solutions for dialysis or organ preservation, and general chapters on plastic containers for blood, syringes and sets for transfusion. The Ph. Eur. Commission is making every effort to proceed with the replacement of this widely used animal test. Typically, where possible, the pyrogen test has been replaced by the bacterial endotoxin test (BET). In a recent revision of the chapter *Guidelines for using the test for bacterial endotoxins (5.1.10)* (Supplement 8.8, July 2016), a recommendation has been introduced to perform a risk assessment when using the bacterial endotoxin test as a pyrogenicity test to confirm the absence of potential contamination by non-endotoxin pyrogens. The *Monocyte-activation test (2.6.30)* is a suitable method for ruling out the presence of non-endotoxin pyrogens in substances or products at release or during the production process (see section 3.2.3).

3.2.3. Monocyte activation tests (MAT)

The monocyte activation test (MAT) is an *in vitro* test which can be used to replace the RPT after suitable validation has been performed. It is used to detect or quantify substances that activate human monocytes to release endogenous mediators such as pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6). The monocyte source used in the test can be whole blood or peripheral blood mononuclear cells (PBMC) from a single or several donors after pooling, with or without cryopreservation storage. Moreover, monocytic continuous cell lines are also available for this test. The general chapter *Monocyte-activation test (2.6.30)* was introduced in Supplement 6.7 (April 2010) following a recommendation resulting from a European Centre for the Validation of Alternative Methods (ECVAM) Workshop [8]. While the MAT offers significant improvements in terms of 3Rs, it had been reported that the test had not been used as widely as expected for quality control purposes since its introduction in the Ph. Eur. As a result, the EDQM carried out a survey in 2013 with the intention of improving the technical content of the chapter, and a revised version of the chapter was published in Supplement 9.2 (July 2017). A key challenge that remains for the EDQM is to be able to provide a reference standard for a non-endotoxin pyrogenic substance. This is now being addressed via a joint project run by the World Health Organization (WHO) and the EDQM Biological Standardisation Programme (BSP) (BSP149) to establish a reference material suitable for this purpose.

The general chapter *Pyrogens* (*2.6.8)* was revised in Supplement 8.8 (July 2016) with the addition of a statement encouraging the replacement of the RPT with the MAT:

> In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test *(2.6.30)*.

Monographs and general chapters which refer to the pyrogen test typically do not refer to the MAT: the reference is made only once in general chapter *2.6.8*. Once a full validation package for the individual substance/preparation/material in question has been made available, replacement of the RPT with the MAT could be envisaged.

3.3. Specific improvements to individual monographs/chapters

Descriptions and details of each revision of Ph. Eur. texts can be found in the EDQM Knowledge Database under 'view history'.

3.3.1. Vaccines for veterinary use

In the absence of a suitable *in vitro* alternative, a first approach to address the 3Rs when a method involving significant suffering or distress of animals (e.g. an LD50 assay) is prescribed in the Ph. Eur. has been to introduce 'humane' end-points. In this spirit, the Ph. Eur. monograph *Rabies vaccine (inactivated) for veterinary use (0451)* was revised in Supplement 6.1 (April 2008). The revision introduced a new section on alternative (i.e. non-lethal) end-points, describing early clinical signs of rabies infection that can be observed and used as an alternative end-point in the potency assay, together with a score chart. Analysts are expected to 'validate' the use of clinical signs as end-points (i.e. show that the use of such alternative end-points yields assay results equivalent to those obtained when a lethal end-point is used) by scoring a sufficient number of batches using both the clinical signs and lethal end-points. Since the test is carried out routinely for the release of vaccine batches, manufacturers have the opportunity to incorporate the alternative scoring without having to perform separate tests for validation.

As of Supplement 7.7 (April 2013), following the decision of the Ph. Eur. Commission, the Target Animal Batch Safety Test (TABST) was deleted from the Ph. Eur. This deletion of the TABST goes a step further than the option, available since 2004, of waiving the use of the TABST for established vaccines. This decision was based on a number of parameters, including poor sensitivity of the test, a very limited number of batches failing the test and observations of field safety issues with batches compliant with the TABST. Taking into account new developments (e.g. improvements in the manufacturing process of veterinary vaccines in recent decades and the introduction of new requirements regarding in-process testing and control of the starting materials), the risk/benefit ratio no longer supported retention of such a test for routine batch release and it was therefore decided to delete it (see [9] and [10] for more details). This change has already greatly reduced the number of animals used for the control of veterinary vaccines, while maintaining the level of safety.

Several recent 3Rs-related amendments in relation to veterinary vaccines were introduced at the same time (9th Edition, January 2017) (see Appendix, Table 1):

- manufacturers were encouraged to use modern *in vitro* methods, such as *Nucleic acid amplification techniques (2.6.21)*, instead of the test for antibody induction in animals to identify inactivated vaccines. According to the general monograph *Vaccines for veterinary use* (*0062*), for inactivated vaccines, the identification test may be combined with the potency test to reduce the number of animals used;
- thanks to the elaboration of a new chapter *Healthy chicken flocks for the production of inactivated vaccines for veterinary use (5.2.13),* which sets quality requirements that provide guarantees with regard to contamination by extraneous agents, together with appropriate validation of the inactivation process (done once in the lifetime of the vaccine),

it was possible to omit the test for specified extraneous agents performed on the final product. The introduction of a reference to chapter *5.2.13* containing requirements for healthy flocks rendered the test for specified extraneous agents previously performed on each final product obsolete, and allowed the deletion of the specified extraneous agents test (using either 10 chickens or 2 pigs) for 6 veterinary vaccine monographs (*0870*, *0959*, *0960*, *0963*, *1202* and *1392*);

- an *in vitro* batch potency test for Leptospira vaccines was introduced;
- a serological assay for rabies vaccines was introduced following the completion of BSP105 ([11]; see also section 4).

Some improvements were also made when the regulatory requirements were reviewed, such as updating the *Infectious chicken anaemia vaccine (live) (2038*) monograph immunogenicity test to replace solitary housing of laying hens and young chickens with social housing in stable groups, which is less stressful for the animals.

Where, in spite of all the efforts made by the Ph. Eur. Commission to promote animal welfare, animal tests still subsist in routine testing, 'door openers' may have been included in Ph. Eur. texts. These indicate that alternative methods can replace the animal test and are to be seen as encouragement for manufacturers to develop their own alternative method. A reference to the type of alternative method may be given as an example. To illustrate this, see the example of *Canine leptospirosis vaccine (inactivated) (0447)* (Ph. Eur. 9th Edition, January 2017) for which a single, universal alternative method could not be developed due to the complexity of the vaccines, but which nevertheless includes as first option a suitably validated *in vitro* batch potency test able to determine the content of one or more antigenic components which are indicators of protection and which are specific for that serovar. This does not preclude manufacturers from developing other types of more appropriate alternative methods (for example, where new techniques are available).

3.3.2. Vaccines for human use

The Ph. Eur. monograph on *Rabies vaccine for human use prepared in cell cultures (0216)* was revised in Supplement 6.1 (April 2008). In the same way as for the monograph *Rabies vaccine (inactivated) for veterinary use (0451)*, and to foster the use of humane end-points in the potency assay, the revision introduced a new section describing early clinical signs of rabies infection that can be observed and used as an alternative end-point, together with a score-chart (see section 3.3.1 for further details). A new general chapter on *Residual pertussis toxin and irreversibility of pertussis toxoid (2.6.33)* was included in Supplement 7.8 (July 2013). It introduced a standard protocol for the Histamine Sensitisation Test in mice (HIST), based on the outcome of a collaborative BSP study (BSP076), with the intention of facilitating the standardisation of the method and therefore reducing the unnecessary use of animals [12]. Based on the results of a more recent collaborative study (BSP114) completed in 2015 [13], the introduction of an *in vitro* Chinese Hamster Ovary (CHO) cell-clustering assay for the determination of residual pertussis toxin as a replacement for the animal HIST is under discussion. The removal of the test for irreversibility of pertussis toxoid has also been proposed.

In vitro methods have, in a number of cases, been introduced as an alternative to or replacement for *in vivo* testing. One example was the introduction in the general chapter *Assay of hepatitis A vaccine (2.7.14)* of a validated ELISA method for determination of the antigen content [14] as an alternative to the serology assay in mice, a change included in Supplement 8.5 (July 2015). Various strategies have also been used to promote both the reduction and refinement of *in vivo* assays, for example through the introduction of serology assays as an alternative to lethal challenge methods for diphtheria, tetanus, acellular pertussis and rabies vaccines; after sufficient experience is gained, these may be used as a simplified model (e.g. a single-dilution model), leading to a reduction in the number of animals needed [15-17].

More recently the Ph. Eur. Commission's attention turned to the current requirements for extraneous agent testing. The aim was to rationalise these requirements without in any way

compromising safety. As part of this initiative, the general chapters *Tests for extraneous agents in viral vaccines for human use (2.6.16)* and *Cell substrates for the production of vaccines for human use (5.2.3)* were revised in Supplement 9.3 (January 2018). The revised general chapter *2.6.16* recommends that the testing strategy for extraneous agents should be established based on a risk assessment and the list of tests must be adapted depending on the extraneous agents that have the potential to contaminate the product. Molecular biology methods may be considered for the detection of specific viruses and/or for the broad detection of viruses. As part of the revisions of both general chapters, the tests in adult mice and guinea pigs were deleted as they were considered redundant due to the presence of other tests providing risk mitigation. In addition, the tests in suckling mice and control eggs are now to be used only if a risk assessment indicates that the tests can provide risk mitigation.

3.3.3. Blood products

A systematic review of animal tests prescribed in monographs for medicinal products derived from human blood and human plasma (hereinafter called 'plasma-derived products') has been undertaken with a view to introducing, wherever possible, provision for the use of an *in vitro* method. This work has been driven by consultations with stakeholders; it is based on the outcome of the EDQM internal workshop on the *in vitro* pyrogen test (2005) and of a survey conducted by the EDQM in 2005 to gather data on the application of different test methods for the replacement of the RPT for plasma-derived products. Notably, data demonstrating equivalency of test methods has enabled the revision of 15 monographs to allow a validated *in vitro* test, such as the bacterial endotoxin test, to be used as an alternative to the RPT (see Appendix, Table 3). Typical wording in monographs is as follows:

> *Pyrogens (2.6.8)* or *Bacterial endotoxins (2.6.14)*. It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

In addition, the European Medicines Agency (EMA) has revised its *Guideline on plasma-derived medicinal products* [18], by introducing a cross-reference to the *Guideline on the replacement of rabbit pyrogen testing by an alternative test for plasma-derived medicinal products* [19]. The revised monographs together with the EMA guideline constitute a powerful combination of tools that will help users to implement a replacement for the RPT.

As a result of the Ph. Eur. Commission's efforts, the majority of Ph. Eur. monographs on plasma-derived products currently promote the use of non-animal alternatives. The Ph. Eur. is continuing to examine whether alternatives to the RPT are available for the remaining monographs, and to complete the 3Rs-driven revision process.

3.3.4. Biological and biotechnological substances

A number of actions had already been undertaken to replace and reduce the use of animal testing in the field of biological and biotechnological products [20-25]. The RPT has been replaced by the bacterial endotoxin test in all but one monograph (see section 7.4), while the ATT was recently deleted from four monographs (see Appendix). Finally, substitution of the assay on isolated rat adrenal cells with a liquid chromatography (LC) method in the *Tetracosactide (0644)* monograph in Supplement 6.3 (January 2009) concluded the replacement of *in vivo* bioassays in monographs for synthetic peptides.

3.4. Challenges

The Ph. Eur. Commission is committed to including validated 3Rs methods in specific monographs wherever possible. In order to include such texts in a monograph, however, there are certain prerequisites, based on scientific principles and the applicability of the method to all or most products on the EU market, which must be met. According to the general principles noted above, the absence of a description of an alternative test in the Ph. Eur. does not preclude the possibility to use suitably validated 3Rs alternative methods developed for individual products provided they are approved by the licensing authority (see EMA Guideline on the principles of regulatory acceptance of 3Rs testing approaches [26]).

Some of the efforts already invested to replace the *in vivo* test in the Ph. Eur. monographs that still contain an animal-based potency assay are described below.

3.4.1. Erythropoietin

The *Erythropoietin concentrated solution (1316)* monograph comprises two potency assays carried out in polycythaemic (method A) and in normocythaemic mice (method B). Several attempts to implement the 3Rs principles in this monograph and to replace these *in vivo* assays have been made in the past.

As the biological activity of erythropoietin *in vivo* is known to critically depend on the level of terminal sialyation of the carbohydrate chains, and is therefore quantitatively related to the isoform distribution, the initial step towards the replacement of the animal test in the monograph was to improve the isoform distribution test. Consequently, a collaborative study was carried out to assess a capillary zone electrophoresis (CZE) method for this purpose [27]. Although the CZE method replaced the isoelectric focusing (IEF) test in the 4th Edition of the Ph. Eur. (implementation date January 2002), its introduction did not justify the deletion of the *in vivo* bioassays.

Subsequently, following a proposal from ECVAM's Scientific Advisory Committee, the inclusion of an *in vitro* activity test in addition to the two *in vivo* assays was proposed [28]. The comparative data gathered over the years was expected to facilitate the eventual replacement of the bioassays in the future. However, further to comments received during the public enquiry phase, this proposal was abandoned as it was considered that more knowledge and experience of *in vitro* assays had to be gathered first.

During the subsequent revision of the monograph another recommendation from ECVAM to delete assay method A leaving method B as the only assay for erythropoietin was carefully considered. The sacrifice of a considerable number of animals that would result from a compulsory revalidation of method B by users currently employing method A was judged unacceptable and unnecessary. Therefore, the Assay section has been maintained with two *in vivo* bioassays until the introduction of an *in vitro* assay replacing them can be executed.

In addition to these activities, the European erythropoietin manufacturers had been contacted on a number of occasions, including two surveys performed in 2005 and 2009, for their assistance in replacing the animal assay. Significant data using available validated *in vitro* methods was gathered during the establishment of the most recent batch of Erythropoietin BRP [29] and its analysis is ongoing. In the meantime, in order to avoid overconsumption and frequent replacement of Erythropoietin BRP, which is calibrated in International Units in a bioassay that requires the use of animals, a separate reference standard, Erythropoietin for physico-chemical tests CRS, was established to be used in CZE, polyacrylamide gel electrophoresis and immunoblotting and in peptide mapping identification tests [30].

3.4.2. Follitropin monographs

The *Follitropin (2285)* and *Follitropin concentrated solution (2286)* monographs were published in the 8th Edition of the Ph. Eur. Despite the efforts invested during the drafting phase, no suitable alternative to the *in vivo* potency test could be found. Hence, the monographs in question contain an *in vivo* assay in which the follicle-stimulating activity of follitropin is estimated in rats.

An assessment of IEF and CZE as possible replacements to the follitropin *in vivo* potency assay had been performed in an international collaborative study co-ordinated by ECVAM [31]. However, no correlation with the International Unit could be found and the methods could not be applied to all follitropin products available in Europe.

An authorised IEF-based method developed by a manufacturer as part of a well-controlled process had also been considered as a potential *in vitro* alternative. However, as it was based on a proprietary analysis tool, it could not constitute a Ph. Eur. method.

3.4.3. Radiopharmaceuticals

Many monographs for radiopharmaceutical preparations were elaborated in the 1970s and 1980s. Animal tests, mainly physiological distribution tests, were included to ensure the desired distribution of the radiopharmaceutical preparation in the body. Physico-chemical tests have evolved with time and are often able to control the composition of the radiopharmaceutical preparation; they can thus be used to replace these physiological distribution tests. However, as with other groups of pharmaceuticals, the demonstration of equivalence of potential alternative techniques to the animal tests would require the sacrifice of many animals.

All monographs on radiopharmaceutical preparations that contained a test involving animals were carefully reviewed. For some, for example radiolabelled colloids, it was considered that current physico-chemical methods alone could not provide adequate quality control and the physiological distribution test could not be replaced or deleted. In other cases the preparations concerned were old products of rather minor economic importance and manufacturers would not invest resources to develop and validate methods to replace the animal test. Academia was not interested either, since research was focused on new products. In the cases of *Technetium (^{99m}Tc) medronate injection (0641)* and *Technetium (^{99m}Tc) etifenin injection (0585)* it proved possible to replace the animal test with a combination of physico-chemical tests.

The revised general monograph *Radiopharmaceutical preparations (0125)* (Ph. Eur. 7.5, July 2012) clearly outlines that 'tests involving animals should be avoided wherever possible'. New monographs on radiopharmaceutical preparations do not contain animal tests.

4. BIOLOGICAL STANDARDISATION PROGRAMME OF THE EDQM

The application of the 3Rs principles in the Ph. Eur. has been greatly facilitated by the Biological Standardisation Programme (BSP) of the EDQM [3]. Since its establishment in 1991, this programme has provided the means to carry out studies to develop and validate methods promoting the application of the 3Rs that are subsequently incorporated into the monographs and chapters of the Ph. Eur. The BSP is co-financed by the EU and the Council of Europe.

The BSP establishes Ph. Eur. working standards (mostly biological reference preparations (BRPs)) and fosters method development in the field of biologicals for human and veterinary use with a focus on method validation for 3Rs purposes. It is overseen by a Steering Committee made up of the chairs of the Ph. Eur. groups of experts involved and the chairs of the European Medicines Agency's Biologics Working Party and Immunologicals Working Party, co-opted experts on specific subject areas, a representative from the EU Commission and the EMA, the Director of the EDQM and an observer from WHO. This Steering Committee takes decisions on the programme of activities and at critical stages of individual projects. The goal is to introduce the validated methods and standards into Ph. Eur. monographs. BSP projects take methods that have undergone proof of concept development and validation in a local context (e.g. by an individual Official Medicines Control Laboratory (OMCL)) or through other projects (e.g. those run by EURL-ECVAM, EPAA, VAC2VAC), and where necessary complete the validation package before using large-scale collaborative studies to demonstrate their general suitability in a wider context [32]. To date 22 projects have been initiated in the interest of 3Rs method development.

A number of projects have been completed in the last 10 years. These include validation of 2 *in vitro* assays to completely replace the use of animals in potency testing of human tetanus immunoglobulins [34-35] and an *in vitro* alternative assay for Hepatitis A vaccine potency [14]. A project to standardise a CHO cell-clustering assay for residual pertussis toxin in acellular pertussis vaccines [13] was completed and, together with a decision by the relevant Ph. Eur. group of experts based on pertinent data, has resulted in a proposal for complete removal of the HIST in mice from general chapter *Residual pertussis toxin and irreversibility of pertussis toxoid* (*2.6.33)*. Other completed projects include a serological assay for acellular pertussis vaccines in guinea pigs which can be combined with serological assays for diphtheria and tetanus components in combined vaccines [15-17], as well as a serological assay for the potency of rabies vaccine (inactivated) for veterinary use [11]. There are several ongoing

projects at different stages of completion, such as validation of an *in vitro* assay (BINACLE: *in vitro* binding and cleavage assay) for the detection of tetanus toxin activity in human and veterinary vaccines for tetanus (BSP136), *in vitro* assays for consistency testing of diphtheria and tetanus antigen content/potency in human vaccines (BSP113), an *in vitro* assay for potency of erythropoietin as a follow up to BSP120, the study to establish Erythropoietin BRP batch 4 [29], a serological potency test for whole-cell pertussis vaccines (BSP104), and in collaboration with the EPAA, the validation of an *in vitro* ELISA assay for antigen content/potency of human rabies vaccines (BSP148) and an *in vitro* replacement for the minimum lethal dose (MLD) and the total combining power (TCP) assays for Clostridium septicum vaccine (BSP130).

The BSP remains open to new proposals within the scope of its activity and encourages all stakeholders to make relevant contributions for consideration.

BSP achievements in the field of the 3Rs are also reported on the EDQM website.[3]

5. EDQM CONFERENCES

Over the years, the EDQM has been eager to facilitate the implementation of 3Rs projects by organising international conferences bringing together experts in the field from regulatory authorities, pharmacopoeias, industry and academia as evidenced by the examples below.

5.1. EDQM International Symposium: Alternatives to Animal Testing – New Approaches in the Development and Control of Biologicals, 23-24 April 2008, Dubrovnik, Croatia

Participants acknowledged that considerable progress had been made in setting non-animal requirements, especially in Europe, but that implementation and regulatory acceptance of 3Rs methods were still key elements that needed further work, in particular for routine application in the control of biologicals. Better transparency and dissemination of existing and future scientific work and achievements should be promoted by publication in appropriate journals and the use of other platforms. It was recognised that Europe had taken a leading role in addressing the challenges and was encouraged to continue to promote new ideas and their application. However, the need for international harmonisation was strongly expressed and supported. Representatives from all the European and international institutions present indicated their willingness to investigate means to improve the situation.

5.2. EDQM International Symposium: Alternatives to Animal Testing, 8-9 September 2011, Strasbourg, France

The aim of the symposium was to share information and experiences of the advances that had been made in this field with regard to the EDQM's BSP and the Ph. Eur. Particular attention was given to the successful completion of a number of EDQM collaborative studies for the validation of 3Rs methods in the fields of human and veterinary vaccines and human blood-derived products, as well as to the Ph. Eur. Commission's efforts to replace the RPT. Aimed at facilitating the practical implementation of the new methods, the symposium provided the opportunity for an in-depth discussion of the new methods and also to prepare for the implementation of Directive 2010/63/EU. The symposium was followed by a thorough evaluation of the impact of the Directive. It was concluded that whenever the Ph. Eur. offered the possibility to carry out either an *in vivo* test or an *in vitro* alternative, the use of the *in vitro* alternative would become obligatory in the EU. Ph. Eur. texts that continued to describe animal methods were found to be compatible with the provisions of the Directive.

5.3. EDQM Workshop on Alternatives to the Leptospirosis Batch Potency Test, 26-27 January 2012, Strasbourg, France

As part of the efforts of the Ph. Eur. Commission to replace *in vivo* with *in vitro* methods, and in a bid to develop alternatives to the batch potency test for leptospirosis vaccines, a workshop

3 https://www.edqm.eu/en/BSP-programme-for-3Rs-1534.html.

targeted at leptospirosis vaccine manufacturers discussed (current and future) alternative methods to the hamster potency test for leptospirosis vaccines, with a view to defining a clear strategy for its replacement. The workshop provided the opportunity for an in-depth discussion of alternative methods and their practical implementation. Participants agreed that a single, universal alternative method could not be developed, due to the complexity of the vaccines (relevance of specific antigens as protective agents, number of serotypes, number of serovars, combinations, presence/absence of adjuvants). However, during the workshop it was shown that an alternative method had already been successfully implemented in Europe, approved by a competent authority, with a further example from the US. These methods use lipopolysaccharide (LPS)-based antigen quantification by ELISA. There was unanimous agreement among the participants present that moving towards complete *in vitro* testing for leptospirosis vaccines is possible and should be promoted.

Further to the EDQM workshop, monographs *Bovine leptospirosis vaccine (inactivated) (1939)* and *Canine leptospirosis vaccine (inactivated) (0447)* were revised for the 9th Edition to introduce the possibility of using alternative methods to the method using guinea-pigs (e.g. LPS-based antigen quantification), thereby contributing to animal welfare (3Rs). Manufacturers are encouraged to develop alternative *in vitro* methods to the animal test for batch release (first option of choice) using appropriate tools such as the monitoring of production consistency and appropriate antigen quantification.

5.4. EDQM International Symposium: The Challenges of Quality Requirements for Fish Vaccines, 10-11 May 2016, Oslo, Norway

The symposium was aimed at discussing the current requirements with a focus on alternative methods, already in use or under development, to replace the challenge batch potency test. The audience discussed the possibility of introducing humane end-points in Ph. Eur. monographs for fish vaccines, and to revise the four monographs already published [*Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids (1521), Cold-water vibriosis vaccine (inactivated) for salmonids (1580), Vibriosis vaccine (inactivated) for salmonids (1581), Yersiniosis vaccine (inactivated) for salmonids (1950)*]. The potential need for new Ph. Eur. monographs, such as a general monograph dedicated to vaccines intended for fish and individual monographs for fish vaccines, for example for the Mediterranean region or for other fish diseases, was also discussed.

Further to the EDQM International Symposium, fish vaccine monographs *1521*, *1580*, *1581* and *1950* were revised for Supplement 9.2 (July 2017) to clarify that alternative methods are not limited to serological methods.

6. PARTNERSHIP WITH REGULATORY AUTHORITIES AND OTHER ORGANISATIONS

Regulatory authorities and OMCLs from the 38 countries signatory to the Convention on the Elaboration of a European Pharmacopoeia as well as observers are key players in the 3Rs achievements. The Ph. Eur. works closely with them and relies on their expertise and motivation to effect important changes. In addition, the EDQM regularly exchanges information with the EMA through its relevant working parties. Particularly relevant for 3Rs, the EDQM participates as an observer to the Joint Committee for Medicinal Products for Veterinary Use/ Committee for Medicinal Products for Human Use Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (EMA Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products – J3RsWG), an Expert Group created in 2010 by the EMA to provide 3Rs advice on scientific/technical matters related to regulatory testing of medicinal products for human and veterinary use.

Through this group, the EMA has published a position on the application of the 3Rs in the testing of medicines, and issued *Recommendations for marketing-authorisation holders on their need to comply with 3Rs methods in the European Pharmacopoeia* [33]. Specific recommendations in line with monograph/chapter revisions have also been established for

hepatitis A vaccine [36], revisions related to extraneous agents testing and cell substrates for vaccines for human use [37], and a series of recommendations on monographs for veterinary vaccines [38, 40] reinforcing the need to apply the 3Rs concept of the Ph. Eur. in a timely manner. This co-operation is an important element in ensuring awareness and implementation of the various 3Rs advances. In addition the EDQM has contributed actively to the activities of the EPAA and interacts with EURL-ECVAM on different topics of common concern.

7. FUTURE PERSPECTIVES

Although significant progress in the field of 3Rs has been achieved over the last 10 years, the Ph. Eur. Commission is aware of the work still to be done and is committed to further incorporating the 3Rs principles in pharmacopoeial texts. Some of the areas identified as a focus for the future are outlined below.

7.1. Vaccines for veterinary use

For veterinary vaccines, further work is ongoing/will be undertaken with a view to replacing as many animal tests as possible, for example:

- Revision of the extraneous agents testing approach to reinforce risk assessment and to be open to any suitable method, including *in vitro* methods; application of the consistency approach to manufacturing, including an overall risk management strategy for starting material and final product testing.
- Deletion of the remaining tests for specified extraneous agents (monographs 0744, 0965, 1953, 1954 and 1943) in light of the new approach for extraneous agents testing described above.
- Revision of the Identification sections for all live vaccine monographs to open to any suitable method. Currently identification is performed with an immunostaining/neutralisation test in cultures (cells or SPF eggs for avian vaccines) using a monospecific antiserum/monoclonal antibodies. A similar exercise had already been performed for all monographs on inactivated vaccines (see section 3.3.1) and is included in the 9th Edition of the Ph. Eur. Moreover, in several recently drafted or revised monographs on inactivated vaccines, reference to the antibody induction test has been removed, thereby allowing the use of any suitable method.
- Revision of the monograph *Clostridium septicum vaccine for veterinary use (0364)* upon finalisation of the BSP study (BSP130) for the replacement of MLD and TCP tests. This exercise may also have an impact on other monographs for toxoids from cytotoxic clostridial toxins (e.g. 0362, 0363).
- Promotion of a move towards *in vitro* methods for the potency testing of fish vaccines.
- The relevance of the test for 'irreversibility of toxoid' described in monograph *Tetanus vaccine for veterinary use (0697)* is under assessment (see section 7.2).

7.2. Vaccines for human use

The potency assays for established human vaccines (e.g. diphtheria and tetanus vaccines) were initially based on lethal challenge methods and were refined over time with the introduction of serology assays and options for single-dilution assays. However, further work is required to achieve complete replacement of these *in vivo* methods by *in vitro* methods and avoid the use of animals for the determination of potency. For rabies vaccines, a lethal challenge method is still described in the monograph as the reference method for potency determination, despite previous initiatives to encourage the use of refined methods based on non-lethal end-points (see 3.3.2). Efforts to validate a suitable *in vitro* assay will be followed closely (see section 4).

The specific toxicity tests applied to vaccines such as tetanus and acellular pertussis vaccines continue to use animals. In this regard, a proposal to remove the test for irreversibility of pertussis toxoid and replace the HIST with a CHO cell-clustering assay in the test for

residual pertussis toxin is being examined (see section 3.3.2). Likewise, the relevance of the test for irreversibility of the tetanus toxoid applied to tetanus vaccines for both human and veterinary use is being questioned and will be further assessed. An endopeptidase assay for determination of tetanus toxin activity (BINACLE) has been developed and is being tested in a collaborative study (BSP136).

Several other BSP projects are under way to advance the development of *in vitro* alternatives to animal methods used for vaccine quality control. These include, but are not limited to, the validation of an antigen content assay for consistency evaluation of diphtheria and tetanus toxoids (BSP113) and the validation of an ELISA potency assay for determination of the potency of human rabies vaccines (BSP148).

7.3. Biological and biotechnological substances

Although a number of attempts have already been made to minimise animal testing and replace the *in vivo* bioassay in the *Erythropoietin concentrated solution (1316)* monograph (see section 3.4.1), introducing an *in vitro* assay alternative has proved difficult to date. One of the reasons is the variable ratios of *in vivo* to *in vitro* bioactivity of erythropoietin from different sources. Moreover, it has not yet been demonstrated that any of the existing cell-based *in vitro* bioassays could be universally applied. Finally, the International Unit of erythropoietin bioactivity, defined by the WHO International Standard, has been established on the basis of and is traceable to the *in vivo* bioassay procedures, further complicating the task of replacement of the *in vivo* assay. Nevertheless, the substantial data on assaying the potency of erythropoietin using both *in vivo* and available *in vitro* assays gathered during recent years constitute a basis for a new discussion.

Similarly, the replacement of the *in vivo* potency assay in follitropin monographs remains a priority. Users are kindly invited to submit any suggestions regarding potential alternative approaches on this topic to the EDQM.

7.4. Test for pyrogens (2.6.8)

In total, 59 Ph. Eur. monographs still refer to the RPT (*2.6.8*). Of those, 2 monographs covering blood products continue to prescribe an RPT as the sole method to test for pyrogens. Similarly, 3 monographs pertaining to containers for pharmaceutical use and 8 antibiotics monographs still refer to an RPT only, while the efforts to remove it from the last RPT-containing monograph on biological and biotechnological substances *(Urokinase 0695)* are ongoing. In the field of human vaccines, 8 monographs continue to only prescribe an RPT, whereas other human vaccine monographs limit the use of this test to the validation of the manufacturing process and whenever revalidation is necessary, through a statement in the Production section.

A review of the need to perform this test will be made and the possibility of replacing it with a specific requirement in the monograph for an appropriate *in vitro* test (e.g. BET or MAT) is envisaged.

8. CONCLUDING REMARKS

As previously [2], the achievements of the Ph. Eur. Commission in the field of animal welfare in the last decade were significant and have had an impact on several hundreds of Ph. Eur. texts. The animal tests that remain in the Ph. Eur., after more than three decades of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) of the Council of Europe, are those that are the most difficult to eliminate. The efforts developed to replace, reduce and refine the use of animals in the Ph. Eur. will therefore have to be intensified as the EDQM continues to encourage and support studies that lead to progress in animal welfare. In order to benefit fully from the current achievements, continued collaboration between the EDQM, regulatory authorities and manufacturers is needed to facilitate implementation. The EDQM will also continue to engage and exchange information with partners outside Europe to foster, as far as possible, common approaches and the acceptance of 3Rs advances on a global level.

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Appendix

Table 1 – *Vaccines for veterinary use – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Vaccines for veterinary use (0062)	R1: general deletion of the target animal batch safety test R1: promotion of consistency of production R1: no test for specified extraneous agents needed any more for inactivated vaccines produced using healthy flocks since introduction of a reference to chapter *5.2.13* on Healthy flocks R2: development safety test performed with 8 animals per group instead of 10/8 birds older than 3 weeks or 10 birds younger than 3 weeks per group instead of 20 (*5.2.6*) R2: for inactivated vaccines, identification by antibody induction in animals replaced by any suitable method, e.g. NAT or combined with the batch potency test (using the same animals for both tests)
Anthrax spore live vaccine for vet. use (0441) Avian infectious bronchitis vaccine (live), freeze-dried (0442) Marek's disease vaccine (live) (0589) Rabies vaccine (live, oral) for foxes and raccoon dogs (0746)	R1: deletion of the batch safety test
Salmonella Enteritidis vaccine (live, oral) for chickens (2520) Salmonella Typhimurium vaccine (live, oral) for chickens (2521) Turkey infectious rhinotracheitis vaccine (live) (2461) *Bordetella bronchiseptica* vaccine (live) for dogs (2525)	R1: no batch safety test required
Aujeszky's disease vaccine for pigs (inactivated) (0744) Bovine viral diarrhoea vaccine (inactivated) (1952) Calf coronavirus diarrhoea vaccine (inactivated) (1953) Calf rotavirus diarrhoea vaccine (inactivated) (1954) Canine adenovirus vaccine (inactivated) (1298) Canine parvovirosis vaccine (inactivated) (0795) Equine herpesvirus vaccine (inactivated) (1613) Feline calicivirosis vaccine (inactivated) (1101) Feline infectious enteritis (feline panleucopenia) vaccine (inactivated) (0794) Feline viral rhinotracheitis vaccine (inactivated) (1207) Mycoplasma gallisepticum vaccine (inactivated) (1942) Porcine enzootic pneumonia vaccine (inactivated) (2448) Porcine parvovirosis vaccine (inactivated) (0965)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10. R2: identification by antibody induction in animals replaced by any suitable method
Rabbit haemorrhagic disease vaccine (inactivated) (2325)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10 R2 conditions for omission of the 2nd inactivation test included R2: identification by antibody induction in animals replaced by any suitable method

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Rabies vaccine (inactivated) for veterinary use (0451)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method R2: Batch potency test by a serological assay (following BSP105 collaborative study) R3: Possibility to replace the lethal end-point by more humane end-points in the potency assay
Aujeszky's disease vaccine (live) for pigs for parenteral administration, freeze-dried (0745)	R1: deletion of the batch safety test R1: antibody induction test replaced by an *in vitro* method to identify the vaccine (infection of susceptible cell cultures instead of animals)
Avian infectious bronchitis vaccine (inactivated) (0959) Avian infectious bursal disease vaccine (inactivated) (0960) Avian paramyxovirus 3 vaccine (inactivated) for turkeys (1392) Egg drop syndrome '76 vaccine (inactivated) (1202) Equine influenza vaccine (inactivated) (0249) Feline chlamydiosis vaccine (inactivated) (2324) Newcastle disease vaccine (inactivated) (0870) Porcine influenza vaccine (inactivated) (0963)	R1: deletion of the batch safety test R1: deletion of the test for specified extraneous agents following introduction of a reference to chapter *5.2.13* on Healthy flocks R2: development safety test performed with 8 birds per group instead of 20 R2: identification by antibody induction in animals replaced by any suitable method
Avian infectious bursal disease (Gumboro disease) vaccine (live), freeze-dried (0587) Fowl-pox live vaccine, freeze-dried (0649) Newcastle disease vaccine (live), freeze-dried (0450)	R1: deletion of the batch safety test R2: development safety test performed with 8 birds older than 3 weeks or 10 birds younger than 3 weeks per group instead of 20
Avian infectious laryngotracheitis vaccine (live), for chickens (1068) Avian viral tenosynovitis vaccine (live) (1956)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens older than 3 weeks or 10 chickens younger than 3 weeks per group instead of 20
Duck plague vaccine (live) (1938) Duck viral hepatitis type I vaccine (live) (1315)	R1: deletion of the batch safety test R2: development safety test performed with 8 ducks older than 3 weeks or 10 ducks younger than 3 weeks per group instead of 20
Infectious chicken anaemia vaccine (live) (2038)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens per group instead of 20 R3: housing of laying hens and young chickens in stable groups of compatible individuals rather than individually
Avian infectious encephalomyelitis vaccine (live) (0588)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens per group instead of 20
Coccidiosis vaccine (live) for chickens (2326)	R1: deletion of the batch safety test R2: development safety test performed with 10 chickens instead of 20
Bovine leptospirosis vaccine (inactivated) (1939)	R1: deletion of the batch safety test R2: development safety test performed with 8 cattle per group instead of 10 R1: introduction of an *in vitro* batch potency test R1: promotion of consistency of production

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Canine leptospirosis vaccine (inactivated) (0447)	R1: introduction of an *in vitro* batch potency test for use with non-adjuvanted vaccines after validation, and extended to all vaccines R1: promotion of consistency of production R1: deletion of the batch safety test R2: development safety test performed with 8 dogs per group instead of 10
Bovine parainfluenza virus vaccine (live), freeze-dried (1176) Bovine respiratory syncytial virus vaccine (live), freeze-dried (1177) Canine distemper vaccine (live), freeze-dried (0448) Canine parainfluenza virus vaccine (live) (1955) Canine parvovirosis vaccine (live) (0964) Distemper vaccine (live) for mustelids, freeze-dried (0449) Feline infectious enteritis (feline panleucopenia) vaccine (live) (0251) Infectious bovine rhinotracheitis vaccine (live), freeze-dried (0696)	R1: deletion of the batch safety test R2: development safety test performed with 5 animals per group (not increased to 8)
Brucellosis vaccine (live) (*Brucella melitensis* Rev. 1 strain), freeze-dried, for veterinary use (0793)	R1: deletion of the batch safety test R2: 'Fifty per cent persistence time' performed on each batch of vaccine using 32 mice replaced by a test for 'residual virulence' performed on the master seed lot and on one representative batch of vaccine. R3: immunogenicity test in sheep replaced by a test in mice
Clostridium botulinum vaccine for veterinary use (0360) *Clostridium chauvoei* vaccine for veterinary use (0361) Feline calicivirosis vaccine (live), freeze-dried (1102) Feline viral rhinotracheitis vaccine (live), freeze-dried (1206) Myxomatosis vaccine (live) for rabbits (1943)	R1: deletion of the batch safety test R2: development safety test performed with 8 animals per group instead of 10
Clostridium novyi (type B) vaccine for veterinary use (0362)	R1: deletion of the batch safety test R2: development safety test performed with 8 animals per group instead of 10 R3: introduction of a serological evaluation of the batch potency test
Clostridium perfringens vaccine for veterinary use (0363) *Clostridium septicum* vaccine for veterinary use (0364)	R1: deletion of the batch safety test R2: waiver for the test for residual toxicity test on the final product by the manufacturer R2: development safety test performed with 8 animals per group instead of 10
Colibacillosis inactivated vaccine, neonatal ruminant (0961) Colibacillosis inactivated vaccine, neonatal piglet (0962)	R1: deletion of the batch safety test R2: development safety test performed with 8 pregnant animals per group instead of 10
Swine-fever vaccine (live) classical, freeze-dried (0065)	R1: deletion of the batch safety test R2: development safety test performed with 8 piglets/ pregnant sows per group instead of 10

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Feline leukaemia vaccine (inactivated) (1321)	R1: deletion of the batch safety test R2: identification by antibody induction in animals replaced by any suitable method
Foot-and-mouth disease (ruminants) vaccine (inactivated) (0063)	R1: deletion of the batch safety test R2: development safety test performed with 8 cattle per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method
Fowl cholera vaccine (inactivated) (1945)	R1: deletion of the batch safety test R2: development safety test performed with 8 birds older than 3 weeks or 10 birds younger than 3 weeks per group instead of 20. R2: identification by antibody induction in animals replaced by any suitable method
Infectious bovine rhinotracheitis vaccine (inactivated) (2674)	R1: promotion of consistency of production R1: introduction of an *in vitro* batch potency test
Mannheimia vaccine (inactivated) for cattle (1944) Mannheimia vaccine (inactivated) for sheep (1946) Neonatal piglet colibacillosis vaccine (inactivated) (0962) Neonatal ruminant colibacillosis vaccine (inactivated) (0961) Pasteurella vaccine (inactivated) for sheep (2072)	R1: deletion of the batch safety test R2: development safety test performed with 8 animals per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method
Porcine actinobacillosis vaccine (inactivated) (1360) Porcine progressive atrophic rhinitis vaccine (inactivated) (1361)	Exception: batch safety test replaced by test for residual toxicity. R2: development safety test performed with 8 animals per group instead of 10
Tetanus vaccine for veterinary use (0697)	Exception: batch safety test replaced by test for residual toxicity. R2: development safety test performed with 8 animals per group instead of 15 R3: introduction of a serological evaluation for the potency test
Salmonella Enteritidis vaccine (inactivated) for chickens (1947) Salmonella Typhimurium vaccine (inactivated) for chickens (2361)	R1: deletion of the batch safety test R2: development safety test performed with 8 chickens older than 3 weeks per group instead of 10 R2: identification by antibody induction in animals replaced by any suitable method
Swine erysipelas vaccine (inactivated) (0064)	R1: deletion of the batch safety test R2: development safety test performed with 8 pigs per group instead of 10 R3: introduction of a serological evaluation for the batch potency test R2: identification by antibody induction in animals replaced by any suitable method
Fish vaccines	
Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids (1521)	R1: deletion of the batch safety test R2: reduction from 200 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Vibriosis (cold-water) vaccine (inactivated) for salmonids (1580)	R1: deletion of the batch safety test R2: reduction from 200 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method
Vibriosis vaccine (inactivated) for salmonids (1581)	R1: deletion of the batch safety test R2: reduction from 200 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method
Yersiniosis vaccine (inactivated) for salmonids (1950)	R1: deletion of the batch safety test R2: reduction from 120 to 60 fish to be used for Immunogenicity R2: identification by antibody induction in animals replaced by any suitable method

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 2 – *Vaccines for human use – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Tests for extraneous agents in viral vaccines for human use (2.6.16) Cell substrates for the production of vaccines for human use (5.2.3)	R1: deletion of the tests in adult mice and guinea pigs. R2: testing strategy for extraneous agents to be established based on a risk assessment. Tests in suckling mice and control eggs to be used only if a risk assessment indicates that the tests provide risk mitigation. R2: allow use of molecular methods for specific or broad detection of viruses
Assay of diphtheria vaccine (adsorbed) (2.7.6)	R3 & R2: introduction of a serology assay as an alternative to challenge, with the possibility to use the same animals for the serological assay of the tetanus vaccine
Assay of tetanus vaccine (adsorbed) (2.7.8)	R2: possibility to use the same animals for the serological assay of the diphtheria vaccine
Assay of pertussis vaccine (acellular) (2.7.16)	R2: possibility to use the same animals for the serological assay of the diphtheria and tetanus vaccines
Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed) (2062)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test
Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed) (1931)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test
Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed) (1932)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: replacement of the rabbit pyrogen test by the bacterial endotoxin test R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed R1: deletion of the abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed) (1933)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test
In vivo assay of poliomyelitis vaccine (inactivated) (2.7.20)	R2: possibility to waive the *in vivo* assay once it has been demonstrated that the D-antigen determination yields the same result. Introduction of guidance on the implementation of D-antigen testing
Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed) (1934)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result
Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated), and haemophilus type b vaccine (adsorbed) (2067)	R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) (2065)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: replacement of the rabbit pyrogen test by the bacterial endotoxin test R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Diphtheria, tetanus, pertussis (whole cell) and poliomyelitis (inactivated) vaccine (adsorbed) (2061)	R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Diphtheria, tetanus, pertussis (whole cell), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) (2066)	R2: replacement of the test for specific toxicity of the diphtheria and tetanus components by a requirement to validate the process so that the product if tested would comply with the test R1: deletion of the abnormal toxicity test R2: possibility to waive the *in vivo* assay for the poliomyelitis component once it has been demonstrated that the D-antigen determination yields the same result R1: replacement of the rabbit pyrogen test by the bacterial endotoxin test R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed
Haemophilus type b conjugate vaccine (1219)	R1: deletion of the requirement to resort to animal models each time the manufacturing process is changed R1: deletion of the abnormal toxicity test
Poliomyelitis vaccine (inactivated) (0214)	R2: possibility to waive the *in vivo* assay once it has been demonstrated that the D-antigen determination yields the same result R1: deletion of the abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Ph. Eur. texts	Revisions
Poliomyelitis vaccine (oral) (0215)	R2: introduction of genome analysis (MAPREC) for screening prior to neurovirulence testing in animals R3: allow the use of transgenic mice to replace monkeys in the neurovirulence assay (for seed lots)
Anthrax vaccine for human use (adsorbed, prepared from culture filtrates) (2188)	R1: deletion of the abnormal toxicity test
Assay of hepatitis A vaccine (2.7.14)	R1: introduction of a validated *in vitro* assay as an alternative to the assay in mice
Hepatitis A (inactivated, adsorbed) and typhoid polysaccharide vaccine (2597)	R1: introduction of a validated *in vitro* assay for hepatitis A potency determination.
Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed) (1526) Hepatitis A vaccine (inactivated, adsorbed) (1107) Hepatitis A vaccine (inactivated, virosome) (1935)	R1: deletion of the abnormal toxicity test
Hepatitis B vaccine (rDNA) (1056)	R1: deletion of the abnormal toxicity test
Human papillomavirus vaccine (rDNA) (2441)	R1: deletion of the abnormal toxicity test
Influenza vaccine (split virion, inactivated) (0158) Influenza vaccine (surface antigen, inactivated) (0869) Influenza vaccine (surface antigen, inactivated, prepared in cell cultures) (2149) Influenza vaccine (surface antigen, inactivated, virosome) (2053) Influenza vaccine (whole virion, inactivated) (0159) Influenza vaccine (whole virion, inactivated, prepared in cell cultures) (2308)	R1: deletion of the abnormal toxicity test
Measles, mumps and rubella vaccine (live) (1057) Measles, mumps, rubella and varicella vaccine (live) (2442)	R1: deletion of the abnormal toxicity test
Measles vaccine (live) (0213) Mumps vaccine (live) (0538) Rubella vaccine (live) (0162) Varicella vaccine (live) (0648)	R2: replacement of the neurovirulence test on seed lots by a requirement to study the neurovirulence during development R1: deletion of the abnormal toxicity test
Meningococcal group C conjugate vaccine (2112) Meningococcal polysaccharide vaccine (0250) Pneumococcal polysaccharide conjugate vaccine (adsorbed) (2150) Pneumococcal polysaccharide vaccine (0966)	R1: deletion of the abnormal toxicity test
Rabies vaccine for human use prepared in cell cultures (0216)	R3: possibility to replace the lethal end-point by more humane end-points in the potency assay R1: promotion of the use of a serology or immunochemical method as an alternative to the assay in mice R1: deletion of the abnormal toxicity test
Shingles (herpes zoster) vaccine (live) (2418)	R1: deletion of the abnormal toxicity test
Tick-borne encephalitis vaccine (inactivated) (1375)	R3: possibility to replace the lethal end-point by more humane end-points in the potency assay R1: deletion of the abnormal toxicity test
Typhoid polysaccharide vaccine (1160) Typhoid vaccine (0156)	R1: deletion of the abnormal toxicity test
Yellow fever vaccine (live) (0537)	R1: deletion of the potency assay in mice R1: deletion of the abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 3 – *Blood products – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Human albumin solution (0255)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human antithrombin III concentrate (0878)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human C1-esterase inhibitor (2818)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor VII (1224)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor VIII (0275)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor IX (1223)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human coagulation factor XI (1644)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human fibrinogen (0024)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human normal immunoglobulin for intramuscular administration (0338)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human normal immunoglobulin for intravenous administration (0918)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human normal immunoglobulin for subcutaneous administration (2788)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human plasma (pooled and treated for virus inactivation) (1646)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human alpha-1-proteinase inhibitor (2387)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human prothrombin complex (0554)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*
Human von Willebrand factor (2298)	R1: addition of bacterial endotoxin test as alternative to rabbit pyrogen test *(2.6.8)*

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 4 – *Biological and biotechnological products – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Tetracosactide (0644)	R1: replacement of animal assay by liquid chromatography
Erythropoietin concentrated solution (1316)	R2: separate standard introduced to minimise the use of Erythropoietin BRP calibrated in International Units
Aprotinin (0580)	R1: deletion of abnormal toxicity test
Aprotinin concentrated solution (0579)	R1: deletion of abnormal toxicity test
Protamine sulfate (0569)	R1: deletion of abnormal toxicity test
Streptokinase concentrated solution (0356)	R1: deletion of abnormal toxicity test
Urokinase (0695)	R1: deletion of pyrogen test (ongoing)

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 5 – *Radiopharmaceuticals – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Technetium (^{99m}Tc) etifenin injection (0585)	R1: replacement of the physiological distribution test by a series of physico-chemical tests.
Technetium (^{99m}Tc) medronate injection (0641)	R1: replacement of the physiological distribution test by a series of physico-chemical tests.

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 6 – *Antibiotics and antimycotics – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Streptomycin sulfate (0053)	R1: deletion of abnormal toxicity test
Dihydrostreptomycin sulfate for veterinary use (0485)	R1: deletion of abnormal toxicity test
Kanamycin acid sulfate (0032)	R1: deletion of abnormal toxicity test
Kanamycin monosulfate (0033)	R1: deletion of abnormal toxicity test
Rifamycin sodium (0432)	R1: deletion of abnormal toxicity test
Griseofulvin (0182)	R1: deletion of abnormal toxicity test
Nystatin (0517)	R1: deletion of abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.

Table 7 – *Other products – 3Rs activity 2007-2017*

Ph. Eur. texts	Revisions
Allergen products (1063)	R1: deletion of abnormal toxicity test
Plastic containers (3.1.1.1, 3.1.1.2, 3.1.13, 3.1.14, 3.2.3, 3.2.4, 3.2.5)	R1: deletion of abnormal toxicity test

R1 = replacement of a test by an *in vitro* test or removal of test.
R2 = reduction in the number of animals required.
R3 = refinement of test to cause less distress, for example by use of more humane end-points.