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Working Group Report

Application of the Three Rs to challenge assays used in vaccine testing: Tenth report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement☆

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ABSTRACT

This report aims to facilitate the implementation of the Three Rs (reduction, refinement and replacement) in the testing of vaccines for regulatory and other purposes. The focus is predominantly on identification of reduction and refinement opportunities in batch potency testing but the principles described are widely applicable to other situations that involve experimental infections of animals. The report should also help to interpret the requirements of the European Pharmacopoeia with regard to the use of alternative tests, humane endpoints and other refinements. Two specific worked examples, for batch potency testing of *Clostridium chauvoei* and canine leptospira, with recommendations for harmonisation of international test requirements for these and other vaccines, are provided as appendices online.

1. Introduction and aims of the report

Testing of veterinary vaccines is a significant area of experimental animal use within European and other countries with a vaccine manufacturing industry. The need to apply the Three Rs of reduction, refinement and replacement [1] to the testing of vaccines for both veterinary and human use, and opportunities to do so, have been discussed by Castle [2], Metz et al. [3], Halder et al. [4] and Hendriksen [5], amongst others, and application of the Three Rs to veterinary vaccines specifically has been reviewed most recently by Cooper and Jennings [6]. The tests that use most animals and cause most suffering, and in which animals may die, are the challenge assays used to assess batch potency of certain vaccines. These involve the induction of disease by infecting animals with pathogens or exposing them to associated toxins. For the tests to be valid, some animals have to show typical signs of disease. This inevitably causes considerable suffering to unvaccinated control animals and to those vaccinated with low vaccine doses which do not protect against disease.

A batch potency test is performed routinely on every batch of a vaccine. The purpose is to demonstrate that the batch to be marketed will be at least as potent as the batch of minimum potency/titre shown to give satisfactory results in key efficacy studies.[1] The test can also provide information on the consistency of the

☆ The UK Joint Working Group on Refinement (JWGR) was established in 1989 by the British Veterinary Association Animal Welfare Foundation (BVAAWF), the Fund for the Replacement of Animals in Medical Experiments (FRAME), the Royal Society for the Prevention of Cruelty to Animals (RSPCA) and the Universities Federation for Animal Welfare (UFAW). The JWGR aims to facilitate the refinement of laboratory animal husbandry and procedures by preparing reports on specific topics, drawing together experts in a particular field to define contemporary best practice and ideals. The Group benefits from the advice of the UK Home Office Inspectorate and representatives of institutions dealing with animal welfare outside the UK. Professor David Morton chairs the Group and the secretariat is provided by the RSPCA. This report on refining challenge assays used in vaccine testing is the tenth in the series (see www.lal.org.uk/education.php).

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[1] Efficacy is investigated during the development of a product to identify its benefits.

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manufacturing process. Alternatives to replace batch potency tests involving challenge are urgently needed on animal welfare grounds. Research has already produced accepted replacements for several batch potency tests including those for tetanus toxoid, inactivated Newcastle disease and swine erysipelas vaccines [7–9]. Test development, validation and acceptance is a very lengthy process, typically taking more than 10 years [10], and in many cases it presents a considerable scientific challenge. A more immediate positive impact on animal welfare could be achieved by refinement of batch potency assays to reduce the suffering involved. Reduction in the numbers of animals that have to be used should also be possible.

Refinement has a beneficial impact on science as well as welfare, since the welfare of experimental animals affects the quality of scientific data produced. For example, when animals are stressed they may appear outwardly 'normal', but are likely to experience subtle, yet uncontrolled physiological and biochemical changes that can influence the variability, reliability and reproducibility of data collected [11,12]. Reducing animal suffering can improve the reliability and reproducibility of data and this can reduce the likelihood that tests will have to be repeated. The variability of results is also reduced, allowing smaller group sizes to be used, saving time, resources and animals. Such savings can offset the cost of developing, validating and implementing refinement, and the cost of obtaining a variation to the standard test monograph.

There is considerable scope for applying the Three Rs to batch potency testing and the report focuses on this. However, the principles described can be applied in all situations that require experimental infections including model development, proof-of-concept, challenge validation, challenge passage, and full scale efficacy studies.

2. Regulatory requirements and the Three Rs

There are two types of national and international legislation that impact on the use of animals for vaccine testing:

(i) regulation of the use of animals in scientific procedures;
(ii) regulation of the production and marketing of vaccines.

The first requires animal use and suffering in scientific procedures to be minimised. The second defines the necessary quality, safety and efficacy test requirements for vaccine products, many of which necessitate the use of animals in such procedures. To reduce the conflict between these different regulations, the principles of humane science, enshrined in the regulation of scientific procedures on animals, need to be carried through into all relevant vaccine testing regulations. However, the latter are not always straight forward to interpret, particularly with respect to the flexibility within the animal test requirements. A clearer understanding of the legislation, and of the roles and responsibilities of the various regulatory and other bodies involved, can help identify where and how implementation of the Three Rs can be achieved. To assist with this, the European situation is described below, with reference to other countries where particularly relevant.

2.1. Regulation of scientific procedures on animals

Scientific procedures that may cause animals pain, suffering or distress are currently regulated in the European Union (EU) by Directive 86/609/EEC[2] [13]. This states that experiments "shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practically available." Furthermore, when choosing test methods, "those which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm… shall be selected." Similar statements are made within Council of Europe Convention ETS 123 [14] on experimental animals, which applies across a wider range of countries. These principles are translated into national legislation, for example in the UK by the requirements of the Animals (Scientific Procedures) Act 1986 [15]. In practice, this means that in all EU countries there is a legal requirement to apply the Three Rs to any regulated work involving the use of animals including the testing of vaccines.

Countries outside Europe have different legislation, implemented in different ways, but the Three Rs are internationally supported and integral to the animal protection legislation of most countries that have legislation controlling animal use, for example, Canada, Australia and the USA. Some countries also require the likely benefits of research proposals to be weighed against the potential harms to the animals used. Manufacturers therefore need to weigh the need for, and benefits of, carrying out tests to assure that their vaccines are safe and efficacious, against the harms to any animals used to provide those assurances.

2.2. Regulation of the production and marketing of vaccines

For a vaccine to be accepted for marketing in Europe, manufacturers must submit an application for a Marketing Authorisation (MA). The requirements for, and guidance on, data that should be generated and presented in the dossier that supports the MA are set out in a number of documents (see Section 2.2.1 and Appendix 3) including EU directives and the European Pharmacopoeia (Ph. Eur.) [16].

The requirements have to be interpreted according to their legal force, and whether they are relevant and can be logically applied to a particular product. In so doing, it is necessary to consider the nature of the method of manufacture and the starting materials used, and to consider carefully what data or routine testing requirements should be applied to the product to ensure batch consistency. This means that, depending on how the product is manufactured, additional tests (animal and/or non-animal) may have to be performed to confirm consistency of production. Equally, some tests (animal or non-animal) may not need to be performed.

2.2.1. The European Pharmacopoeia

European Pharmacopoeia monographs set mandatory requirements for products that are on the market in countries that are signatories to the European Pharmacopoeia Convention.[3] The Ph. Eur. comprises:

- *General notices* which clarify how to interpret various sections of the pharmacopoeia and individual monographs;
- *General chapters* which give methods that are used for multiple monographs;
- *General monographs* which are applicable to a wide range of products; and
- *Specific monographs* which are applicable to products of a particular type (as stated in the Ph. Eur. Definition section).

Requirements that are common across a range of products are not usually repeated in each product-specific monograph. The specific monographs must therefore be read in conjunction with

[2] This Directive was under revision at the time of writing in 2010.

[3] To date, there are 36 signatories to the Convention, in addition to the European Union itself.

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the relevant general monographs, for example monograph 0062, Vaccines for Veterinary Use, and with an understanding of the contents of the General Notices of the Ph. Eur. (Chapter 1.1).

If there is no specific monograph for a product, it will need to comply with the general pharmacopoeial standards. In the case of a veterinary vaccine, it will therefore have to comply with monograph 0062, Vaccines for Veterinary Use.

2.2.1.1. The Three Rs in the European Pharmacopoeia. Since the signing in 1986 of the European Convention on the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, the Ph. Eur. has carried out a programme of work to reduce, refine and remove the use of animals in its texts [17,18]. The Ph. Eur. actively encourages the implementation of the Three Rs through the following statements.

General notices:

"…..This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product. The manufacturer may obtain assurance that a product is of Pharmacopoeia quality from data derived, for example, from validation studies of the manufacturing process and from in-process controls."

"……The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative."

General Monograph on Vaccines for Veterinary Use (0062):

"….In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering"

This means that there is flexibility in demonstrating compliance with the requirements of the Ph. Eur.; manufacturers do not have to perform all test methods, but should demonstrate that the product would comply if subjected to these tests. Since manufacturers and the Competent Authorities have a duty to ensure that animal usage is kept to a minimum and animal health and welfare legislation is upheld, both need to critically assess whether a test is necessary, and whether reduction and refinement options could be applied. Competent Authorities should also encourage the development of alternative methods which lead to a reduction, refinement or replacement of animal use, and data from these should be accepted when suitably validated.

2.2.1.1.1. Humane endpoints. The general monograph 0062 Vaccines for Veterinary Use, also makes it clear that the principle of humane endpoints (see Section 3.6 of this report) must be applied in the tests conducted:

"…..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. The criteria for judging tests in monographs must be applied in light of this. For example, if it is indicated that an animal is considered to be positive, infected etc. when typical clinical signs occur then as soon as it is clear that the result will not be affected the animal in question shall be either humanely killed or given suitable treatment to prevent unnecessary suffering.

Specific definitions of humane endpoints are not usually included in test descriptions within specific monographs. However, an example of where this has been done is supplement 6.4 of the Ph. Eur., which contains revisions of the monographs *Rabies vaccine for human use prepared in cell cultures (0216)* and *Rabies vaccine (inactivated) for veterinary use (0451).* These include a section on alternative endpoints describing typical clinical signs to be noted and a typical score chart. The analyst is expected to 'validate' the endpoint for a sufficient number of batches by scoring the test in the usual way, but also using the alternative endpoint. Since the test is carried out routinely for release of batches of vaccine, manufacturers have the opportunity to do the alternative scoring without having to do additional tests for validation. This approach is the one that is most likely to lead to the use of alternative endpoints for other vaccines.

2.2.1.2. The Three Rs and batch potency testing. It is made clear in the General Notices, General Monographs and Specific Monographs of the Ph. Eur. that alternative batch potency tests from the examples given in the Specific Monographs are acceptable. Any such test has to be validated and give assurance to the manufacturers and the Competent Authority that the batch is of suitable quality and meets the requirements of the Ph. Eur. and the standards agreed through the MA. Only in rare cases of doubt or dispute over whether the batch of product is of pharmacopoeial quality, does the test have to be performed exactly as described in the monograph.

2.2.2. Replacement alternatives in batch potency testing

Currently, a promising approach to the replacement of individual batch potency tests is *in vitro* antigen quality and quantification assays as part of the consistency approach [19]. This requires proof of consistency of production, and aims to demonstrate that each new batch of vaccine produced is of a similar quality to a vaccine batch of the same provenance, and is of proven efficacy and safety. The strategy involves demonstrating consistency using a battery of physico-chemical, immuno-chemical and *in vitro* methods [20].

An example of where this has been successful is the antigen quantification assay as a replacement for the rabies (NIH) potency test. This is accepted by the regulatory authorities on condition of demonstrated validity. Generally, antigen quantification tests are based on the use of monoclonal antibodies quantifying relevant antigen epitopes; they have limited value when vaccines are adjuvanted. However, even then it can be possible, as there is now an accepted *in vitro* potency test for oil-adjuvanted Newcastle disease vaccines [8].

2.2.3. Options for validation of alternative methods

The most appropriate approach to validation of alternative potency test methods will depend on how broad the applicability is for the alternative assay. Two possible options are:

(i) For a *manufacturer-specific alternative method which is to form part of the dossier for a manufacturer-specific vaccine*, a suitable in-house validation study will probably be acceptable to the relevant regulatory authorities. To enhance the chances of such methods being accepted by regulators within Europe,

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scientific advice can be sought from the European Medicines Agency (EMEA) or from National Competent Authorities, something that is regularly encouraged in the UK.

(ii) For a *vaccine-specific alternative method* where it is intended that the alternative test be accepted into a regulatory monograph or guideline, more formal validation rather than just a successful in-house study is likely to be required. Organisations such as the European Centre for the Validation of Alternative Methods (ECVAM) and the Biological Standardisation Programme of the European Directorate for the Quality of Medicines and HealthCare (EDQM) work towards validating alternative methods to be included in regulatory guidelines and monographs. There is a defined procedure for such validation, which is outlined in Appendix 3 (Section A3.2.).

There is no guarantee that an alternative method will be accepted and manufacturers have to pay a fee for each licence variation for the introduction of any change in the assays they submit. Together, these factors create a strong disincentive for industry to explore and validate alternative methods. To facilitate the acceptance of alternatives and avoid conflicts of opinion between different Competent Authorities, manufacturers should communicate with all relevant parties at an early stage. (The roles, responsibilities and interactions of the various institutions and regulatory authorities involved in the regulation of vaccines and development of test guidelines within Europe are illustrated in Appendix 3, Fig. 1) It would also help if Competent Authorities would waive the fee for licence variations that implement the Three Rs, as done by the UK Veterinary Medicines Directorate (VMD).

2.2.4. Recent three Rs initiatives

There are two recent Three Rs initiatives within Europe. The Committee for Medicinal Products for Veterinary Use (CVMP) is exploring ways to establish stronger EU ties with EDQM and ECVAM and to consider how advice concerning Three Rs issues for the development, manufacture and testing of veterinary medicinal products could be provided. Phased assessment of the registration dossier is also being considered. This would help to minimise the number of animals used in clinical safety and efficacy studies, and also avoid animals being used to validate a method if there are indications at an early stage that the method may not be accepted for regulatory purposes. This initiative is at an early stage and any significant change to the way in which veterinary pharmaceuticals are authorised may have to wait for the next review of the legislation, due in 2014.

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint Three Rs initiative from the European Commission, which was formed in 2005. It consists of individual companies and trade associations representing the chemicals, soaps and detergents, cosmetics, crop protection, pharmaceutical, bio-industry and animal health sectors, together with the following Commission services: Directorate Generals Enterprise and Industry, Research and Development, SANCO, Environment, and the Joint Research Centre (ECVAM). In 2010, the EPAA in collaboration with ECVAM organised a workshop on the consistency approach to vaccine quality control and the JWGR encourages them to pursue further vaccine-related Three Rs activities as well.

2.3. Regulations in other countries

Other countries and market areas where vaccines are manufactured either have their own pharmacopoeias and/or regulatory requirements, for example, the US Code of Federal Regulations Title 9 (9CFR) and Japanese Pharmacopoeia (Japanese Veterinary Biologicals Product Standards), or they base their requirements on those of other countries. As these regulations have developed independently, there are some differences in the detail of test methods, test requirements, animal numbers and endpoints. Harmonisation of international guidelines, taking the most refined methods as the standard, would have a very positive impact on animal welfare and obviate the need for repetition of tests for different markets. Appendices 1 and 2 illustrate how this could be done for *Clostridium chauvoei* and canine leptospira vaccines and provide a template for applying the principles to other vaccines.

Although full international harmonisation may take a long time, harmonisation even between some of the major markets, for example US, EU and Japan, would facilitate mutual acceptance of data and bring real Three Rs benefits.

3. Practical application of refinement and reduction

To maximise application of refinement and reduction in batch potency and other tests, it is important to critically review every aspect of experimental design and test procedures, and every aspect of animals' life-time experiences (see Table 1). This includes factors such as housing and care, transport, and handling in addition to the potentially adverse effects of vaccination and challenge.

3.1. Materials and equipment

Good preparation and storage of materials will maximise efficiency and reproducibility of tests and minimise the number of animals that have to be used. Minimising the need to passage challenge materials and to check virulence in animals also helps to reduce animal use.

The same principle applies to equipment and data management systems. Adequate maintenance of these increases the chance that test results are reliable, accurate and precise, and reduces the likelihood of repeat testing being needed, again minimising animal use. Requirements are stipulated in good manufacturing practice (GMP) standards and these should be applied by vaccine manufacturers and others involved indirectly, such as animal suppliers and contract research organisations.

Where possible, equipment, materials and services should be obtained from sources which operate to recognised quality assured standards such as the ISO standards. Alternatively, some small scale suppliers may be willing to participate in specific quality assurance (QA) audits.

3.1.1. Preparation, maintenance and storage of vaccine challenge materials and reagents

Whenever possible, bacterial and viral challenge strains should not be maintained by routine *in vivo* passage. As well as the additional use of animals, this can lead to increased variability in the biological nature and virulence of the strain, and each passage increases the likelihood of contamination with extraneous agents which could influence the outcome of subsequent challenges. Ideally, a master seed/working seed system should be used to maintain challenge stocks to minimise variation.

The best storage conditions for each toxin, or bacterial or viral strain, should be ascertained from the available literature and storage viability tested. Factors to be considered include: the form of storage (for example, chilled liquid, frozen or freeze-dried); storage temperature; toxin concentration; whether (for some bacteria) they should be stored as vegetative cells or spores, and the cell density; types of excipients and percentage inclusion; and the method of freezing and thawing.

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Table 1
Factors to consider in relation to refinement and reduction.

Materials and equipment	• Use of appropriate well-maintained and, where relevant, sterile equipment. • Careful preparation, maintenance and storage of materials; and consideration of their nature (e.g. irritancy, tissue compatibility, sterility, temperature) when administered.
Criteria for selection of animals	• Selection of an appropriate species and strain of animals with consideration of other factors such as age, weight and sex. • Use of a consistent source of high health status animals.
Animal husbandry and care	• Implementation of animal housing and care that takes into account the physical and behavioural needs of the animals as well as the need to be able to monitor them without too much disturbance. • Use of sympathetic handling and restraint procedures.
Numbers of animals and statistical design	• Application of an appropriate experimental and statistical design with well justified numbers of animals. • Timing of challenge to facilitate monitoring (in relation to the animals' time budget[a] and staff availability).
Administration of substances	• Use of the most refined methods including: - use of an appropriate gauge needle (i.e. the smallest gauge appropriate to the species, route and substance administered); - selection of the least invasive route likely to cause least trauma and pain to the animals; - selection of an appropriate and least harmful site/s for administration and suitable preparation of the site to facilitate accurate administration first time; - use of aseptic technique; - exploration of opportunities to administer reduced volumes.
Humane endpoints	• Description and implementation of humane endpoints to minimise level and duration of suffering.
Monitoring animals	• Careful, regular and timely monitoring of animals for adverse effects including those associated with the administration procedure itself. • Use of anaesthetic and analgesics to reduce pain.
Staff	• Sufficient, appropriately trained and competent staff who can implement all of the above.

[a] The relative amounts of time that an animal spends performing different behaviours.

The effects of storage on the activity of toxins or the viability/virulence of bacterial or viral challenges should be checked; frequently initially, then less so if they appear to be stable in storage. The results should be recorded and monitored to identify any trends.

3.1.2. Challenge validation

All challenge assays involve the use of unvaccinated animals to confirm the toxicity or virulence of the challenge material used. If challenge tests are performed relatively frequently, i.e. once or more each year, no other validation of the material should be needed. If challenge tests are less frequent, it may be necessary to validate the challenge annually. For most viral challenges, plaque assay counts on a suitable cell line can be used for validation of test material. For bacterial challenges, viable counts on agar will have to be supported by less frequent *in vivo* challenges. For toxins, if possible, an *in vitro* method such as a cell line assay should be used for routine validation and animals should only be used when part of the challenge test.

The performance of the challenge material during testing should be recorded and checked to detect any changes in toxicity or virulence. Where a reduction in virulence of the master seed is detected, it may be necessary to passage the bacterium or virus through the relevant animal species to retain the virulence of the challenge. The way this is done should be optimised to keep the numbers of animals and level of suffering to a minimum.

3.1.3. Calibration and maintenance of equipment

Test equipment and data recording and management systems need to be validated and maintained properly, so that data are measured and recorded accurately and precisely. High quality records of such maintenance need to be kept and audited regularly. Adequate records should also be kept for each batch test run, for inspection in the event of aberrant test results.

Facilities should validate new equipment and carry out regular calibrations and conformity checks on existing equipment. Ideally, this should be linked to national or international standards. Subcontractors used to maintain equipment should also have some form of quality control system that gives confidence that they are operating to suitable standards. Ideally both subcontractors and suppliers should be audited to ensure they comply with the requirements of GMP and QA.

Examples of equipment that should be regularly inspected and calibrated include: measuring devices such as micro-pipettes, thermometers and telemetric implants; storage equipment such as incubators, freezers and refrigerators; and environmental monitoring equipment. Building management systems, including temperature, humidity and lighting are also important as they can have a profound effect on the welfare of animals.

Staff should be adequately trained, experienced and competent to conduct measurements and undertake data recording and management.

These comments apply equally to test equipment used in the development and use of alternative *in vitro* tests, for example ELISA readers and associated software programmes and data storage systems.

3.2. Selection of animals

Species, strain, source: selection of the appropriate species and strain of animal, suitably susceptible to the pathogen or toxin and responsive to the test vaccine, is important for the generation of reliable and reproducible scientific data. The strain and source of animals should be consistent to reduce any possibility that variability of results could be related to genetic factors. The use of a regular and reliable source will avoid any need to assess new strains and use more animals.

Age and weight: experimental groups should comprise animals of similar age and weight at critical time points, for example at vaccination and challenge. This allows consistency in the monitoring of progression of clinical signs, which can make it easier to define earlier endpoints and minimise variability.

Sex: the sex of animals is unlikely to affect the data obtained, but may have an indirect affect on animal welfare if it affects the ability

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to group-house social animals. Thus, for species where it is easier to group-house females than males there is an animal welfare benefit to using females. However, the overall benefit is lost, if as a consequence, males are killed and wasted. In short-term studies, or in studies with juveniles, it should be possible to house animals in mixed sex groups.

Health status: animals should be of high health status and, in the case of the common laboratory species, preferably specific pathogen free (SPF), to ensure that experimental results are not influenced or complicated by sub-clinical background pathology, and to assure consistency.

Good suppliers of the common laboratory species monitor the health status of their breeding colonies. This involves periodic evaluation of a representative sample of the colony using serological, microbiological and other pathological tests to ensure that the colony is free of relevant specified pathogens and to claim SPF status. Specified pathogens, methods of detection and frequency of sampling are described in recommendations made by the Federation of European Laboratory Animal Science Associations (FELASA) [21–23].

Farm animal species from different agricultural sources are likely to vary in health status. It is therefore good practice for all animals in each test to be bought from the same source, preferably one that is closed to entry of new stock. This will ensure consistency in test results and reduce the potential for spread of endemic disease. Disease can be especially severe and problematic in young animals (for example, the occurrence of diarrhoea and pneumonia in calves), particularly if animals from different sources are mixed. Vaccination and preventive treatments on farm prior to delivery may therefore be necessary.

It is important to check the veterinary health plans in place at the source, the health monitoring strategies, and whether both the source as a whole and the individual animals are free from disease. Inspection by the testing establishment veterinarian is advisable as is investigation by a veterinary pathologist of any culls or losses.

Any use of specially prepared animals, such as colostrum-deprived or gnotobiotic animals, requires special husbandry measures such as irradiated food or antibiotics in drinking water. The health and welfare of such animals will require close monitoring. Their use should be considered carefully to assess whether data obtained are relevant and valid with respect to the expected vaccine efficacy in normal animals, particularly in the case of ruminants where much of the development of the immune system occurs after birth, and the role of the gut associated lymphoid tissue is very important.

Fish are increasingly used in vaccine studies. Generally, these animals are not reared in SPF conditions. Consequently, indicators of abnormal behaviour and/or clinical signs of disease need to be defined and their health and welfare status should be monitored on arrival and during housing in the laboratory.

Acclimatisation: a period of acclimatisation to the new environment is recommended for all animals as this allows them to recover fully from any adverse effects of transport, mixing of individuals, and changes in diet, environment and handlers. Acclimatisation also allows for the incubation of diseases, most of which are likely to show within 3 to 4 days of arrival, and are often complicated by a change or variation in diet. This is particularly important where animals are to join an established group.

An acclimatisation period of around one week allows animals that are unsuitable for any reason to be identified before being used in a study. This period is generally considered within industry to be the minimum necessary for animals used for regulatory testing (although it may be precluded in some instances, for example in the evaluation of vaccine safety in day-old chicks). It is also the time recommended by Obernier and Baldwin [24] in their review of the literature on the physiological acclimatisation period for laboratory animals after transport.

Allocation: animals should be randomly assigned to test groups to minimise selection bias.

3.3. Animal husbandry and care

The many factors to be addressed in the husbandry and care of animals are well documented in various guidelines [14,25]. Species dependent requirements for facilities, environment and care described in such guidelines should be set as the minimum standard, with opportunities for improvement and further refinement encouraged, explored and implemented.

The principles of optimal care and husbandry apply to all uses of animals for scientific purpose and are not specific to testing vaccines. However, there are points that require particular attention in vaccine challenge tests, especially where features of housing and care and the animals' response to these (for example, levels of activity, social interactions, interaction with objects in the environment) are used to help observe and implement humane endpoints. Factors to consider include:

Social housing: animals should be group-housed unless there are compelling scientific or veterinary reasons not to do so. If individual animals in a test group might die or reach a humane endpoint at different times, for example in control groups in challenge tests, care should be taken to ensure that a single remaining animal is not left alone for any longer than absolutely essential.

Housing: housing should satisfy animals' physiological, behavioural and psychological needs and provide comfort and security. Disturbance should be kept to a minimum to avoid affecting the animals' behaviour, particularly when this is used as a determinant of humane endpoints. However, this has to be balanced with the need to facilitate the regular and detailed observation of animals that is essential in challenge tests.

Flooring and bedding: solid flooring together with appropriate bedding material is particularly important for animals who may experience pain or ill-health during the course of challenge infection. Bedding material should be selected that improves welfare, but does not make animals too difficult to observe. It may even be possible to use materials that help to reveal an endpoint, for example white shredded paper to show haematuria.

Physical environment: modifying environmental conditions, such as ambient temperature, may help improve the welfare of sick animals. Modification of lighting patterns, i.e. use of reverse-lighting for species that are nocturnally active, enables animals to be observed when at their most active during normal staff working hours. This allows more meaningful observation of their behaviour and removes the need to disturb them during their inactive period.

Diet and water: diets should be consistent and of assured quality as changes may have an impact within or between tests; water supply should be similarly monitored and its quality maintained. Any recommended supplementation or medication of feeds should be assessed for potential impact on test outcomes.

Animals suffering from certain harmful effects of a challenge may show reduced food or water intake. It may be possible to counter this by the addition of ingredients such as fresh vegetables, such as sweet-corn or carrot, fruit, such as apples or grapes, and grains, and to increase water intake by moistening foodstuffs.

Enrichment: environmental enrichment encourages expression of a wider range of behaviours than is possible in a barren cage. This increases the scope for scoring relevant clinical signs because animals often change the level of interaction with enrichment items when they experience adverse effects, and this may occur before other detectable clinical signs are present. For example,

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animals that normally rapidly eat some form of treat, or interact with a play object, may reduce such activity following the onset of ill-health, or they may increase their activity or hide. (Note some enrichment items may make it harder to observe animals as they can obscure them from view, but translucent or tinted structures can help to overcome this problem). Observations of this nature can be relevant for any animal species and can easily be scored through time-specific behaviour sampling methods, or even through some form of telemetry or activity monitor.

Environmental enrichment must be appropriate for the species, strain, type of accommodation, and nature of the test protocol. Enrichment should be designed and monitored to ensure it has a beneficial effect for the animals. Experienced staff should be able to judge improvements in the condition or demeanour of animals, or any reduction in adverse behaviour such as fighting, hair plucking or tail chewing. It is also important to show that there are no adverse effects on the outcome of tests.

3.4. Numbers of animals and statistical design

The design of regulatory vaccine tests should ensure that results provide an accurate assessment of test material using the lowest number of animals possible and causing the minimum pain, distress and suffering to animals.

3.4.1. Test numbers in regulatory requirements

There is considerable disparity regarding group sizes for particular species for individual vaccines specified in the Ph. Eur. and its equivalent in other countries. Tests that use species such as birds, mice or fish typically specify much larger numbers of animals than those that use dogs, cats or cattle. For example, in the 9CFR requirements for *Pasteurella multocida* vaccines, the potency of bovine vaccine in calves requires 10 vaccinates and 5 controls, whereas for the avian vaccine in chickens, 20 vaccinates and 10 controls are specified. There is also disparity between the numbers of animals required in European and American regulations for tests on the same vaccine (see examples in Appendices 1 and 2).

It is difficult to see how these disparities can be scientifically justified and it is important to explore opportunities to reduce numbers and ensure group sizes have a good scientific and statistical basis. It is quite probable that the populations of rodents, birds and fish from which test groups are derived are more homogenous than those of cats, dogs or farm animals, so it may be argued that the group sizes required for the former should be no larger than for the latter. If the numbers of birds, mice and fish required could be reduced to the numbers required for tests using dogs, cats, or farm animals for example, a considerable reduction in the total numbers of animals used would be obtained. Further reductions in the numbers are possible if more than one batch of vaccine is tested simultaneously with each test sharing a single common control group.

A re-evaluation of the current test requirements and harmonisation to achieve a consistent minimum number of animals both within and between individual pharmacopoeias is therefore recommended by the JWGR, together with a simple fast-track process to get revised test requirements implemented quickly.

3.4.2. Statistical design

Aside from the regulatory requirements, it is good practice to re-examine the statistical design of all tests carried out in-house at regular intervals as this provides the historical data to allow more accurate statistical analysis and assessment of the need for controls. Determination of the number of animals required for a test system is dependent on many factors, including the predictive value of test systems and the type of measurements made i.e. whether these are continuous or binomial. It requires specialist knowledge of the relevant statistical and epidemiological principles involved and it is recommended that an expert in statistics is consulted.

3.4.2.1. Predictive value of test systems. The number of animals to be used in a test system is driven by the required accuracy of the test result, expressed as the *predictive value*. This concept applies at the level of the individual animal in the test, and to the test system overall. There are two types of predictive value:

- *Positive predictive value*: the probability that if a test result is positive, the material tested is truly positive.
- *Negative predictive value*: the probability that if a test result is negative, the material tested is truly negative.

Predictive values are partly dependent on the *sensitivity and specificity* of the individual test or test system:

- *Sensitivity*: the probability that, if a test material is positive, a test or test system will return a positive result.
- *Specificity*: the probability that, if a test material is negative, a test or test system will return a negative result.

The positive predictive value of a test or test system can be increased by increasing the test or test system specificity, or by increasing the probability of the test material being truly positive prior to testing. The negative predictive value of a test or test system result can be increased by increasing the test or test system sensitivity or increasing the probability of the test material being truly negative prior to testing.

The sensitivity and specificity of an individual test or test system can often be modified by changing cut-off points[4] or endpoints.

Predictive values are also dependent on the probability of the test material being truly negative or positive known as the priori. This value can often be estimated based on previous experience with the test material, or on information about the test material.

3.4.2.2. Type of measurements. Continuous data: when the test outcome of interest is continuous data, appropriate sample sizes can be determined using well established methods and software requiring input of:

- the probability of making type 1 and type 2 errors[5];
- the minimum difference of interest between a treatment group and control group;
- the variability of the data.

Binomial data: the situation with binomial data for example, yes/no or positive/negative results for individual animals, is different and determination of the number of test animals needed requires several steps:

Step 1 Determine the sensitivity and specificity of the individual animal test.

Step 2 Define the threshold result for the test system to pass or fail the test article. The threshold result can be set so that the proportion of treated animals and the proportion of control animals that reach the test endpoint are fixed and do not vary. Alternatively, the threshold result can be the ratio of

[4] Cut-off point: an arbitrary dividing line between + and −, or between responder and non-responder.

[5] Type 1 error: rejection of null hypothesis when it is actually true. Type 2 error: acceptance of null hypothesis when it is false.

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the proportion of treated animals reaching the test endpoint, divided by the proportion of control animals which reach the test endpoint, known as the relative risk or risk ratio.

Step 3 With the sensitivity and specificity of a test for the individual animal known, calculate the probability of a truly positive animal returning a positive result and a truly negative animal returning a negative result. The binomial distribution can then be used to partition treated and control animals into positive and negative results.

Step 4 Check compliance of the results with the outcome specifications declared in Step 2. If compliance is not achieved, either the number of animals can be increased, or the sensitivity and specificity of the test for the individual animal may need to be reviewed. This step will need to be repeated until compliance can be achieved.

3.5. Administration of test material

Administration procedures themselves have the potential to cause adverse effects in addition to any effects from the challenge material. Opportunities for refining such procedures are described in an earlier JWGR report [26] and other useful references are Diehl et al. [27] and Wolfensohn and Lloyd [28].

Aseptic practice should be observed to limit the potential for local infection at injection sites and contamination of test materials, which may interfere with the immune response. Other factors including needle size, differing tissue sensitivity in different areas of the body, and temperature and composition of the inoculum, can all influence the degree of pain and discomfort experienced by an animal and should all be considered and optimised (see Table 1).

Some vaccines, for example certain clostridial vaccines, can cause transitory discomfort and possible longer-term irritation, self-mutilation and abscessation. In such cases, the deposition of smaller volumes over more than one site should be considered. This reduces the likelihood of discomfort and tissue pressure necrosis, and will allow the presentation of a greater surface area of animal/vaccine interface to elicit an immune response. However, these advantages need to be weighed against the discomfort of additional needle sticks.

3.6. Humane endpoints

Endpoints in challenge assays are traditionally some of the most severe; the performance of a test vaccine is assessed against claims for that vaccine and if it is claimed to protect against a lethal challenge, the endpoint of the test system may be death in challenged animals. Such endpoints are likely to be of the most substantial severity and result in the greatest pain and distress. They are clearly not humane, and are also unpleasant for animal care staff to observe.

Death as an endpoint can be strongly questioned on scientific as well as animal welfare grounds. It is only an indirect scientific measure, because animals in vaccine tests frequently die from secondary and tertiary effects rather than the infection challenge itself. Deaths of animals challenged with microorganisms are usually the result of secondary or tertiary effects of infection. For example, animals given a neurotropic microorganism such as rabies virus might show secondary neurotoxic effects such as convulsions, muscle weakness and incoordination. This may prevent them from reaching water, in which case they may die from the tertiary effects of dehydration and heart failure due to increased viscosity of the blood, neither of which is directly related to the infectious agent.

An alternative approach, preferable for both animal welfare and scientific reasons, is to define an accurate scientific endpoint that is a surrogate measure of the claim for the test material, using the occurrence of one or more clinical signs known to precede death. This results in far less suffering and is the principle underlying the development of humane endpoints, defined by the OECD [29] as "the earliest indicator in an animal experiment of substantial pain, distress, suffering, or impending death". The aim is to stop an experiment at the earliest point at which the scientific objectives are achieved in order to avoid animals suffering unnecessarily.[6]

The need to develop more humane endpoints in vaccine potency testing has been argued for many years (e.g. [2,31]). Valid humane endpoints need to be defined for every challenge potency test in the Ph. Eur., and any requirement to exceed a humane endpoint and cause significant suffering, or death, should be stringently questioned.

3.6.1. Defining humane endpoints for batch potency tests

The objective of a challenge assay used for batch potency testing is to demonstrate with a reasonable level of confidence that (i) the challenge is sufficient to cause disease, and (ii) the vaccine protects against disease. The standard tests require that unvaccinated animals and/or animals immunised with unprotective vaccines show specific signs of challenge or die within the observation period. More humane endpoints can be developed by identifying reliable, reproducible, predictive clinical signs, or non-clinical physiological markers such as levels of protective antibodies, that provide sufficient information about the potency of the vaccine and its ability to protect animals at specific doses. These indicators should then be validated against the traditional endpoint. The endpoints selected need to have the highest reduction in suffering with respect to both its duration and intensity [31].

3.6.1.1. Identifying suitable signs. The first step is to carefully observe animals through all stages in the development of disease following infection, recording any clinical signs that occur. These may be physiological, such as weight loss or diarrhoea, or behavioural, including changes in the animals' social behaviour, use of enrichment items such as nesting material or chew blocks, or food and water consumption.

Some signs, for example weight loss and body temperature, are easily measurable and can be objectively assessed. Others, such as convulsions, dyspnoea, piloerection and social behaviours may not be so easily measurable, but they can still provide reliable information and be related to the scientific outcome measure. It may be possible to assign numerical 'scores' to these (see below). Where measurement of physiological parameters requires sampling of body fluids such as blood or urine (for example, for assay of haematological and biochemical indicators of organ function or failure, or monitoring blood leukocyte count for decline as an indication of infection) there are additional factors to consider. Firstly, if such measures are used to help predict and refine endpoints, then the speed of analysis is important. Secondly, invasive monitoring techniques can in themselves cause suffering or stress. The benefits that they provide in terms of improved animal monitoring and implementation of more humane endpoints therefore needs to be weighed against their potential for causing additional stress and discomfort, particularly in smaller species. This also applies to the measurement of physiological parameters using implanted telemetry devices, transponders or chips. The implantation procedures can cause suffering, so the harms and benefits of implanting devices should be carefully thought through and the most refined approach used [32].

[6] This approach has also been reported for the rodent protection test used to confirm *in vivo* efficacy of novel antiviral, antibacterial and antifungal agents [30].

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The reliability of each sign needs to be assessed in relation to its utility for reliably predicting the required scientific outcomes and whether it might give false positive or false negative information. Using a combination of signs may improve predictability, especially if behavioural and physiological data can be correlated. For example, when testing the potency of a whole cell pertussis vaccine, scoring clinical signs together with weight loss and reduced body temperature signal an inevitable deterioration more reliably than the individual signs alone. The selected humane endpoints are then validated by observing whether or not the animals continue to morbidity or death and the data are analysed to ensure they are sufficiently robust from a statistical point of view.

The success of a humane endpoint trial can be measured in animal welfare terms by calculating the reduction in the number of days over which the animals suffer. For example, the whole cell pertussis potency assay above uses a mouse body temperature of 34.5 °C as the humane endpoint. This occurs at a mean of 2 days (range 1–7 days) earlier than the normal experimental endpoints of death or severe suffering requiring euthanasia [31].

3.6.1.2. Monitoring animals and avoiding observer variation. The next step is to consider how animals should be monitored and how variation between human observers can be minimised. This is important because differences in observational skills and interpretation can lead to relevant clinical signs being discarded and endpoints being applied inconsistently.

Animals should be assessed at times and frequencies that will best help to identify the early onset of harmful effects. The timing of challenge and subsequent observations therefore needs to take into account the following factors.

3.6.1.2.1. Normal circadian activity patterns of the animals. Important clinical signs are more likely to be observed if animals are monitored when they are likely to be most active, or performing specific behaviours such as feeding. The optimal time for assessing animals will depend on the species and strain-specific behaviour patterns and time budgets, in conjunction with husbandry routines. It is best to observe them at approximately the same times each day.

The frequency and duration of direct cage-side observations should be carefully considered, with input from animal care staff. The use of behaviour recognition software can improve measurement of animal behaviour in a variety of settings, and can make 24 h monitoring and analysis a possibility.

3.6.1.2.2. Predicted onset and duration of clinical signs of disease. The observations made when defining humane endpoints should enable the time between the challenge and the onset of adverse effects to be predicted. If animals display clinical signs and then recover, it should also be possible to determine approximately how long animals are likely to experience adverse effects. Monitoring protocols should then be set up that ensure animals are observed when they are especially likely to be suffering. This information can also be used to time the challenge so that these critical periods coincide with maximum staff availability.

3.6.1.2.3. Staff availability. There needs to be sufficient staff available to monitor all animals individually. A challenge should be administered so that the critical endpoint phase is expected to occur during the working day and not during a weekend or holiday. The person responsible for the test should be present or rapidly available during this period. Ideally, the study director should be contactable, but if this is not possible, a trained member of staff must be available to make decisions to help ensure that humane endpoints are effectively implemented (see also Section 3.7).

3.6.1.2.4. Avoiding observer variation. Observer variation can be reduced by good training and teamwork, and by ensuring that clinical signs are clearly described and accurately recorded in welfare assessment sheets that are tailored to the type of test and the type of product. The use of video to record clinical signs and aspects of scoring is an invaluable aid, both for helping uniformity of scoring between personnel and for training.

Structured welfare assessment sheets that include a list of agreed clinical signs [33] should be available to all relevant staff and preferably be included in standard operating procedures (SOPs). They should be discussed with all concerned, including the principle investigator and veterinary and animal care staff, before starting work, and be reviewed at regular intervals to see whether signs have been effectively predicted and whether any humane endpoint might be brought forward even further. They should also be considered by ethical review processes or animal care and use committees, so that others with additional expertise may contribute.

One approach to reducing variation when staff use welfare assessment sheets is to simply record signs as being either present or absent, with no quantitative judgements. If necessary, an allowance can also be made for uncertainty (a record such as 'possibly present'), that would highlight the need for closer observation, for example where loose stools precede diarrhoea, or slight coughing precedes pneumonia. Signs can also be given numerical scores, but this involves making value judgements and needs careful definition and agreement to ensure consistency between observers.

The chosen method of data recording needs to take into account the clinical signs to be recorded, observer consistency, ease of use, clarity and effectiveness. Examples of commonly used recording schemes include: paper records, computer collation of data and direct entry into hand-held devices such as palm tops. All systems for data recording should be accurate, contemporaneous and attributable. Results should be reproducible and data should be stored in a form suitable for archiving and which is traceable.

3.6.1.2.5. Avoiding observer bias. Observer bias occurs where observers are aware of different treatments and may, unwittingly, make biased decisions based upon that knowledge. It is important therefore, wherever possible, to randomise the distribution of cages in a study so that the observer is unaware of the treatment group in each cage. This 'blinded' approach has the added benefit that any environmental impact associated with cage location, for example high or low light levels, should be neutralised across the different groups.

3.6.2. Use of analgesia and anaesthesia

The use of suitable analgesics and, where appropriate, anaesthetics, should be considered when it is expected and predicted that the challenge process could be painful at any stage. It is theoretically possible to anaesthetise an animal throughout an experiment of short duration, but many infections have incubation periods of several days and take time to produce clinical signs. In any case, anaesthesia may not be appropriate as some of the more common clinical signs are related more to distress than pain, for example through inability to reach the water bottle due to muscle weakness.

Analgesics may be useful for some types of challenge that cause pain at certain stages (for example, with *Cl. chauvoei* or rabies). Their value will depend on the time course of the infection and analgesic regimes should take account of the expected period of discomfort and pain after the challenge has been administered, with doses repeated as necessary. If analgesia is used, the type of analgesic, dose and treatment regime need to be empirically examined to assess not only whether it is effective and beneficial, but also to confirm that it does not materially interfere with the course of the disease or identification of humane endpoints. Similarly, analgesia should not have a significant affect on the robustness of the test's ability to discriminate between effective and non-effective batches of vaccine.

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Table 2
Training requirements.

Training requirement/topics	Relevant Staff					
	Animal technicians	Senior animal technicians	Veterinarians	Test supervisors	Other laboratory staff	QC staff
Scope, purpose and requirements of relevant regulations, guidelines and monographs and their application to the conduct of vaccine testing challenge assays		√	√	√	√	√
Husbandry and care of the species of animals used, including appropriate environmental enrichment	√	√	√	√		
Normal species specific behaviour and likely strain differences	√	√	√	√		
Correct application of most refined techniques for administration of test substances, challenge organisms and analgesics by different routes	√	√	√			
Possible adverse effects associated with administration procedures	√	√	√			
Expected outcomes of tests - effects on behaviour and health caused by the test substances and challenge organisms used	√	√	√	√		
The nature and level of pain, suffering or distress in the relevant species and methods of assessment	√	√	√	√		
Use of relevant systems (e.g. score sheets) for recording observations	√	√	√	√		
Actions to be taken in the event of adverse reactions, suffering or ill-health	√	√	√	√		
Definition, determination and implementation of humane end-points	√	√	√	√		
Appropriate methods of euthanasia and of confirmation of death	√	√	√			
Collection and storage of data in accordance with GMP, GLP, QA	√	√	√	√	√	√
Handling, storing and validating test and challenge materials	√	√	√	√	√	
Hazards associated with handling test and challenge materials	√	√	√	√	√	

In summary, the decision to use analgesics and anaesthetics, and even to provide palliative care, should depend on validated studies and critical observation of whether this actually reduces suffering for the animal, either in duration or intensity.

3.7. Staff issues

A team of well trained, highly competent staff is integral to implementation of the Three Rs. All relevant staff should have the opportunity to be involved in the planning, development and review of tests, procedures and systems and be encouraged to contribute ideas on the Three Rs and animal welfare. Input and feedback between all those involved will create an environment where opportunities for implementing the Three Rs are actively sought throughout the process of vaccine research, development and testing.

3.7.1. Training

Training is fundamental to the provision of a high standard of animal welfare, implementation of the Three Rs, delivery of high quality, valid test results, and compliance with legal and product registration requirements. In all aspects of vaccine testing it is therefore essential that staff should be well trained and competent in the procedures and activities they perform. This will be well worth the resources required i.e. financial costs, time, and the expertise of trainers and supervisors.

All staff should have a personal development plan tailored to their job which should include a training record, and regular review of training needs. Training should cover both knowledge and practical skills. Staff may not be allowed to carry out certain regulated procedures before they are fully authorised, so much of their initial training will be theoretical. Theory is important, but can only provide an introduction to the set of practical skills required, so this needs to be followed by a period of supervision or apprenticeship until competence in all necessary practical skills is attained. Competency will require formal assessment, allowing that different individuals will not all require the same sets of skills, and will take different times to attain them.

The learning process should not be considered to end once initial training and formal assessment has occurred, a personal licence or authorisation has been gained, or upon the completion of the supervision period. Ongoing performance and results should be monitored for consistency and for conformity with expected standards and results. Continuing staff development is also important and personal development requirements should be considered for all individuals. The provision of continuous professional development (CPD) or refresher training will encourage the maintenance of current competencies, and the addition of new ones [34].

There are several useful publications that describe the competencies, and associated training and supervision needs of staff involved in the care and use of animals generally [35–37] and many countries have a system of training provision linked to assured quality standards.

3.7.1.1. Vaccine testing–specific training. As with the husbandry and care of animals, there are certain topics that are particularly relevant to vaccine challenge assays and these are likely to require more specific in-house training. These topics are listed in Table 2 alongside the categories of staff for which they are particularly important.

3.7.2. Additional staff issues

There are additional staff related issues that can play a part in refinement which should be considered when planning and conducting a programme of vaccine testing.

Staff numbers should be maintained at levels that allow adequate time for test programmes to be conducted correctly. This is essential for the maintenance of scientific quality, animal welfare, health and safety of staff, and compliance with legal requirements. It will also avoid the unnecessary animal use that results from repetition of unsatisfactory tests. Consistent staffing will give similar benefits, leading to less variability in observations, procedures and results, and possibly less stress to test animals.

When scheduling challenge tests, critical time points should coincide with the availability of staff best able to assess health, determine humane endpoints, and take decisions on the course of the test. For example, the time at which a challenge is administered should be planned such that any predictable adverse effects occur during normal working hours. If this is not possible, additional staffing must be provided to ensure frequent

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observation of animals at these critical points, thereby minimising any suffering. A responsible person such as the study director, veterinarian or senior animal technician must always be easily contactable.

4. Summary and recommendations

There is considerable scope for applying the Three Rs to vaccine batch potency testing. Although the test requirements are driven by international regulations such as the Ph. Eur., so too is the need to implement the Three Rs with respect to the tests specified. Everyone involved with defining and implementing such regulations including regulators within Competent Authorities, manufacturers, study directors, test facility managers and animal care staff, can all contribute in some way using the principles and ideas within this report. Although this focuses specifically on applying the Three Rs in vaccine batch potency testing, the principles can be applied to vaccine studies more widely, including to model development, proof-of-concept, challenge validation, challenge passage, and efficacy studies.

The practical recommendations below are separated into three categories aimed at companies and individual staff, Competent Authorities, and the Ph. Eur., 9CFR and their equivalents.

4.1. Practical refinement that companies and individual staff can do now

- All staff involved in the planning, development, conduct and review of tests, procedures and systems should be encouraged to read this report as a 'thought starter' to facilitate implementation of reduction, refinement and, where feasible, replacement. Input and feedback between all those involved will create an environment where opportunities for implementing the Three Rs are sought throughout the challenge assay process and the development of novel vaccines.
- As part of this process, relevant staff should periodically come together to consider how the Three Rs could be applied in practice to every aspect of experimental design and test procedures, and every aspect of the animals' life-time experience for the vaccines they work with. Section 3 of this report provides the background to facilitate a review of:
  - materials and equipment;
  - selection criteria for animals;
  - animal housing, care, handling and transport;
  - acclimatisation of animals;
  - numbers of animals, statistics and experimental design;
  - administration of test materials;
  - reduction of the adverse effects of challenge;
  - development of humane endpoints;
  - monitoring animals, use of anaesthesia and analgesia; and
  - staff training.
- High standards, for example GMP or equivalent, should be applied to all aspects of vaccine testing, since production of good quality data reduces the likelihood of having to repeat tests.
- Specific examples of reduction and refinement that can immediately be applied to two vaccines, *Cl. chauveoi* and canine leptospira, are given in Appendices 1 and 2, respectively. These examples also provide a template for similar review of test requirements and methods for other vaccines. The JWGR recommends that research teams run the vaccines they are working with through the template to explore the reduction and refinement opportunities.
- One significant and immediate reduction in the level and duration of pain and suffering to which a test animal is exposed can be made by the identification of humane endpoints for test systems. A valid humane endpoint needs to be defined for every batch potency vaccine test involving challenge. Any requirement in the Ph. Eur., and its equivalents to exceed this, in particular to require death as an endpoint, should then be challenged by those involved with the tests.
- To facilitate the acceptance of alternative methods, whether refinement, reduction or replacement, and avoid conflicts of opinion between different Competent Authorities, manufacturers should communicate with all relevant parties (see, Appendix 3, Fig. 1) at an early stage during the development and validation of alternative methods.
- The EPAA should include more vaccine-related Three Rs activities in its remit.

4.2. Actions for Competent Authorities

- Competent Authorities should encourage the development of alternative methods and data from these alternatives should be accepted when suitably validated, and where this leads to a reduction, refinement or replacement of animal use.
- Competent Authorities that regulate animal experiments have a duty to ensure that animal usage is kept to a minimum and animal health and welfare legislation is upheld, so they must critically assess whether the tests they are asked to authorise are necessary, and whether reduction and refinement options could be applied.
- Competent Authorities should waive the fee for licence variation when satisfactory alternative methods that demonstrably improve animal welfare are proposed and/or used, and provide a simple, fast, harmonised system for approval of such methods.

4.3. Actions for the European Pharmacopoeia, the 9CFR and equivalent bodies

- Improving awareness of the opportunities for applying the Three Rs within the regulatory requirements for testing vaccines is key to the implementation of each 'R' in practice, but it is currently not easy to interpret test requirements and determine how flexible these are. Greater clarity of the Ph. Eur. text is needed, together with provision of additional user-friendly guidance.
- Wider knowledge of the test requirements in countries outside of the EU and USA would also be helpful with greater interaction between the regulatory bodies concerned. This would determine where harmonisation or mutual acceptance is possible and identify where further reduction, refinement or replacement opportunities could be developed and implemented.
- Harmonisation between the Ph. Eur., the 9CFR and other national equivalents is necessary. In particular, the JWGR recommends that current test requirements are re-evaluated to define consistent, statistically justifiable minimum numbers of animals in test and control groups. Where a need for harmonisation is identified, a request should be sent to interested parties for example, Group 15 V of the Ph. Eur.
- Specific recommendations for revision of the *Cl. chauvoei* and canine leptospira vaccine monographs are included in Appendices 1 and 2.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biologicals.2010.04.004.

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Review

Appendices to Working Group report: Application of the Three Rs to challenge assays used in vaccine testing: tenth report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement

Publication reference:

Abstract

The full version of the Joint Working Group on Refinement report aims to facilitate the implementation of the Three Rs (reduction, refinement and replacement) [1] in the testing of vaccines for regulatory and other purposes. The focus is predominantly on identification of reduction and refinement opportunities in batch potency testing but the principles described are widely applicable to other situations that involve experimental infections of animals. The report should also help to interpret the requirements of the European Pharmacopeia with regard to the use of alternative tests, humane endpoints and other refinements.

These three appendices provide supplementary information as follows:

- Appendix 1 and Appendix 2 are worked examples of the application of the Three Rs (mainly reduction and refinement) in the batch potency testing of *Clostridium chauvoei* and canine leptospira vaccines respectively. The text and tables provide recommendations for harmonisation of international test requirements for these vaccines and provide a template to facilitate a similar approach for others. The appendices encompass the general principles in the published report and should be read in conjunction with this.
- Appendix 3 provides additional information on regulatory requirements in Europe.

Appendix 1. : Refinement of the *Clostridium chauvoei* challenge potency test

Background

Clostridium chauvoei is the causative organism of Blackleg, an emphysematous, necrotising myositis disease of cattle, sheep and several other species. The mechanism of the infection is not clearly understood, but *Cl. chauvoei* is known to produce numerous toxins. Vaccines for Blackleg comprise unpurified formalin treated whole cultures or, in a few cases, culture supernatants.

Batch potency tests are carried out in guinea-pigs and current regulations state that vaccinated animals must survive for 5 days after challenge with virulent organisms. However, they suffer substantially and are severely debilitated for the whole of this period. This is likely to be a consequence of the irritant and necrotic nature of the challenge material which contains calcium chloride and is deposited deep into the thigh muscles. The unvaccinated controls will develop clinical signs and die. Replacing or refining the batch potency test for this vaccine would therefore have major benefits for animal welfare.

The vaccines are generally very effective even though the specific protective antigens are still to be determined. Until these are known, it will not be possible to develop a serological test to assess the potency of vaccine batches and, therefore a challenge potency test remains necessary. The form of the challenge potency test varies not only by region and requirement, i.e. 9CFR and Ph. Eur., but also by manufacturer. These variations can include factors such as the volume of vaccine administered, preparation and volume of the challenge, modulators, clinical scoring, humane endpoints and monitoring regimes.

This appendix presents proposals to harmonise, where applicable, the varying requirements in 9CRF and Ph. Eur., and provide guidance to produce a test which uses the minimum number of animals, with the highest level of refinement and the most humane endpoints. The current requirements of each of the two regions are set out in Table A1 under 6 separate headings relating to: selection of animals; housing and husbandry; vaccination details; challenge material; challenge and humane endpoints; and test results. The recommended approach, including recommendations for refinement, harmonisation of test requirements, revision of the test, or mutual acceptance of data, is set out in the right-hand column of the table. Specific recommendations for harmonisation and mutual acceptance of data are summarised below.

Recommendations

1. Numbers of animals

While the Ph. Eur. requires 10 guinea-pigs to be vaccinated with the vaccine on test, the 9CFR allows this number to be reduced to 8. Testing organisations should be encouraged to use the lowest number if they can provide evidence that the results are still reliable. In addition, where an organisation can provide adequate supporting data, the Regulatory Authorities should be encouraged to accept tests using only 8 vaccinates.

The purpose of the animals in the unvaccinated control group is solely to confirm the virulence of the challenge. With this in mind, it may be possible for testing organisations to agree with the Regulatory Authorities to use fewer than 5 challenge control guinea-pigs without compromising the validity of the test. Monitoring the results in the control group over a period of time should provide evidence to support this and manufacturers could collaborate to achieve acceptance of this approach. Further reductions in the numbers are possible if more than one batch of vaccine is tested simultaneously with each test sharing a single common control group.

2. Weight

Adoption of the 9CFR's wider weight range, of 300 to 500 g, would increase the proportion of available animals that could be used in any one test. This would mean that fewer guinea-pigs would have to be bred to provide sufficient animals of a suitable weight.

3. Vaccine dose/volume

While the 9CFR recommends vaccination of the test animals with 1/5 of the standard dose the Ph. Eur. states only that the volume of vaccine used must not be greater than the recommended minimum dose. The Ph. Eur. could consider including a recommendation on the volume of vaccine to be given in order to encourage operators to use the lowest reasonable volume, which should be no more than 1.0 ml.

4. Schedule

Both the Ph. Eur. and 9CFR should consider that, provided that the testing is performed to a consistent schedule within each

organisation, the second dose of vaccine could be administered 21 to 28 days after the first. Similarly the challenge could be performed 14 to 15 days after the second vaccination. This would avoid having to repeat tests to satisfy different Regulatory Authorities

5. Challenge dose

The 9CFR specifies that the challenge should contain approximately 100LD_{50}. A relatively large number of animals would have to be used to determine this dose level and to confirm it for each challenge. The Ph. Eur. requirement that the challenge dose should be virulent enough that none of the control guinea pigs survive is much easier to attain and requires the use of fewer animals. The volume should be no more than 0.5 ml and ideally only 0.1 ml.

6. Humane end-points

Both the Ph. Eur. and the 9CFR specify death as the endpoint. This is unnecessary and it is recommended that the texts are revised to reflect the acceptance of more humane endpoints, perhaps with reference to the relevant sections of this appendix.

7. Pass and validity criteria

There are several differences between the Ph. Eur. and the 9CFR regarding what constitutes a pass, fail or invalid test. The requirements could be harmonised to use the lowest number of animals and the following figures are proposed. At least 7 out of 8 vaccinates must survive for 3 days after challenge for the vaccine to pass. If only 6 survive a retest is required, fewer than 6 and the vaccine fails. On the retest, at least 6 vaccinates must survive for the vaccine to pass. For the controls, not more than one out of 5 should survive for more than 3 days after challenge for the test to be valid.

Table A1
Opportunities for reduction and refinement within the batch potency test for clostridial vaccines

	Regulatory Requirements	Recommended Approach and Possible Monograph Harmonisation and/or Revision
Animal selection criteria		
Species	Both Ph. Eur. and 9CFR monographs refer to guinea-pigs or target species which are goats, cattle and sheep	Currently, potency assessment is by challenge test and is done only in the guinea-pig.
Strain	Not specified in monographs	Use a strain demonstrated to be susceptible to challenge and that is capable of mounting a protective immune response.
Source	Not specified in monographs	Use a reliable and authorised source that can supply large enough numbers of good quality animals on a regular basis.
Sex	Not specified in monographs	Use mixed sexes, if possible, to allow most efficient use of available animals.
Health status	Not specified in monographs	As a minimum the animals must have been assessed as in good health
Weight	Ph. Eur. monograph: 350-450 g 9CFR monograph: 300-500 g	300-500 g.
Age	Not specified in monographs	Dependent on weight.
Numbers	Ph. Eur. monograph: 10 vaccinates, 5 controls 9CFR monograph: 8 to 10 vaccinates, 5 controls	As a harmonisation, 8 vaccinates and 5 controls are recommended. It is also possible that the number of controls could be further reduced if the test material is monitored over time, and manufacturers could collaborate to achieve acceptance of this approach.
Housing and husbandry		
Acclimatisation	Not specified in monographs	Animals that are brought in need to have time to recover from transport stress and to acclimatise to anything new such as new social groups, diet and handlers. This requires them to spend a minimum period of 7 days in their groups and accommodation prior to start of the procedure.
Housing	Not specified in monographs	If at all possible animals should be group-housed both before and after challenge. There is also no reason why controls and vaccinates cannot be housed together. If it is likely that guinea-pigs will still be on test when they become sexually active, at 12 weeks of age, it may be necessary to consider the use of single sex groups.
Enrichment	Not specified in monographs	Appropriate environmental enrichment, which has been shown to have no adverse effects upon the outcome of tests, should be provided.
Diet	Not specified in monographs	The guinea-pig lacks the ability to synthesise ascorbic acid (Vitamin C), an essential co-factor in its immune system. Therefore, adequate quantities of the vitamin should be given routinely in the diet and drinking water to optimise the animals' immune responses.
Identification	Not specified in monographs	Animals should be individually identified for recording purposes by a non-invasive method such as dye marking.
Vaccination details		
Route	Both monographs: sub-cutaneous	Care must be taken to ensure that the vaccine is accurately administered sub-cutaneously.
Dose volume	Ph. Eur. monograph: a quantity of vaccine not greater than the dose stated on the product label as the minimum dose 9CFR monograph: 1/5th of recommended dose	As a refinement, a maximum volume of no more than 1.0 ml should be injected at one site. As a harmonisation 1/5th of the recommended dose should be used.
Schedule	Ph. Eur. monograph: 2nd vaccination is 28 days later 9CFR monograph: 2nd vaccination is 21 to 23 days later	As a harmonisation the 2nd vaccination is administered 21 to 28 days after the first.

Table A1 (*continued*)

	Regulatory Requirements	Recommended Approach and Possible Monograph Harmonisation and/or Revision
Observations	Not specified in monographs	Daily, before and after vaccination. It is also good practice to observe the animals 20 – 30 minutes after vaccination to check that there are no immediate adverse effects due to vaccination.
Challenge material		
Selection	Ph. Eur. monograph: a virulent strain. Should be heterologous with the vaccine strain(s) 9CFR monograph: APHIS supply the specific challenge material	The challenge strain should be heterologous with respect to the vaccine strain(s)
Storage	Not mentioned in monographs APHIS challenge material is freeze-dried spores	The challenge can be stored as vegetative cells or spores. It may be stored frozen, freeze-dried or in suspension at -20°C or 2 to 8°C. The most reproducible challenge is considered to be spores stored at -20°C in 50% glycerol or freeze-dried.
Preparation	Not mentioned in monographs	If necessary the challenge may be rehydrated and/or diluted in a suitable buffer such as physiological saline. Any modulator should be added just prior to the start of injections.
Maintenance	Not mentioned in monographs	The reconstituted challenge should be prepared not long before challenge and stored at 2 to 8°C. The challenge material should be allowed to warm up to ambient temperature immediately prior to injection.
Challenge and humane endpoints		
Route	Both monographs: Intramuscular	The injection should be deep intra-muscular into the thigh, the side chosen in accordance with the preference of the challenge operator. It is important that the operator is well trained and familiar with the technique so that the dose is delivered correctly. Post-mortem examinations should be performed on any control animal that survives for 72 hours, to ensure that the challenge has been administered correctly.
Volume	Minimum and maximum challenge volumes are not specified in any monographs.	The challenge should be contained in the smallest volume that is consistent with the generation of the required effect. The volume of the challenge should preferably be 100 µl and certainly should not exceed 0.5 ml.
Dose	Ph. Eur. monograph: a suitable quantity of a virulent culture, or spore suspension, to kill the 5 control animals 9CFR monograph: approximately $100LD_{50.}$	The challenge dose should contain a suitable quantity of a virulent culture, or of a spore suspension, of *Cl. chauvoei* in a calcium chloride solution to have the potential to cause the death of 100% of the control animals.
Schedule	Ph. Eur. monograph: 14 days after 2nd vaccination 9CFR monograph: 14 to 15 days after 2nd vaccination	As a harmonisation the guinea-pigs should be challenged 14 to 15 days after the last vaccination.
Modulators/activators	Ph. Eur. monograph: can use a modulator or activator, such as calcium chloride, to initiate the disease (concentration not specified) 9CFR monograph: there is no mention of modulators in the monograph but the USDA working protocol uses 5% w/v calcium chloride in the challenge dose	In the case of challenge where calcium chloride is used as a modulator oractivator, it should be used at a concentration of approximately 5% w/v in the final challenge dose to ensure reproducible initiation of the disease.
Analgesia	Not mentioned in monographs	All animals should be given a long-lasting anaesthetic e.g. buprenorphine immediately prior to challenge. This may be repeated as necessary, with due regard to licensing, safety and efficacy in the guinea-pig.
Observation/ monitoring	Not mentioned in monographs	The time of peak effect can vary with the type and form of challenge, in most cases this is 24 to 48 hours after challenge. Challenged animals should therefore be examined for the presence and progression of clinical signs indicative of *Cl. Chauvoei* infection at least four times a day from approximately 20 hours after challenge and for the subsequent day. Survivors are then monitored at least twice a day for the remainder of the test or until they reach the humane endpoint.
Examination	Not mentioned in monographs	As well as the general health and behaviour of individual animals, the examination should concentrate on the site of challenge and the underside of the guinea-pig. Examination and monitoring should be done by someone competent in identifying clinical signs and who knows what action to take when these occur. Observation criteria should be defined and agreed by the test and animal care team.
Clinical signs	Not mentioned in monographs	Expected clinical signs are: - deeply coloured (purple/black "bruised") swollen hind leg, extending from the injected challenge site outwards along the flank; - oedema, depilation and exudation of overlying areas of the thigh or flank; - paleness of facial mucous membranes. - reluctance to move; - stiffness in the whole body.
Endpoints	Not mentioned in monographs	Unprotected animals begin to show outward signs of infection approximately 18 hours after challenge and can rapidly progress to a moribund state within the next 6 to 30 hours. Animals showing obvious progression of clinical signs, e.g. advancing sub-cutaneous discolouration, over a period of two to eight hours should be euthanased as moribund, i.e. a decision can normally be made regarding euthanasia of a moribund animal from 24 to 48 hours post challenge. Vaccinated animals may show partial protection and the progression of the same signs may be delayed, but a decision on whether an animal is moribund or is protected and will recover can be made within 72 hours of challenge.

(*continued on next page*)

Table A1 (*continued*)

	Regulatory Requirements	Recommended Approach and Possible Monograph Harmonisation and/or Revision
Test results		
Pass criteria	Ph. Eur. monograph: at least 9 out of 10 vaccinates must survive for 5 days after challenge. 8 survivors only means a retest, fewer than 8 is a fail. On the retest at least 9 vaccinates must survive. 9CFR monograph: at least 7 out of 8 vaccinates must survive for 3 days after challenge. 6 survivors means a retest, fewer than 6 is a fail. On the retest at least 6 vaccinates must survive.	As a harmonisation, use the lowest figures i.e. at least 7 out of 8 vaccinates must survive for 3 days after challenge. If only 6 survive this will mean a retest is required; fewer than 6 is a fail. On the retest, at least 6 vaccinates must survive.
Validity criteria	Ph. Eur. monograph: all 5 controls must die within 3 days of challenge 9CFR monograph: at least 4 of the 5 controls must die within 3 days of challenge	As a harmonisation, no more than one of the 5 controls must survive for more than 3 days after challenge.

Appendix 2. : Refinement of the canine leptospira vaccine hamster potency test

Background

Canine leptospira vaccines are inactivated preparations of whole organisms and/or antigen extracts of one or more serovars of *Leptospira interrogans*. Vaccines often contain more than one serovar and these may differ depending on those of local epidemiological importance.

In Europe, it is a requirement that batches of inactivated canine leptospira vaccines are tested for potency prior to release according to the method specified in the Ph. Eur. This states that, for each serovar contained in the vaccine, 5 hamsters are vaccinated with 1/40th of the dose for dogs and then, 15 to 20 days later, these hamsters plus 5 unvaccinated controls are challenged with a virulent leptospira of the same serovar as that contained in the vaccine. At least 4 vaccinates must survive the test and 4 unvaccinated challenge controls should die showing typical signs of leptospira. Many vaccines are bivalent so often 20 hamsters are required for each test. Many thousands of hamsters are used worldwide each year for the routine batch release of these vaccines.

Clearly, this is a severe test with ramifications for the welfare of hamsters, and therefore it warrants a high priority for replacement. There have been attempts over many years [38] to refine or replace the challenge test using two main approaches. The first is a refinement, aimed at developing a serological test that assesses potency by measuring the antibody response to vaccination rather than challenge. This has been used successfully to carry out potency tests on cattle leptospira vaccines [39], but it has proved difficult to develop a similar test for canine leptospira vaccines. This is partly because the protective antigens, and therefore the relevant antibodies to quantify, are not well understood. In addition, each serovar contained in the vaccine may require a separate, validated serological test so that the antibody response (potency) to each may be individually assessed. The second approach is to measure antigen content. The technical challenge is to separate the antigen from the adjuvant and then to develop a method for quantifying the antigen. Again, it is necessary to distinguish serovars. Several groups are working on this [38] and in the long term this approach may be accepted as a replacement.

Although both approaches are specified as alternatives in the Ph.Eur, it also states that the tests must be validated if they are to be used as discussed above, and the development and validation of such alternatives is both technically demanding and expensive. In addition, because of concerns that the antigen content test does not take into account the effects of the adjuvant, this method is not recommended in the Ph. Eur. for vaccines containing an adjuvant. It may therefore be some time before the challenge potency test is replaced. In the meantime, those carrying it out can do much to reduce animal suffering by introducing more humane endpoints.

The aim of Table A2 is to demonstrate how the test can be refined by implementing the principles from the general text of the JWGR report. The current requirements of the 9CFR and Ph. Eur. are set out under 6 separate headings relating to: selection of animals; housing and husbandry; vaccination details; challenge material; challenge and humane endpoints; and test results. The recommended approach, including recommendations for refinement, harmonisation of test requirements, revision of the test, or mutual acceptance of data, is set out in the right-hand column of the table. Since there is considerable scope for harmonisation between the test requirements of the Ph.Eur and 9CFR, the specific recommendations for harmonisation and for mutual acceptance of data are summarised below.

Recommendations

1. Numbers of animals

It is unclear why the 9CFR requires 10 hamsters in test and control groups, and the Ph. Eur. requires 5 hamsters. Testing organisations should be encouraged to use less than 10 animals if they can provide evidence that this is reliable. However, the test is undoubtedly difficult to perform reliably and consistently and it is possible that using 10 hamsters improves both. Ironically therefore, using more hamsters on each test occasion may in the long run reduce the overall number used, if it reduces the need for repeat testing. The two stage approach offered by the 9CFR which allows accumulated numbers, may reduce ambiguity between retests and in the long-run reduce the number of hamsters used. The Ph. Eur. could consider whether this approach would be advantageous.

Alternatively, it may be possible for companies to agree with the Regulatory Authorities to use fewer than the specified number of challenge control hamsters without compromising the validity of the test. Monitoring the results of the controls over a period of time should provide evidence to facilitate this. Outside the EU, Competent Authorities should be encouraged to accept results using fewer than 10 hamsters where the company can provide adequate data. Further reductions in the numbers are possible if more than one batch of vaccine is tested simultaneously with each test sharing a single common control group.

To fully resolve the issue, a comparison between the tests is warranted with an emphasis on defining a statistically justifiable number of hamsters, balancing the lowest numbers with appropriate test reliability.

2. Weight and age

There is scope for harmonisation between the Ph. Eur. and 9CFR which would facilitate mutual acceptance of results. If the weight

range specified in the Ph. Eur. were adopted, it would increase the proportion of hamsters available that could be used in a single test.

3. Routes

An evaluation of the two vaccination routes specified in the 9CFR should be undertaken to see if the route makes any difference to the outcome of the test. If not, or at least if the differences are clear, it should be possible to adopt just one route or facilitate mutual acceptance of data.

4. Dose/volume

The Ph. Eur. should consider including a recommendation on the volume of vaccine to be inoculated to encourage operators to use the lowest reasonable volume.

The Regulatory Authorities should consider whether mutual acceptance of data is an option. The reason for the differences in the dose/volume of vaccine specified in the Ph. Eur. and 9CFR may be historical. If a common dose/volume could be agreed it would facilitate mutual acceptance between Europe and the US.

5. Challenge material

Repeated back-titration of the challenge should be discouraged. This depends on establishing a reliable method of producing challenge material from stored bacteria rather than maintaining and preparing it in hamsters and preparing fresh each time.

6. Humane endpoints

Both the Ph. Eur. And the 9CFR specify death as the endpoint. This is unnecessary since it is possible to determine the critical phase of the test, monitor the animals carefully with reference to an agreed clinical score table (an example of which is given at the end of this appendix), and implement a more humane endpoint. It is therefore recommended that the texts should be revised to reflect more humane endpoints.

7. Pass/fail criteria

Apart from minor differences between the methods such as times and volumes, the Ph. Eur. and 9CFR have similar pass/fail criteria. Both methods require that 80% of the vaccinates survive and 80% of hamsters in the challenge groups should die. Mutual acceptance of data between Europe and the US should therefore be possible.

Table A2
Opportunities for reduction and refinement within the batch potency test for leptospira vaccines

	Regulatory requirements	Recommended approach and possible monograph harmonisation and/or revision
Animal selection criteria		
Species	Hamsters	
Strain	Not specified	Preferably in-bred; out-bred animals can give variable results. What is important is that the strain chosen gives consistent results when testing the product.
Source	Not specified	Use a reliable and authorised supplier. Any change of supplier must be validated as the change in hamster could affect the timing of the endpoint.
Sex	Not specified	Most people use females, but males are also used for example, in Australia, where it is not possible to import females. Gender is probably not a significant factor, but it is best to be consistent so multi-site companies need to consider this as a potential factor when comparing results from different sites.
Health status	Ph. Eur.: healthy hamsters that do not have antibodies to the principal serovars of *Leptospira interrogans* and are from a regularly tested and leptopira-free source 9CFR: not specified	High health status animals should be used as some extraneous infections may affect test results, or at least add variability. Colonies must be regularly monitored for a range of possible infections according to FELASA guidelines. Authorised suppliers do this and provide the information to clients. It is important that certificates are checked by the receiving facility to verify that no infection or health issues are present in the colony that may affect experimental data. Not all suppliers test for antibodies to leptospira, so this should be done initially when choosing a supplier and periodically thereafter. It is advisable for users to visit and audit suppliers.
Weight	The Ph. Eur.: not specified 9CFR: 50-90 g	The Ph. Eur. is more flexible than the 9CFR because it specifies an age rather than a weight, which means the allowable weight range is greater (likely to be 50 to 180 g up to 3 months of age). This gives the operator a wider window in which to use the hamsters, thereby avoiding potential wastage of animals. There is scope for harmonisation between the Ph. Eur. and 9CFR which would facilitate mutual acceptance of results obtained in European and US facilities.
Age	Ph. Eur.: not more than 3 months old 9CFR: not specified	See weight.

(*continued on next page*)

Table A2 (*continued*)

	Regulatory requirements	Recommended approach and possible monograph harmonisation and/or revision
Numbers	Ph. Eur.: 5 test and 5 controls 9CFR: 10 test and 10 controls, but a repeat test is allowed in which the cumulative totals are taken into consideration, so potentially 20 test + 20 controls.	Further reductions in the numbers are possible if more than one batch of vaccine is tested simultaneously with each test sharing a single common control group. The justification for using 10 rather than 5 hamsters per test is not clear. It may be possible to use fewer and still obtain reliable results. It may also be possible to agree with the Regulatory Authorities to use fewer than the specified number of challenge controls. Monitoring results from the controls should provide evidence to facilitate this. Many laboratories regularly, if not routinely, back-titrate the challenge material each time. This uses even more hamsters and should be avoided. Once a reliable method for preparing the challenge from stored cultures is established, routine back-titration should be unnecessary.
Sampling (pre-study)	Not specified	Generally, this is not necessary other than checking supplier certificates as above. However, the hamsters should be examined on receipt and before starting the test to ensure they are healthy. Check for freedom from antibodies if the supplier does not routinely do so.
Housing and husbandry		
Acclimatisation	Not specified	Animals that are brought in need to have time to recover from transport stress and to acclimatise to anything new such as new social groups, diet and handlers. They need to spend a minimum period of 7 days in their groups and accommodation prior to the start of procedures.
Housing	Not specified	The Ph. Eur. specifies 5 hamsters per group and it should be possible to house these together. If carrying out the 9CFR test, they may be housed in pairs or groups of 5. Hamsters are nocturnal so reverse lighting may help when observing them for clinical signs and determining the end-point as the hamsters tend to be more active when it is dark.
Enrichment	Not specified	Appropriate environmental enrichment should be provided, for example a cardboard box or tube, nesting material (for example hay) and a refuge.
Diet	Not specified	The diet should be consistent and quality controlled.
Identification	Not specified	If hamsters are housed in groups, it is necessary to identify each animal so they can be monitored individually. Microchips may facilitate the identification of individuals.
Vaccination schedule		
Route	Ph. Eur.: subcutaneous. 9CFR: subcutaneous or intramuscular	A refinement is to only use the subcutaneous route since this is the least severe, but it may be necessary to evaluate the two different routes of vaccination before this can be agreed.
Dose volume	Ph. Eur.: 1/40th of a dog dose. 9CFR: 0.25 ml containing 1/80th of a dog dose	It is a refinement and good practice to keep volumes as small as possible, (particularly with the intra-muscular route). The maximum volume injected should not exceed 1.0 ml by the subcutaneous route and 0.1 ml by the intramuscular route. It is recommended that 9CFR specify a smaller volume. The reason for the difference between the specifications is probably historical. If a common dose volume could be agreed it should facilitate mutual acceptance between Europe and the US.
Observations	Not specified	Animals should be observed daily before and after vaccination. It is good practice to observe the animals 20-30 minutes after vaccination, in practice before leaving the animal house, to check that there are no immediate adverse effects.
Challenge material		
Selection	Ph. Eur.: The serovar(s) against which protective immunity is claimed 9CFR: There are different monographs for each serovar; the challenge strain must be the same serovar as the vaccine	To develop a more humane endpoint, the serovar selected must be able to produce identifiable and reproducible effects that may be used instead of death as the end-point within the specified timescale.
Storage	Not specified	It should be possible to store challenge organisms in liquid nitrogen for at least 10 years [40]. This avoids the need for continual passage in hamsters which introduces variation and is expensive and time consuming. However, the maintenance of virulence can be problematic and it may be necessary on occasion to undertake passage in hamsters. New stocks may be grown in medium and filtered through a 0.22 micron filter to remove possible contaminating bacteria before laying seeds down in liquid nitrogen. DMSO or glycerol are normally included to maintain viability during freezing.

Table A2 (*continued*)

	Regulatory requirements	Recommended approach and possible monograph harmonisation and/or revision
Preparation	Not specified	A vial from liquid nitrogen storage can be grown *in vitro* and if necessary, passaged *in vitro* to ensure actively growing organisms and obtain sufficient quantity. The cell count can be adjusted to the required number of organisms per inoculum to fulfil the test requirements.
Enumeration	Not specified	The number of live, good quality organisms can be assessed using a Thoma counting chamber.
Maintenance	Not specified	From time to time it may be necessary to undertake passages in hamsters to retain virulence, but this should be kept to a minimum.
Evaluation	Not specified	Challenge cultures should be checked for quality when they are counted. They should be actively motile, of uniform size, i.e. not many long forms, and not contaminated with other bacteria. Challenge material should be monitored over time to ensure its virulence does not wain.
Challenge and humane endpoints		
Route	Ph. Eur.: intraperitoneal route 9CFR: intraperitoneal route	The intraperitoneal route of administration is known to be variable so consideration should first be given as to whether this is really the best route to use. It is essential that the operator is well trained and familiar with the technique so that the dose is delivered correctly. Detailed explanation of how to do it should be written into SOPs to facilitate consistency.
Volume	Not specified	It is good practice to keep volumes as small as possible. The maximum volume injected i.p. should be 1.0 ml.
Dose	Ph. Eur.: Sufficient quantity to result in 4 of 5 positive animals in the unvaccinated control group within 14 days of challenge 9CFR: 10-10,000 hamster LD_{50}	The challenge dose needs to be determined by titration in hamsters to ensure it contains a suitable quantity of virulent bacteria. However, if the challenge titre can be correlated to cell counts, it should be possible to base future dilution of the challenge material on cell counts rather than doing back-titrations each time. Even so, it may be necessary to re-titrate stocks in hamsters every 6 to 12 months.
Schedule	Ph. Eur.: 15 to 20 days after vaccination 9CFR: 14 to 18 days after vaccination Ph. Eur.: The test ends 14 days after the 4th control hamster dies 9CFR: The test ends 14 days after challenge	Challenge should be administered so that the critical endpoint phase occurs when it is most cost-effective to provide the close monitoring that is required, for example during the working week and not at weekends and holidays. The critical time when animals become ill will vary from facility to facility and with the challenge strain used, but will be from 3 to10 days after challenge. With experience the critical time can be narrowed down to a 2 to 3 day period.
Modulators/activators	None	
Analgesia	Not specified	There are no ill effects associated directly with the challenge procedure itself, so analgesics are not useful in this respect.
Observations/ monitoring	Not specified	It is good practice to observe the animals 20 to 30 minutes after challenge, in practice before leaving the animal house, to verify that there are no immediate adverse effects of the challenge procedure. Monitoring should be increased to 3 to 4 times daily during the critical period after challenge when the hamsters are likely to become ill. This will vary from test facility to test facility, but is commonly 3 to 10 days after challenge. Arrangements should be made for observing animals outside of normal working hours. Monitoring should be done by someone competent in identifying clinical signs and who knows what action to take when these occur. Observation criteria should be defined and agreed by the test and animal care team.
Examination	Not specified	A suggested clinical score sheet is provided at the end of this appendix. Hamsters should not be handled after challenge both from a health and safety perspective and because it interferes with observation of their behaviour which is used in clinical scoring.
Clinical signs	Not specified	A suggested clinical score sheet which lists clinical signs is provided at the end of this appendix.

(*continued on next page*)

Table A2 (*continued*)

	Regulatory requirements	Recommended approach and possible monograph harmonisation and/or revision
Endpoints	Ph. Eur.: Death 9CFR: Death	If left, hamsters normally die 3 to10 days after challenge, but this will vary from facility to facility and with the challenge strain. Each facility must therefore determine time to death for themselves. However, death is avoidable in the majority of hamsters and therefore it is not an acceptable endpoint. If observation records are maintained it is straightforward to identify a set of clinical signs that equates to inevitable death. An increased frequency of monitoring over the critical period will facilitate this. Companies carrying out the test should agree a more humane endpoint with the Competent Authorities. Reference to more humane endpoints in the Ph. Eur. and 9CFR monographs is needed.
Test results		
Pass criteria	Ph. Eur: The hamsters are monitored for 14 days after challenge. For the vaccine to pass, at least 4 of 5 vaccinated hamsters must remain in good health for 14 days after the 4 challenge controls die. 9CFR: The hamsters are monitored for 14 days after challenge. For the vaccine to pass, at least 8 of 10 vaccinates must survive. If 5 or more vaccinates die the vaccine fails. If 3 or 4 of 10 vaccinates die, a second stage may be undertaken. In this case, if the cumulative number of vaccinates from both stages that die is 5 or less, the vaccine passes. If the cumulative number of dead hamsters is 6 or more, the vaccine fails.	There is scope for harmonisation between the Ph. Eur. and the 9CFR. Both methods require 80% of the vaccinates to survive. Both methods would seem very similar in terms of their pass/fail criteria and the regulatory authorities should consider whether mutual acceptance of data is an option. Currently, both the Ph. Eur. and the 9CFR specify death as the endpoint. This is unnecessary and it is recommended that the texts be revised to reflect more humane endpoints, perhaps with reference to an agreed clinical score table, an example of which is given below. It is unclear why the 9CFR requires 10 hamsters and the Ph. Eur. only 5 per group. However, the test is difficult to perform reliably and consistently and the two-stage approach offered by the 9CFR may offer more flexibility, reduce ambiguity between re-tests and in the long-run reduce the number of hamsters used. A comparison between the tests is required to provide a firm statistical basis for the numbers used.
Validity criteria	Ph. Eur.: For the test to be valid, 4 of 5 challenge controls should die within 14 days of inoculation. 9CFR: For a valid test, at least 8 of 10 of the challenge controls must die within 14 days of inoculation.	Again, there is scope for harmonisation between the Ph. Eur. and the 9CFR. Both methods require 80% of the challenge controls to die, and the same comments for the pass criteria regarding humane endpoints, harmonisation and mutual acceptance of data, apply to the validity criteria.

Clinical signs used to determine a humane endpoint for *Leptospira canicola* and *Leptospira icterohaemorrhagiae* challenge

The table below provides guidance on the possible effects of inoculation with *L. canicola* or *L. icterohaemorrhagiae* and the actions taken on observation of the clinical signs listed. A record of observations and scores should be made for each test to monitor trends and help determine endpoints for individual hamsters. A more refined welfare assessment record sheet is being developed.

Sample record sheet

Clinical Signs	Record	Action
• Normal behaviour	0	None required
• Arched back with slightly rough coat	1	Observe again within 2 hours
• Dull sunken eyes • Moderately rough coat • Subdued but will respond when stimulated	2	Animals sick; observe again in 2 hours. If signs persist at the end of the working day consult the designated 'responsible person' to decide on subsequent monitoring/action.
• Unstable on feet • Subdued, will not respond when stimulated • Nasal bleeding • Blood in urine • Prostration • Permanently closed eyes	3	Animals very sick; euthanase
• Dead	4	

Hamsters should be observed at least twice daily and more frequently at the peak of clinical disease. This is usually 3 to 10 days after challenge. Once the peak has been established, the challenge can be timed so that the critical phase occurs when observations can be made most easily for both animals and staff.

Appendix 3. : Additional information on regulatory requirements in Europe

A3.1 Documents containing requirements and guidelines on the data that should be generated and presented in the dossier that supports the Marketing Authorisation (MA) (note, this list is not exhaustive)

The basis for regulation and control of veterinary pharmaceuticals within Europe is Directives 81/851 and 81/852. Veterinary vaccines became subject to the same directives from 1993 (through Directive 92/18/EU), with a few modifications to the regulatory requirements and a new section to cater for the different technical requirements, currently set out in Directive 2001/82/EC as amended by 2004/28/EC Annex 1, Title II.

A3.1.1 Regulatory requirements

- Annex 1, Title II of Directive 2001/82/EC as amended by 2004/28/EC: http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol5_en.htm
- European Pharmacopoeia (Ph. Eur.): http://www.edqm.eu
  - General Notices
  - Relevant General Texts
  - General Monograph on Vaccines for Veterinary Use (Ph. Eur. 0062)
  - Relevant specific monographs

[1] For biologicals, these groups of experts are as follows:

- Group 15 – Human Sera and Vaccines
- Group 15 V – Veterinary Sera and Vaccines
- Group 6 – Biological Substances
- Group 6B – Human Blood Products

A.3.1.2 Guidance

- Position paper on batch potency testing of immunological veterinary medicinal products (CVMP/IWP/038/97): http://www.emea.europa.eu/htms/vet/vetguidelines/immunologicals.htm
- EudraLex Volume 5 (Notice to Applicants and Regulatory Guidelines for Medicinal products for Veterinary use): http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol6_en.htm
- EudraLex Volume 7 (Scientific Guidelines for Medicinal Products for Veterinary Use): http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol7_en.htm
  - General requirements for the production and control of live mammalian bacterial and viral vaccines for veterinary use (Eudralex Volume 7B)
  - General requirements for the production and control of inactivated mammalian bacterial and viral vaccines for veterinary use (Eudralex Volume 7B)

A.3.2 Formal validation of alternative methods

A request for a revision of the methods described for biologicals in the Ph. Eur. must be sent to the European Directorate for the Quality of Medicines (EDQM) which will consider whether there is sufficient information to change the monograph. The decision to prepare a new monograph for a particular type of product, or to revise an existing monograph, is taken by the European Pharmacopoeia Commission when such a request is received. A draft of the new or revised monograph is then prepared by a group of experts of the Ph Eur.[1], sent to all the National Pharmacopoeia Authorities and the International Federation for Animal Health (IFAH), and published in the European Forum Pharmeuropa. Comments are invited within a given time and are considered by the relevant expert group. If major changes are made to the document at this stage, the revised draft may be sent out for consultation again. The final draft is sent to the European Pharmacopoeia Commission for approval. The approved version is published in the next supplement of the Ph. Eur. approximately 6 months later, and comes into force approximately 6 months after that.

Monograph revision is not undertaken before formal validation of the alternative method has been carried out. Validation studies can be initiated using the Biological Standardisation Programme of the European Directorate for the Quality of Medicines and Health Care (EDQM). This pursues the goals of establishing common European reference preparations, and of standardising the methods used for the quality control of biologicals, with the explicit aim of developing alternative methods whenever possible. Validation studies can also be performed by ECVAM: an example of a method validated by ECVAM and now accepted by the Ph. Eur. as an alternative to the challenge test, is the serological potency assay for inactivated swine erysipelas vaccine. EDQM and ECVAM have also collaborated in a number of projects to achieve the 3Rs, for example for the introduction of a serological evaluation as an alternative to toxin neutralisation test for the batch potency test of Clostridial vaccines.

A.3.3 Relationships between EU regulatory authorities involved in regulation of veterinary vaccines

Figure A1 illustrates the roles, responsibilities and interactions of the various institutions and regulatory authorities involved in the regulation of vaccines and the development of test guidelines within Europe.

Specific guidelines within Europe on various aspects of pharmaceutical development are published by the European Commission in the EudraLex, *The Rules Governing Medicinal Products in the European Union*. This is done through the Commission's Directorate-General (DG) Enterprise and Industry. The guidelines are developed by the Committee for Medicinal Products for Human Use (CHMP) and the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency (EMEA), often in co-operation with specific CHMP/CVMP working parties or other expert groups. For example, the Immunologicals Working Party (IWP) advises the CVMP on the elaboration and revision on guidelines on immunological products. Other regulatory guidance in the form of position papers or reflection papers is also published by the EMEA.

The administrative work related to the European Pharmacopoeia is the responsibility of the European Directorate for the Quality of Medicines and HealthCare (EDQM) which is a Directorate of the Council of Europe, a separate body from the European Union. The EDQM also validates alternative methods through its Biological Standardisation Programme.

International organisations such as the World Health Organisation (WHO) and the World Organisation for Animal Health (OIE) also publish guidelines for the quality control of immunologicals.

The CHMP and the CVMP of the EMEA provide technical and scientific support for International Conference on Harmonisation (ICH) and Veterinary International Co-operation on Harmonisation (VICH) activities. VICH is a trilateral (EU-Japan-USA) programme aimed at harmonising technical requirements for veterinary product registration. The VICH was established under the auspices of the OIE and the European Commission and EMEA are members (along with IFAH Europe).

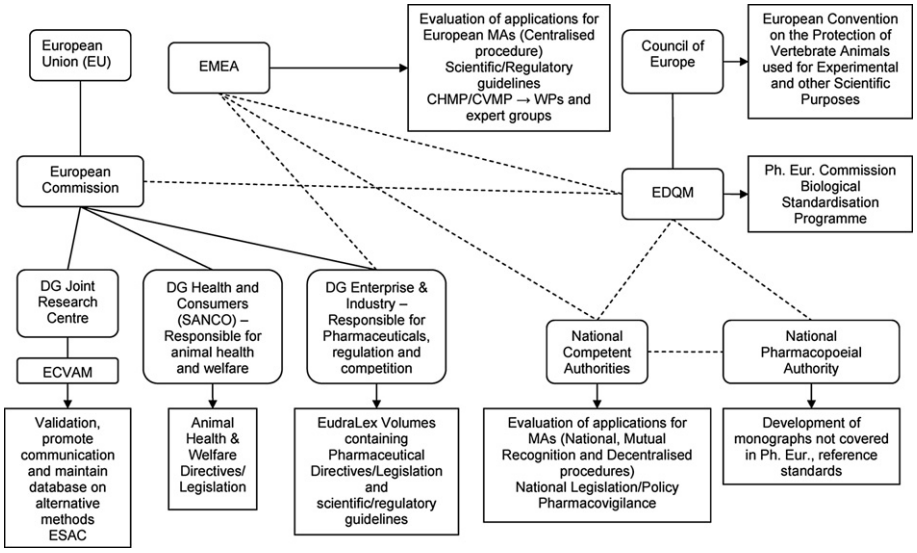

Figure A1. Relationship between EU regulatory authorities involved in regulation of vaccines.

Key

Solid line indicates direct responsibility, broken line indicates interaction. Rounded boxes indicate institution/organisation, square boxes indicate functions/publications of the associated institution/organisation.

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