Three Rs Potential in the Development and Quality Control of Immunobiologicals

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Content

Summary

Immunobiologicals (vaccines, immunoglobulins and –sera) are considered to be the most cost-effective tools in the prevention of infectious diseases. Their importance will further increase due to various eradication programmes of the WHO and EU and the emergence of new infectious diseases or the re-emergence of diseases as diphtheria and tuberculosis. The production and quality control of immunobiologicals are regulated by monographs and guidelines, which are issued by international or national Pharmacopoeias (e.g. Ph. Eur.), international organisations (e.g. WHO, O.I.E.) and international regulatory bodies (e.g. EMEA). Their purpose is to assure the quality of the product, i.e. its safety and potency. It is estimated that 10 millions of laboratory animals are world-wide used for the production and quality control of immunobiologicals, of which 80% are needed for the safety and potency testing of the finished product (batch control).

In recent decades, the use of Three Rs principles has been recognised by the above mentioned organisations and various national competent authorities and had been incorporated into general monographs and guidelines. Several tests with questionable relevance have been deleted from Ph. Eur. monographs (e.g. abnormal toxicity test, extraneous agents testing of viral vaccines for carnivores) or are now carried out during production. Reduction of the number of animals used could be achieved by introducing single-dilution tests. A large number of immunochemical tests have been developed, which could completely or partly replace the use of animals for potency testing, however, only a few have been validated so far (e.g. ToBI and ELISA for potency testing of human and veterinary tetanus vaccine; ELISA for potency testing of erysipelas vaccine). Regulatory acceptance of validated alternative methods is still a critical step. In particular, the period between successful validation and the implementation appears to be far too long. Reasons for this could be the slow process of multinational agreement to revise pharmacopoeial monographs and guidelines, and the time-consuming and expensive production of sufficient reference material (antigen, sera etc) for the new test systems.

The shift in the quality control concept from reliance on final batch testing to the concept of consistency of production offers the opportunity to reduce the numbers of animals being used and promote the use of alternative methods. Emphasis is put on a combination of in vitro tests, which could make it possible to monitor batch-to-batch consistency. This new concept of quality

control is already in place for the new well-defined vaccines. In most cases, non-animal methods are used for monitoring consistency at critical steps in the production and testing of a vaccine. Whether the concept of consistency of production could be also applied to the conventional, less-defined products, should be investigated.
Only little progress has been achieved with regard to international harmonisation. Most of the manufacturers produce for the world market, so harmonisation of the requirements or mutual recognition of tests would help to reduce the use of animals.
There is agreement that for the time being animals will still be needed for the development of vaccines in order to gain best knowledge on the disease, the pathogen and the specific immune response, including: pathogenesis, identification of the protective antigens, the way the antigen is processed, the dynamics of the immune response, the induction of memory, and the selection of the best adjuvant. With regard to routine batch release of conventional products, a number of Three Rs approaches are already available and should further be developed and validated. Whereas routine batch release of new products should be based on in vitro methods already established during their development.

Zusammenfassung: Das 3R Potenzial bei der Entwicklung und Qualitätskontrolle von Immunobiologika
Immunobiologika (Impfstoffe, Immunglobuline und –seren) sind die kostengünstigste Möglichkeit, Infektionskrankheiten vorzubeugen. Ihre Bedeutung wird noch weiter zunehmen, vor allem im Hinblick auf die verschiedenen Bekämpfungsprogramme der WHO und EU, dem Auftauchen von neuen Infektionskrankheiten oder der Zunahme von Krankheiten wie Diphtherie und Tuberkulose.
Die Produktion und Qualitätskontrolle von Immunobiologika werden durch Monographien und Richtlinien bestimmt, die internationale oder nationale Arzneibücher (z.B. Ph. Eur.), internationale Organisationen (z.B. WHO, O.I.E.) sowie internationale Behörden (z.B. EMEA) erstellen und herausgeben. Sie dienen der Qualitätssicherung der Produkte, d.h. der Überprüfung der Unbedenklichkeit und Wirksamkeit. Man schätzt, dass pro Jahr 10 Millionen Tiere für die Produktion und Qualitätskontrolle von Immunobiologika verbraucht werden, davon 80% für die Überprüfung der Unbedenklichkeit und Wirksamkeit des Endprodukts (Chargenprüfung).
In den vergangenen Jahrzehnten haben die oben genannten Organisationen und verschiedene nationale Behörden die Bedeutung der 3R erkannt und ihre Prinzipien in die allgemeinen Monographien und Richtlinien aufgenommen. Einige Tests mit fraglicher Relevanz wurden aus den Ph. Eur. Monographien gestrichen (z.B. Anomale Toxizität, Fremdvirusausschluss bei viralen Lebendimpfstoffen für Carnivoren) oder werden jetzt während der Produktion durchgeführt. Die Einführung von Ein-Punkt Tests führte ebenfalls zur Reduzierung der Tierzahlen. Eine Reihe von Alternativmethoden wurde bereits entwickelt, die die Wirksamkeitsprüfung am Tier ganz oder teilweise ersetzen könnte, aber nur wenige wurden bis jetzt validiert (z.B. der ToBI-Test und ein ELISA für die Wirksamkeitsprüfung von Tetanusimpfstoffen für Mensch und Tier; ein ELISA für die Wirksamkeitsprüfung von Rotlaufimpfstoffen). Die behördliche Akzeptanz von validierten Alternativmethoden erweist sich immer noch als kritischer Schritt. So erscheint die Zeitspanne von der erfolgreich abgeschlossenen Validierung bis zum Vollzug in gesetzliche Vorschriften als viel zu lang. Gründe hierfür mögen der langsame multinationale Einigungsprozess zur Revidierung von Monographien und Richtlinien sowie die zeitraubende und kostenintensive Herstellung von Referenzmaterialien (Antigene, Seren, usw.) für die neuen Methoden sein.
Die Änderung des Konzepts der Qualitätskontrolle von Immunobiologika von der reinen Endproduktkontrolle hin zur Kontrolle der Produktionskonsistenz eröffnet die Möglichkeit, die Tierzahlen weiter zu reduzieren und den Einsatz von Alternativmethoden zu fördern. Hierbei wird auf eine Kombination von in vitro Tests gesetzt, die es ermöglichen sollen, die Konsistenz zwischen Chargen zu überprüfen. Das neue Konzept der Qualitätskontrolle wird bereits bei neuen, gut definierten Produkten eingesetzt, und in den meisten Fällen werden tierversuchsfreie Methoden zur Überwachung der Konsistenz besonders kritischer Schritte in der Produktion und Überprüfung von Impfstoffen angewendet. Inwiefern sich dieses Konzept auch für konventionelle, weniger gut definierte Produkte eignet, sollte untersucht werden.
Nur wenig Erfolg wurde bei der internationalen Harmonisierung erzielt. Nachdem jedoch die meisten Hersteller für den Weltmarkt produzieren, würde die Harmonisierung von Vorschriften oder die gegenseitige Anerkennung von Tests dazu beitragen, die Tierzahlen zu reduzieren.
Auch in Zukunft werden Tiere für die Entwicklung von Impfstoffen gebraucht, vor allem um mehr über eine Krankheit zu erfahren, das pathogene Agens und die spezifische Immunantwort zu erforschen, einschliesslich der Pathogenese, des protektiven Antigens, der Aufbereitung des Antigens, der Dynamik der Immunantwort, der Induktion des Immungedächtnisses und der Auswahl des besten Adjuvants.
Was die Chargenprüfung der konventionellen Immunobiologika anbelangt, sind bereits eine Reihe von Alternativmethoden vorhanden, die weiter entwickelt und validiert werden sollten.
Zur Chargenprüfung von neuen Immunobiologika sollten bereits während der Produktentwicklung in vitro Methoden entwickelt und etabliert werden.

Keywords: vaccines, immunosera, quality control, Three Rs methods, regulatory acceptance

1 Introduction

It is estimated that more than 10 million animals a year are used worldwide for the development, production and quality control of immunobiologicals. Figures from the Netherlands show that about 20% of the animals are used for the development of new or improved products and about 80% in the routine quality control of batches of immunobiologicals (Hendriksen, 2000). The use of animals for the actual production of vaccines is restricted to very few cases such as suckling mice rabies vaccines in developing countries, monkey kidney cells for some polio virus propagation, rabbits for rabbit viral haemorrhagic disease vaccine, chickens for avian coccidiosis vaccine and cattle for lungworm disease.

Production and quality control is regulated by monographs or guidelines, which are issued by international or national Pharmacopoeias (e.g. European Pharmacopoeia [*Ph. Eur.*]), international organisations (e.g. World Health Organization [WHO], Office International des Epizooties [O.I.E.]) and international regulatory bodies (e.g. European Medicines Evaluation Agency [EMEA]). These specify tests, which can be divided into two categories: safety and efficacy tests during licensing, and safety and potency tests for batch quality control. Efficacy and potency tests ensure that the product induces protective immunity after administration whilst safety tests ensure that the product does not induce abnormal adverse reactions.

Some batch safety tests (target animal test for veterinary vaccines, abnormal toxicity test or the extraneous agent testing of poultry vaccines) are of questionable relevance and other safety tests (e.g. neurovirulence testing of live poliovirus vaccines in monkeys) raise very serious ethical concerns.

With regard to batch potency testing, most live vaccines are tested with *in vitro* methods and do not require animals. However, the testing of inactivated vaccines often requires large numbers of animals: e.g. more than 100 animals per batch are required for the potency testing of diphtheria, tetanus, whole pertussis, erysipelas and rabies vaccines. In addition to the large number of animals used, the potency testing of inactivated vaccines is often based on a vaccination and challenge test (e.g. clostridial, erysipelas, leptospiral vaccines) which involves considerable pain and suffering for the animals since, on average, 50% of the animals will succumb to the challenge and may die from the effects of toxicity or infection. In recent years, national control authorities, industry and regulatory bodies have made great efforts to develop, standardise and validate alternatives to these vaccination-challenge tests, and also to refine these tests and promote the use of humane endpoints.

There are two main approaches for the replacement of challenge tests: a) antigen quantitation which completely replaces the animal test (e.g. ELISA tests for rabies, hepatitis B, leptospiral, Newcastle disease vaccines); and b) the replacement of the challenge procedure with an immunological technique which allows the measurement of the appropriate response to vaccination. In many cases this is a simple serological model in which antibodies induced by the vaccine in the animal are quantified using immunochemical techniques such as neutralisation tests in cell cultures (e.g. Diphtheria toxoid, *Clostridium (C.) septicum, C. novyi* and *C. perfringens* vaccines), ELISA procedures (e.g. pertussis, botulinum, tetanus, erysipelas, leptospiral, *C. septicum, C. novyi* and *C. perfringens* vaccines), modified ELISA methods (ToBI test for tetanus vaccines), or a host of other techniques. In some cases, *in vitro* methods for the evaluation of cell-mediated responses may also be applied.

2 Concepts of quality and safety control of immunobiologicals

2.1 History of quality control

Initially, when the first immunobiologicals such as Jenner's smallpox vaccine, Pasteur's vaccines or erysipelas antiserum were invented more than a century ago, they were not submitted to quality control. As a result the vaccines sometimes contained an insufficiently inactivated virulent strain, were contaminated with other pathogens, or were of insufficient potency. It soon became apparent that large differences in quality between batches of the same vaccine could occur and, in consequence, the first governmental regulations for batch quality control were introduced.

Historically, the way regulatory requirements for immunobiologicals have developed, has been somewhat disasterled (Tab. 1).

Tab. 1: Immunobiological-related accidents

			Year	Cases	Deaths
Toxin in vaccine	Diphtheria	Dallas	1929	96	10
		Concord	1924	21	n.d.
		Bridgewater	1924	22	n.d.
		Baden	1924	28	7
		Kyoto	1948	600	68
Incomplete inactivation	Polio	Cutter incident	1955	260	5
Contamination with toxin	Tetanus in D-antiserum	St. Louis	1901	20	14
Wrong culture	BCG	Lübeck	1930	135	72

n.d. = no data available
Source: Hendriksen, 1996

Without animal experiments the quality of vaccines would not have been as good as it is today, and their successful use on such a large scale would never have been possible.

2.2 Standards and reference preparations

For traditional vaccines such as DTP, rabies or erysipelas, the potency is expressed as a relative value. This is achieved by comparing the potency of the test vaccine to a reference vaccine. The WHO, the *Ph. Eur.* or national control authorities provide such standard or reference preparations. Comparison of the test and the reference vaccine is based on multi-dilution tests, which are carried out for licensing and as batch

potency test. This approach uses large numbers of animals; however, this has been recognised and some of the monographs now allow the use of single-dilution test (see Progress and Criticism).

It is also evident that with the development of new vaccines, the control authorities and manufacturers have to deal more and more with product-specific aspects. The use of one single universal worldwide standard, against which it is valid to test each product against, might no longer be possible. For newer vaccines, for example *Haemophilus influenzae* b, recombinant Hepatitis B vaccine, acellular pertussis vaccine, and certainly the new vaccine combinations the tendency is for manufacturers to use clinical standards to test consistency of production, i.e. a clinical standard is a batch of a vaccine, which has been used in clinical trials and shown to be efficacious (Dobbelaer, 2000).

2.3 Uniqueness of each vaccine batch versus consistency of production

Biologicals are derived from living organisms in a batch-wise procedure, which means that their characteristics can vary from batch to batch. Therefore, each batch produced in one production run is considered as unique and undergoes strict quality control with emphasis on testing the finished product testing.

In recent years, it had been emphasised that consistency of production is essential and quality control should monitor critical steps during production and control of a biological rather than rely on control of the final batch (Griffiths, 1996). This concept is mainly applied to new, well-defined biologicals; however, it could also be introduced into the production of conventional, less-defined products (Hendriksen et al., 1998; Lucken, 1999; Leenaars et al., 2001). Consistency of production means that each batch of a product is of the same quality and is within the same specifications as a batch, which has been shown to be safe and efficacious in human trials or in the target animal species. Generally, alternative methods such as physiochemical or immunochemical methods are better able to monitor consistency than *in vivo* (e.g. vaccination-challenge procedures) tests. This is because of the parameters measured (e.g. antibody response versus lethality) and the additional inherent variability of the classical challenge models (Hendriksen et al., 1998).

The shift in the quality control concept from reliance on the final batch testing to the concept of consistency of production offers the opportunity to reduce the numbers of animals being used and promote the use of alternative methods. Emphasis is put on a combination of *in vitro* tests which could make it possible to monitor batch-to-batch consistency even for traditional vaccines like tetanus and diphtheria toxoids (Leenaars et al., 2001).

3 Regulation and acceptance

3.1 National and international control authorities

In Europe, competent control authorities regulate the authorisation and batch release, based on the *Ph. Eur.* and national pharmacopoeiae. If there is no monograph for a particular product or the monographs do not specify exactly the method to be used, the competent control authority sets the requirements to be fulfilled or requests a certain method to be used during the licensing procedure.

Since the establishment of the EMEA (European Medicines Evaluation Agency, London, UK, www.emea.eu.int/) in 1995, a centralised authorisation procedure is compulsory for biotech products in the 15 Member States (MS) of the European Union. This centralised procedure can also be used for new and innovative products. Other medicinal products can still undergo the decentralised procedure in one of the MS. The EMEA relies on two scientific committees, the Committee for Proprietary Medicinal Products (CPMP) and the Committee for Veterinary Medicinal Products (CVMP), each of which comprise 30 members nominated by the 15 MS. The CPMP and the CVMP set up guidelines for medicinal products, often in cooperation with specific CPMP/CVMP working parties (WP). The Biotech-WP provides specific expertise for the CPMP on human immunological products. The Immunological Veterinary Medicinal WP (IWP) advises the CVMP for example on general policy issues such as the elaboration and revision of guidelines on immunological products. The guidelines for the testing of medicinal products are included in *The Rules Governing Medicinal Products in the European Union* (European Union, 1999). In 1997, the Safety-WP adopted a position paper on the replacement of animal studies by *in vitro* models (EMEA, 1997), which addresses the feasibility of replacing *in vivo* studies with *in vitro* investigations in the preclinical development of medicinal products and gives advices on their validation and incorporation into CPMP Notes for guidance.

Fig. 1: Organigramme EMEA (European Medicines Evaluation Agency)

EXECUTIVE DIRECTOR
Financial controller, a.i.
Directorate

PRE-AUTHORISATION EVALUATION OF MEDICINES FOR HUMAN USE
Scientific advice and orphan drugs
Quality of medicines
Safety and efficacy of medicines

POST-AUTHORISATION EVALUATION OF MEDICINES FOR HUMAN USE
Regulatory affairs and organisational support
Pharmacovigilance and post-authorisation safety and efficacy of medicine

VETERINARY MEDICINES AND IT
Veterinary marketing authorisation procedures
Safety of veterinary medicines
Information technology

ADMINISTRATION
Personnel, budget and facilities
Accounting

Commission services at the EMEA in London
ETOMEP

TECHNICAL COORDINATION
Inspections
Document management and publishing
Conference services

3.2 National and international pharmacopoeias

In Europe, requirements for pharmaceutical products are laid down in the *Ph. Eur.* Since its elaboration in 1964, 28 European countries (including the European

Union) have signed the Convention of the *Ph. Eur.* Another 18 European and Non-European countries (including the WHO) are observers.

The *Ph. Eur.* includes general notices, methods of analysis (e.g. biological tests), general texts (e.g. general texts on vaccines), general monographs (e.g. vaccines for veterinary use) and specific monographs. A specific monograph is divided into the following sections: Definition, Production, Identification, Tests, Storage and Labelling. The Production section applies to the manufacturer and stipulates usually extensive safety and immunogenicity testing, which is, however, only performed once in the lifetime of a product during the licensing procedure. The Tests section applies to the manufacturers and to the control authorities, and the tests specified here have to be carried out on each batch of a product by the manufacturer but not necessarily by the control authority.

The *Ph. Eur.* Commission has 21 Groups of Experts, of which Group of Experts 15 (Sera and Vaccines) and Group of Experts 15V (Veterinary Sera and Vaccines) are responsible for the drafting of monographs on vaccines, antisera and antitoxins in collaboration with the *Ph. Eur.* secretariat. Group of Experts 6 (Biological Substances) and Group of Experts 6B (Human Blood and Blood Products) may also draft monographs on immunobiologicals such as hormones, immunoglobulins or other blood-derived products. The draft monographs are published in *Pharmeuropa* for public consultation. The expert group reviews the monographs in the light of the comments received, and they are finally adopted by the *Ph. Eur.* Commission.

Countries, which have signed the Convention of the *Ph. Eur.* are legally obliged to implement the texts of the *Ph. Eur.* into national legislation.

Apart from the *Ph. Eur.* Secretariat, the European Directorate for the Quality of Medicines (EDQM) of the *Ph. Eur.* comprises the division for publications, the laboratory and Division IV, which is responsible for the biological standardisation programme and the European network of OMCLs.

3.3 European guidelines and Council Directives

Within the European Union, the quality control of vaccines is regulated – in addition to the *Ph. Eur.* - by the following Council Directives and guidelines:

- for human vaccines by Council Directive 89/342/EEC extending the scope of Council Directives 65/65/EEC and 75/319/EEC and laying down additional provisions for immunological medicinal products consisting of vaccines, toxins or serums and allergens;
- for veterinary vaccines by Council Directive 90/677/EEC extending the scope of Council Directive 81/851/EEC on the approximation of the laws of the Member States relating veterinary medicinal products and laying down additional provisions for immunological veterinary medicinal products;
- "The Rules Governing Medicinal Products in the European Union" incorporates testing guidelines issued by the EU (EU, 1999).

According to the Directives and guidelines, quality control of each batch of a vaccine produced in one production run is mandatory in order to assure its safety and immunogenicity.

3.4 International organisations

There are two international organisations, which publish guidelines for the quality control of immunobiologicals: the WHO and the O.I.E.

3.4.1 WHO

The WHO was created in 1948 as a specialised agency of the United Nations. The objective of the WHO is "the attainment by all peoples of the highest possible level of health". In a wide range of functions, two are specifically addressed to vaccines and biologicals: to stimulate and advance work on the prevention and control of epidemic, endemic and other diseases; and to establish and stimulate the establishment of international standards for biological, pharmaceutical and similar products. It is essential that governmental institutions and international organisations co-operate with the WHO in developing and promoting harmonisation of vaccine standards (Vannier et al., 1997).

The International Biological Reference Preparations (IBRP) are established by the WHO Expert Committee on Biological Standardization (ECBS), which meets annually and addresses medicinal products (among them vaccines) for human use but also veterinary vaccines against diseases of zoonotic relevance. The use of IBRPs contributes to the reduction of animal tests by using harmonised test requirements and thus avoiding retesting. The actual list of IBRPs was recently published (WHO, 2000).

The Vaccines & Biologicals Department of the WHO regularly publishes and updates guidelines for international vaccine standardisation in its Technical Reports Series. Within the framework of the Global Training Network on Vaccine Quality, it regularly organises training courses on vaccine quality control for developing countries, which also address the standardisation/optimisation of the use and the reduction of laboratory animals for the production and quality control of vaccines.

3.4.2 O.I.E

The O.I.E. issues the Manual of Standards for Diagnostic Tests and Vaccines, which is edited by its Standards Commission and distributed world-wide (O.I.E., 2000). It contains recommendations for (a) "prescribed tests" for diagnosis, and (b) requirements for vaccines for list A and B diseases. List A diseases are transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal products. List B diseases are transmissible diseases that are considered to be of socio-economic and/or public health importance within countries and that are significant in the international trade of animals and animal products.

The chapter on vaccines includes information on recommended vaccines, data on seed management, characteristics of the vaccine strains, culture conditions, validation of vaccines, manufacturing, in-process controls, sterility tests, safety tests, and potency tests. The O.I.E. distributes this information and publishes the annual reports of the O.I.E. Standards Commission, which undoubtedly contribute to international harmonisation (Blancou and Truszczynski, 1997).

In 1994, the O.I.E. set up an Ad hoc Group on the harmonisation of veterinary medicines, which was the first step towards the creation of the "Veterinary International Cooperation on Harmonisation" (VICH).

3.5 International harmonisation

Europe

The European Commission represents the fifteen Member States of the EU. The Commission is working, through harmonisation of technical requirements and procedures, to achieve a single market in pharmaceuticals, which would allow free movement of products throughout the EU. The CPMP and CVMP of the EMEA provide technical and scientific support for International Conference on Harmonisation (ICH) and VICH activities.

The International Conference on Harmonisation

The ICH was established in 1990. It is a joint activity of regulators and industry as equal partners in the scientific and technical discussions of the testing procedures, which are required to ensure and assess the safety, quality and efficacy of medicines for human use. The focus of ICH is on the technical requirements for medicinal products containing new drugs. The six founder members of the ICH are the European Commission representing the 15 EU MS, the European Federation of Pharmaceutical Industries' Associations (EFPIA), the Japanese Ministry of Health and Welfare, the Japan Pharmaceutical Manufacturers Association, the US Food and Drug Administration, and the Pharmaceutical Research and Manufacturers of America. There are three observers, the WHO, the European Free Trade Area (represented at ICH by Switzerland) and Canada. The ICH secretariat is run by the International Federation of Pharmaceutical Manufacturers Association (IFPMA), which is a federation of member associations representing the research-based pharmaceutical industry and other manufacturers of prescription medicines in 56 countries throughout the world. IFPMA has closely been associated with ICH, since its inception to ensure contact with the research-based industry, outside the ICH regions. IFPMA has two seats on the ICH Steering Committee.

Veterinary International Cooperation on Harmonisation

The Veterinary International Cooperation on Harmonisation (VICH) was launched in 1996. VICH focuses on harmonising registration requirements for veterinary medicinal products in the EU, USA and Japan. Countries not involved in the VICH are kept informed on its progress through the O.I.E.

A Working Group on Target Animal Safety of veterinary medicines was recently established and had its first meeting in November 2000.

3.6 Legal and ethical background to the Three Rs

There is a legal and ethical obligation for the countries, which have signed the Convention of the Council of Europe and in particular, for the MS of the European Union. Both, the European Convention for the Protection of Animals Used for Experimental and other scientific purposes, ETS 123 (Council of Europe, 1986) and Directive 86/609/EEC (EEC, 1986) claim that

- *"an (animal) experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonable and practicably available"; (replacement)*
- *"in a choice between experiments, those which use the minimum number of animals ... cause the least pain, suffering, distress, and lasting harm and which are most likely to provide satisfactory results shall be selected"; (reduction and refinement) and*
- *"all experiments shall be designed to avoid distress and unnecessary pain and suffering to experimental animals"; (refinement).*

In addition, Directive 86/609/EEC states in Article 23 that the European Commission and the MS should initiate Three Rs studies.

4 Development and validation of alternatives

4.1 General aspects

4.1.1 Who develops and validates?

In the last fifteen years, a number of alternatives have been developed for the quality and safety control of immunobiologicals by national control authorities, academia, and manufacturers. In principle, there are two different approaches: the development of product-specific methods, which are specifically designed and validated by manufacturers for their products, and the development of reference methods, which can be used for a product group e.g. tetanus or rabies vaccines.

Alternative reference methods, which might replace, for example, a pharmacopoeial test, are validated in an international collaborative study. Within Europe, the Council of Europe and the European Commission have initiated the Biological Standardisation Programme (BSP), which is dedicated to validate alternative methods (Council of Europe, 1996a; Buchheit, 2001). In recent years, the European Centre for the Validation of Alternative Methods (ECVAM, Institute of Health and Consumer Protection; JRC, I-Ispra) established by the European Commission also got involved in the validation of alternative methods for the testing of biologicals. Several worldwide validation studies have been run under the auspices of WHO, e.g. validation of a transgenic mouse model and a molecular biological test to replace the neurovirulence testing of oral poliomyelitis using monkeys.

4.1.2 Who is sponsoring?

Financial support to the development was mainly given by national governments (e.g. BMBF, Swiss National Science Foundation), semi-governmental institutions (e.g. ZON), national institutes (e.g. RIVM), European authorities (e.g. European Commission research programmes) or international organisation (WHO, Council of Europe), national alternative centres, foundations (e.g. set, Stiftung 3R, DZ, FFVFF) and industry (e.g. In-VITRO). International validation studies are mostly sponsored on a European level and international level, e.g. by the BSP of EDQM, the European Comission (e.g. ECVAM) and the WHO.

4.2 Special aspects

The following two sections cover animal tests and possible alternatives to the batch potency and batch safety testing of vaccines and immunosera. In some cases, certain classes of vaccines (e.g.

clostridial vaccines) or vaccines for a certain species (e.g. avian vaccines) are reviewed together. Annex 1 and Annex 2 include tables, which summarise the numbers of animals needed for batch safety and batch potency testing, list distress categories and refer to the chapters.

4.2.1 Vaccines and immunosera for human use

4.2.1.1 Bacterial vaccines

BCG vaccine

Safety

There are two safety tests stipulated, which involve the use of animals: the test for virulent mycobacteria (subcutaneous or intramuscularly injection of 6 guinea pigs) and the test for excessive dermal reactivity (intradermal injection of test and reference vaccine into two groups of 6 guinea pigs). Suitable alternatives to these animal tests are not available.

Cholera vaccine

Potency

The *Ph. Eur.* monograph on cholera vaccine includes the test for antibody production, which is a potency test based on immunisation of 6 animals (guinea pigs, rabbits, mice) and estimation of serum antibodies with suitable methods. Alternatives, which could replace this serological model, e.g. antigen quantification, are not available.

Diphtheria vaccine

Safety

Both, the WHO and the *Ph. Eur.* stipulate tests for diphtheria toxin to be carried out on the final bulk (absence of toxin; irreversibility of toxoid) and the final lot (specific toxicity). Currently, there are three methods used, which are two animal tests involving subcutaneous or intradermal inoculation of five respectively one guinea pig, and an *in vitro* method using cell cultures.

In 2000, the monograph on diphtheria vaccine and vaccines containing a diphtheria component was revised (Council of Europe, 2000a). It is intended to combine the test for absence of toxin and irreversibility of toxin and to use cell cultures for the detection of diphtheria toxin. The test for specific toxicity on the final bulk and the final lot will be deleted.

The use of Vero cells for the detection of diphtheria toxin was first described by Abreo and Stainer (1985); and further optimised and standardised by van der Gun et al. (1999); however, a validation study is still required.

A rapid enzyme immunoassay has been developed for diagnostic purposes, which might also be used for diphtheria vaccines (Engler and Efstratiou, 1999).

Potency

The *Ph. Eur.* includes two methods for the potency testing of diphtheria vaccines, both are classical multi-dilution challenge assays. The first method is carried out with least 6 groups of guinea pigs (exact number not stated in the monograph, in practice 8-12 animals) are immunised and subcutaneously (lethally) challenged. The second test is based on intradermal challenge of 5 immunised guinea pigs and evaluation of the dermal reaction. The WHO requires either the intradermal challenge or a serological method used for the estimation of diphtheria antibodies in the serum of immunised mice or guinea pigs. Until today, only the WHO permits the use of a single-dilution assay once the consistency of production and testing has been established; the revised *Ph. Eur.* text 2.7.6. Assay of Diphtheria Vaccine (absorbed) (Council of Europe, 2000b) now includes the option to use a single-dilution assay.

The most promising serological method is the Vero cell test, which is already allowed by WHO (WHO, 1995). Validation of this method is foreseen in the BSP of EDQM. Other possibilities for the estimation of serum antibodies are the ToBI test (Hendriksen et al., 1989), an ELISA procedure (Moon et al., 1999) and two types of double antigen immunoassays, of which one could also detect tetanus antibodies (Aggerbeck et al., 1996; Kristiansen et al., 1997; Azhari et al., 1999).

Haemophilus type B conjugate vaccine

Safety

A pyrogen test is carried out on the final bulk for safety control purposes (see 4.2.1.5 Test for pyrogens).

Potency

The potency testing of haemophilus vaccines is based on a serological animal model: a group of eight mice is immunised with the test vaccine and after a given period the serum antibodies are estimated and compared to those of a group of eight non-immunised mice. For liquid products, this test is not carried out on the final lot but only on the final bulk.

There is a need to evaluate whether the number of animals required could be reduced, in particular, the number of control animals appears rather high.

Meningococcal polysaccharide vaccine

Safety

A pyrogen test is carried out for safety control purposes (see 4.2.1.5 Test for pyrogens).

Acellular pertussis vaccines (ACPVs)

Safety

The *Ph. Eur.* monograph on acellular pertussis vaccine stipulates two safety tests to be carried out on the final lot, the test for absence of residual pertussis toxin and the test for reversibility of toxoid. The so-called histamine-sensitisation (HS) test is carried out in both cases: five mice are immunised with ACPV, five control animals are injected with diluent, and after a given period intraperitoneally challenged with histamine. An *in vivo* alternative to the HS test is the leukocytosis promotion (LP) test, which is considered not to be consistently reliable (Corbel et al., 1999). The *in vitro* alternative, the Chinese hamster ovary (CHO) clustering test, can be used for the testing of bulk components but not for absorbed vaccines, since the adjuvant might interfere with the cells.

Potency

Potency testing of ACPV is based on a serological multi-dilution test: 6 groups of mice are immunised with dilutions of the test vaccine and reference vaccine and after a given period the animals are bled and the serum antibodies are estimated with an immunochemical method. The tester can choose the number of animals per group, which should be suitable to meet the requirements for a valid test. The monograph allows the use of a single-dilution test provided that the tester has gained sufficient experience with the method.

Whole cell pertussis vaccine (WCPVs)

Specific toxicity testing

According to the current *Ph. Eur.* requirements, the specific toxicity of WCPV is

tested with the mouse weight gain (MWG) test, which can be regarded as a non-specific test estimating overall toxicity. At least 10 mice are injected with the vaccine to be tested; their weight is recorded on day 3 and day 7 and compared to the weight of a control group. The recently revised *Ph. Eur.* monograph stipulates the use of only 5 guinea pigs according to the proposal of Weisser and Hechler (1997) (Council of Europe, 2000c).

Bordetella pertussis is known to produce at least five toxins; however, it is not clear to what extent they cause reaction to the vaccine after injection into humans. A number of animal and non-animal test systems have been developed for the detection of specific toxins (Tab. 2) and four of them were recently compared in a large-scale collaborative study (van Straaten-van de Kappelle et al., 1997). From this study it was concluded that the MWG test is not the most suitable test to detect pertussis toxin activity and that the HS test and the LP test might be more suitable for this purpose. However, the authors underline the importance of further optimisation and standardisation of the test systems. They emphasised that the *Limulus* amebocyte lysate (LAL) test should be used to measure endotoxin levels, whereas the CHO clustering test could only be used for adjuvant-free vaccines.

Since the HS test is based on a challenge procedure with histamine and subsequent death of the animals in the presence of pertussis toxin, it is a more severe procedure than the LP test.

Potency

The potency testing of WCPVs is a classic multiple-dilution challenge test (Kendrick test): groups of at least 16 mice are immunised with serial dilutions of the test vaccine and a reference preparation. 14-17 days after immunisation, the animals are intracerebrally challenged with live pertussis bacteria, observed for 14 days and the survival rates are evaluated. This intracerebral mouse protection test is stipulated (with minor differences) by all international requirements. It uses large numbers of animals and inflicts severe distress on the mice. The precision and reproducibility of the test is poor (reviewed in Weisser and Hechler, 1997).

Modification of the animal model seems to be possible, thus the intracerebral challenge could be replaced with aerosol challenge, which is less distressing for the animals and is not based on the lethal endpoint (Canthaboo et al., 1999a).

The most promising alternative method developed is a whole cell ELISA, which estimates pertussis antibodies in the serum of immunised mice and avoids intracerebral challenge. A collaborative study with five participating laboratories revealed that the whole cell ELISA is a suitable method for the potency testing of WCPVs (van der Ark et al., 2000).

Canthaboo et al. (1999b) report an alternative method, which is based on the estimation of nitric oxide induction in macrophages of mice immunised with WCPV. This method aims to replace the intracerebral challenge, however, it is still under development.

Pneumococcal polysaccharide vaccine

Safety

A pyrogen test is carried out for safety control purposes (see 4.2.1.5 Test for pyrogens).

Tetanus vaccine

Safety

The current *Ph.Eur.* monographs stipulates three animal tests to be carried out for the detection of tetanus toxin, which are carried out on the toxoid bulk (absence of toxin, irreversibility of toxoid) and on the final bulk (specific toxicity). The freedom from residual and reversible tetanus toxicity of the toxoid bulk is tested in guinea pigs or mice. Weisser and Hechler (1997) have outlined possibilities for refinement and reduction of these animal tests. The revised *Ph. Eur.* monograph includes significant changes which are the combination of the two tests on the toxoid bulk carried out in guinea pigs and the deletion of the specific toxicity test on the final bulk (Council of Europe, 2000d).

A promising *in vitro* approach for the detection of tetanus toxin, an endopeptidase test, has recently be developed (Ekong and Sesardic, 1999; Sesardic et al., 2000a); however, further standardisation and validation are still required.

Potency

According to the requirements of the *Ph. Eur.* monograph, a classic multi-dilution vaccination challenge test has to be performed for the potency testing of human tetanus vaccines. Guinea pigs or mice can be used for this purpose. A series of at least three dilutions of the vaccine and the reference preparation are administered subcutaneously. The exact number of animals to be used per group is not stated, but the monograph prescribes that it must be sufficient to meet the statistical requirements. Four weeks after immunisation, the animals are challenged with either a lethal or a paralytic dose of tetanus toxin, and after one week of observation, the survival rate is analysed. The challenge with the tetanus toxin causes severe suffering to the animals, as at least 50% of the animals die of tetanus or develop paralysis. The recently revised *Ph. Eur.* text 2.7.8. Assay of tetanus vaccine (absorbed) foresees the use of a single-dilution assay provided that the tester has sufficient experience with the method for a given product (Council of Europe, 2000e).

Tab. 2: Alternatives to the mouse weight gain test

Method	Toxin	Animals	Status	Reference
Histamine-Sensitisation (HS) test	Pertussis	Multi-dilution test in mice*	Allowed by WHO	van Straaten-van de Kappelle et al., 1997
Leukocytosis Promotion (LP) test	Pertussis	Multi-dilution test in mice	Allowed by WHO	van Straaten-van de Kappelle et al., 1992
Limulus Amebocyte Lysate (LAL) test	Endotoxin (LPS)	-	Allowed by WHO	van Straaten-van de Kappelle et al., 1997
Chinese Hamster Ovary (CHO) clustering test**	Pertussis	-	Allowed by WHO; Used for PT detection in acellular pertussis vaccines	Fujiwara and Iwasa, 1989

* = HS test is used as a single-dilution test for the quality control of acellular pertussis vaccines
** = only for adjuvant-free vaccines

This will significantly reduce the number of animals required for the potency testing.

During the last decade, two serological test systems, an ELISA procedure and the toxin binding inhibition test (ToBI) were developed, which measure the level of tetanus antibodies in the sera of the immunised animals (Hendriksen et al., 1991; Hendriksen et al., 1994).

A validation study was initiated in 1996 with the financial support of ECVAM and EDQM. The main objectives of the validation study were to replace the toxin challenge with *in vitro* estimation of tetanus antibodies; to replace the multi-dilution (quantitative) test with a single-dilution (qualitative) test; and to use guinea pigs instead of mice for the immunisation with tetanus vaccine (Winsnes et al., 1999). The results indicate a very good correlation between the antibody concentration assessed by the two serological methods and death/survival of the guinea pigs after the challenge: the predictive value for the ToBI test is 94% (range 92-97%, for six laboratories) and 92% for the ELISA method (range 91-95%, for six laboratories). Antibody concentrations determined by ELISA and ToBI were generally in the same range (Council of Europe, 2000f). It is hoped that the ELISA and the ToBI test will be allowed for batch potency testing soon. WHO already permits the use of serological tests for batch potency testing of human tetanus vaccines provided that it has been validated for vaccines of the same type (WHO, 1995).

Typhoid vaccines

The *Ph. Eur.* contains three monographs on typhoid vaccines, which are typhoid polysaccharide vaccines, oral live typhoid vaccines (strain TY 21 A) and typhoid vaccines. Only the latter stipulates tests in animals; however, it is no longer relevant.

4.2.1.2 Viral vaccines

Hepatitis vaccines

Potency

The three *Ph. Eur.* monographs on hepatitis vaccine A, hepatitis B and the combined product stipulate that potency testing should be carried out *in vivo* or *in vitro*. The *in vivo* potency test is a serological test which is carried out in mice or guinea pigs. The *in vitro* test is based on immunochemical determination of the antigen content and has to be approved by the National Control Authority. Descamps et al. (1999) report that the use of antigen quantification for batch release reduced the number of mice by 60% at one of the main hepatitis vaccine manufacturer. However, not all competent authorities accept the *in vitro* method and therefore, animals are still used. In the meantime, the WHO has also modified its requirements on hepatitis B vaccines and allows the use of antigen quantification for batch release (WHO, 1999a).

Influenza vaccines

The *Ph. Eur.* includes three monographs on influenza vaccines, which are inactivated split virion, surface antigen and whole virion influenza vaccines. The monographs stipulate a test for inactivation to be carried out in fertilised chicken eggs. There is a need to evaluate whether cell cultures would be suitable for this purpose, since at least influenza virus strain B grows on MDCK cells.

Poliomyelitis vaccines

Production

Poliomyelitis vaccines deserve a special consideration since monkeys might be used for their production and quality control (neurovirulence testing of oral poliomyelitis vaccine). Some vaccine manufacturers use primary and subcultured monkey kidney cells for the propagation of the vaccine virus, other use human diploid cell lines or Vero cells. It has been discussed whether the use of primary monkey kidney cells should cease. However, in the light of the WHO poliomyelitis eradication campaign and the decreasing need of poliomyelitis vaccine, it was been claimed that far more monkeys would be needed to re-establish consistency of production than would be saved by change of cell substrate.

Inactivated poliovirus vaccines (IPV)

Potency

The *Ph. Eur.* monograph includes three multi-dilution serological animal models for the potency testing of IPV. Since a test with the reference vaccine is carried out in parallel, at least 60 chicks, guinea pigs or rats are required per test. The rat potency test, which has been evaluated in a collaborative study, is now recommended by *Ph. Eur.* (Wood and Heath, 2000).

The potency test can be omitted on the final lot provided that it had been performed with satisfactory results on the final bulk.

For potency testing, it is then sufficient to quantify the antigen content.

Live oral poliomyelitis vaccines (OPV)

Neurovirulence test

Live attenuated virus vaccines are tested for neurovirulence in monkeys. The aim of the test is to confirm that the attenuated vaccine virus strain has not reverted to neurovirulence. In the case of OPVs, this test is carried out on each master seed lot, working seed lot and on each monovalent bulk. Monkeys (for example, *Macaca fascicularis, Cercopithecus aethiops*) are intraspinally injected with the test or reference preparation and killed after a given observation period and the central nervous system is checked for the specific neuronal lesions of poliovirus. According to the *Ph. Eur.* and WHO requirements, at least 80 monkeys are needed for the quality control of a trivalent bulk.

Alternative tests have been developed and are, at least for poliovirus type 3, close to regulatory acceptance (Wood, 1999). The alternatives are the MAPREC test (molecular analysis by PCR and restriction enzyme cleavage; Chumakov et al., 1991), which detects neurovirulence specific mutations, and a neurovirulence test in transgenic mice (TgPVR21 mice), which express the human cellular receptor for polioviruses (Koike, 1991). Wood and Macadam (1997) and Dragunsky et al. (1996) have reviewed the specifications of these test systems.

The MAPREC test for poliovirus type 3 is already an option in the WHO requirements, which suggest that only preparations, which pass the MAPREC test, should be tested in monkeys in order to detect other mutations. WHO International Standard and Reference Preparation and a SOP are now available for poliovirus type 3 but not yet for type 1 and 2 (Wood, 1999). The WHO has endorsed the MAPREC test as the *in vitro* test of preference for the quality control of poliovirus type 3 (Wood et al., 2000).

The WHO has organised a collaborative study to validate the transgenic mouse model for neurovirulence testing

of poliovirus type 3 vaccines (Wood, 1999) and the TgPVR21 mouse model is now recommended as an alternative to the test in monkeys (Wood et al., 2000). Studies on the suitability of TgPVR21 mice for neurovirulence testing of poliovirus type 1 and 2 are in progress.

Horie et al. (1998) modified the MAPREC test and developed the NON-RI MAPREC, which does not involve the use of radioisotopes. A further molecular test has been developed by Kaul et al. (1998), who used Taq Man PCR for the quantification of neurovirulence specific mutations. Proudnikov et al. (2000) used a new technology of hybridisation for the detection and quantification of neurovirulent mutants in OPVs, which can be used for screening samples for known mutants as well as for new mutations.

Rabies vaccine

Potency

According to the WHO requirements and the *Ph. Eur.* monograph, the potency of inactivated rabies vaccines for human use is estimated with the NIH test. The NIH test is a multi-dilution challenge test: at least 6 groups of mice are immunised with serial dilutions of test and reference preparation. The animals are intracerebrally challenged with virulent rabies virus and observed for signs of rabies for 14 days. The NIH test requires a high number of animals (up to 170 mice per batch, Weisser and Hechler, 1997) and causes severe distress to them. The use of non-lethal endpoints should be considered (see 4.2.3 Humane endpoints). Various alternative methods have been developed which could replace the NIH test. These methods are either based on serology or antigen quantification and some are listed in Table 3.

Recently, a validation study has been performed involving seven laboratories: the potency of five commercially available human and veterinary rabies vaccines and one reference preparation was tested with an ELISA procedure (Rooijakkers et al., 1996), which estimates rabies virus antigens, glycoprotein (G) and nucleoprotein (N). All the participating laboratories carried out the requested assays and generated valid data. The results clearly show that the reproducibility of the two ELISA kits is by far superior to that of the NIH test (unpublished data).

Tab. 3: Alternative methods for the poteny testing of rabies vaccines

Method	Animals	Reference	Status
Antibody estimation			
Rapid fluorescent focus inhibition test	Yes (5 mice)	Smith et al., 1973 Council of Europe, 1998a	WHO method; *Ph. Eur.* method
ELISA	Yes	Joffret et al, 1991	Commercially available
Antigen quantification			
Single radial diffusion	No	Ferguson et al., 1984 Vogel et al., 1989	WHO method Accepted in Austria
Antibody binding test	Depending on the method used	Arko et al., 1973	WHO method
ELISA (G antigen)	No	Gamoh et al., 1996	
ELISA (G and N protein)	No	Rooijakkers et al., 1996	Validated (unpublished data)

4.2.1.3 Immunosera/Antitoxins

C. botulinum antitoxin for human use

Potency

The *Ph. Eur.* stipulates a classical toxin neutralisation test in mice for the potency testing of botulinum antitoxin, which requires large numbers of animals (at least, 150 mice per batch). Possibilities for reduction and refinement have been reviewed by Weisser and Hechler (1997). An *in vitro* method for the detection of neutralising antibodies against botulinum toxin type A has recently be reported (Martin and Sesardic, 1999; Sesardic et al., 2000b), which is dependent on the activity of a botulinum type A protease on a synthetic substrate.

Diphtheria antitoxin

Potency

The potency testing of diphtheria antitoxin is based on an *in vivo* intradermal toxin neutralisation test in guinea pigs or rabbits. Serial dilutions of the test antitoxin are mixed with a given dose of diphtheria toxin and each dilution is intradermally injected into two animals. Weisser and Hechler (1997) suggested that the total number of animals could be reduced by using a number of injection sites on each animal. Thus, one animal would be sufficient for the complete titration of the test antitoxin and the reference preparation. With regard to replacement, there is a need to evaluate whether any of the *in vitro* methods developed for the potency testing of diphtheria vaccines could also be used for the potency testing of diphtheria antitoxins (e.g. Vero cell test).

European viper venom antiserum

Potency

European viper venom antiserum is protective against the venom of five viper species and the potency of each test antiserum has to be tested against the five venoms. The PD50 value for each venom is determined with an *in vivo* toxin neutralisation test in mice. Weisser and Hechler (1997) report that around 400 mice are needed per batch. In addition, about 50% of the mice are not protected against the venom and suffer extremely.

A number of alternative methods have been developed, however, all of them for the potency testing of non-European snake venom. Most of the alternatives are based on *in vitro* neutralisation of specific snake venom effects, e.g. antiprocoagulating, myonecrotic, haematolytic or proteolytic effects, on cell cultures (da Silva et al., 1982; Warrell et al., 1986; Guitterrez et al., 1988; Laing et al., 1992; Gowda and Middlebrook, 1993; de Araújo et al., 1999). Various immunochemical procedures have been assessed for the detection of snake venom antibodies in the sera of patients and in antisera (Theakston, 1983); however, no studies are reported on the suitability of these methods for the potency testing of European snake venom antiserum. In the light of the large number of animals required per batch and the severe distress inflicted on the animals, efforts should be made to investigate whether immunochemical methods or *in vitro* neutralisation could be used for the potency testing of European snake venom.

Gas-gangrene antitoxins

Potency

The *Ph. Eur.* includes four monographs on gas-gangrene antitoxins, which are Gas-gangrene Antitoxin *(C. perfringens)*, Gas-gangrene Antitoxin *(C. septicum)*, Gas-gangrene Antitoxin *(C. novyi)* and Mixed Gas-gangrene Antitoxin. The potency testing of gas-gangrene antitoxins is based on *in vivo* toxin neutralisation in mice or "other suitable animals". Serial dilutions of the test antitoxin are mixed with a given dose of toxin and each dilution is intramuscularly *(C. novyi)* or intravenously *(C. perfringens, C. septicum)* injected into a group of 6 animals. Weisser and Hechler (1997) reported that the total number of animals needed for the potency testing of a batch of mixed gas-gangrene antitoxin is about 150. They recommend that the number of animals per group should be reduced from six to between one to three.

Alternative methods could either be based on *in vitro* neutralisation of the cytopathic effects caused by clostridial toxins (Knight et al., 1986 and 1990; Bette et al., 1989; and Tab. 6) or modification of immunochemical methods, which have been developed for the potency testing of clostridial vaccines (Tab. 6).

Tetanus immunoglobulins and antisera for human use

Potency

The *Ph. Eur.* monographs *Human Tetanus Immunoglobulin* and *Tetanus Antiserum for Human Use* (equine origin) and *Tetanus Antiserum for Veterinary Use* (equine origin) stipulate a toxin neutralisation test in mice for the potency testing of these products. The monograph *Human Tetanus Immunoglobulin* has recently been revised and the use of validated serological *in vitro* methods is now allowed; however, no reference method is proposed or described, and the *in vivo* test is still used.

Studies performed at the PEI had shown that the EIA and the RIE are promising methods (Mainka and Haase, 1995; Zott, 1996) for replacement of the *in vivo* test. With the financial support of ECVAM, the PEI initiated the standardisation of these two methods and modified the ToBI test for the potency testing of tetanus immunoglobulins and antisera, which proved to be the most suitable method for prevalidation (Ebert et al., 1998).

Coded samples of tetanus antisera and immunoglobulins including samples of inferior quality were tested in six laboratories. Comparison of the ToBI data with the *in vivo* data (provided by the manufacturers) showed a high agreement in the ranking of the potencies of the samples. The best results were obtained for the human tetanus immunoglobulins: interlaboratory variation was <15% and the ranking of the samples was identical in all laboratories. It is therefore concluded that a formal validation study of the ToBI test for the potency testing of human tetanus immunoglobulins should be initiated. With regard to the tetanus antisera for human and veterinary use, it should be considered whether the monograph on tetanus antiserum for human use should be deleted since tetanus antiserum of equine origin is no longer used for humans or whether the two monographs should be harmonised and the requirements on the quality and safety of the products be updated to reflect the present state-of-the-art. Nevertheless, validation of the ToBI test for the potency testing of tetanus antisera is recommended. Provided that the ToBI test could be incorporated into the *Ph. Eur.* monographs, at least 2.000 animals per year could be saved in Germany (Weisser and Hechler, 1997).

Kolbe and Clough (1999) developed a competitive ELISA for the estimation of tetanus antibodies in equine tetanus antiserum for veterinary use. They compared the ELISA results of nine batches of tetanus antiserum with the *in vivo* results obtained with the toxin neutralisation test in guinea pigs (US requirements) and found a good correlation. However, data on the repeatability and the reproducibility of results from other laboratories were not provided.

4.2.1.4 Tuberculins for human use

There are two monographs on human tuberculins in the *Ph. Eur.*, which stipulate the use of animals for toxicity (two guinea pigs), sensitisation (3 guinea pigs) and potency (6 guinea pigs) testing. The test for identification is also carried out in animals but can be combined with the potency testing.

The test for live mycobacteria was recently deleted and replaced by new and more sensitive culture methods (Council of Europe, 2000g).

Weisser and Hechler (1997) question the relevance of the toxicity test since it does not provide any further information. Alternatives to the test for sensitisation are not available, however, the number of control animals could be reduced from three to one. There have been several attempts to develop alternatives for the potency testing of tuberculins, however, none has been validated. The most promising approach is the lymphocyte stimulation test (Hasloev et al., 1986), which measures *in vitro* the cytokine release of T-cells in response to mycobacteria. Weisser and Hechler (1997) suggest that potency could be tested in human volunteers, since in house reference preparations are also calibrated in humans against the international reference. Further possibilities could be the use of *in vitro* skin models.

For the time being, the design of the current potency test in animals could be refined (e.g. individual evaluation of each animal) and potency testing should only be carried out during production and on the final bulk, which would significantly reduce the total number of animals needed.

4.2.1.5 Test for pyrogens

The *Ph. Eur.* stipulates the test for pyrogens for a number of immunobiologicals (Tab. 4). It assesses the fever reaction in three rabbits induced by pyrogens.

A well-established alternative to the pyrogen test in rabbits is the *in vitro* Limulus amebocyte lysate (LAL) assay, which has already replaced the rabbit test in a number of *Ph. Eur.* monographs (test for bacterial endotoxins). However, it can only detect cell wall components of gram-negative bacteria but not pyrogenicity caused by gram-positive bacteria, fungi or other possible pyrogens. With regard to the products listed in Table 4, it should be possible to use the test for bacterial endotoxins for pyrogenicity testing of meningococcal vaccines, since the pyrogens to be detected are endotoxins. The monograph on hepatitis B vaccines explicitly states that a validated test for bacterial endotoxins could replace the rabbit pyrogen test.

The fact that the *Ph. Eur.* has established a new Expert Group for Alternative Pyrogen Testing reflects the importance of these issues.

A number of *in vitro* tests based on the human fever reaction have been devel-

Tab. 4: *Ph. Eur.* monographs on immunobiologicals stipulating the test for pyrogens in rabbits

Haemophilus type B conjugate vaccine
Meningococcal polysaccharide vaccine
Pneumococcal polysaccharide vaccine
Hepatitis B vaccine (rDNA)
Rabies vaccine for human use prepared in cell cultures
Tick-borne encephalitis vaccine
Human normal immunoglobulin (applies also to all "special" human immunoglobulins, e.g.
Human tetanus immunoglobulin
Human normal immunoglobulin for intravenous use

oped (Tab. 5). The *in vitro* approaches use leukocyte cell lines, isolated primary blood cells or whole blood and measure the release of fever mediators in response to pyrogens. A collaborative study is in progress, which aims to validate the most suitable model(s) for pyrogenicity testing of immunobiologicals, pharmaceuticals, medical devices etc. (Hartung et al., 2001).

4.2.2 Vaccine and immunosera for veterinary use

4.2.2.1 The target animal safety test

The target animal safety test (TAST) is required by *Ph. Eur.* monographs and various EU guidelines on veterinary vaccines. It is carried out on the finished product and should detect non-specific contamination. At least two animals of the target species are injected with the 2-fold (inactivated vaccines) or 10-fold (live vaccines) recommended dose of the vaccine to be tested. None of the animals should show abnormal or systemic reactions during a given observation period. Significant differences in the numbers of animals required (mammals: 2 animals; poultry: at least 10 animals), the administration scheme and the period of observation are evident between the monographs.

During the last few years, the relevance of the TAST has more and more been questioned, because the introduction of Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) into the manufacturing of vaccines has significantly increased the safety and quality of the products, and some of the animal tests carried out for purity and safety purposes appear now to be superfluous (Roberts and Lucken, 1996; Zeegers et al., 1997; Pastoret et al., 1997; Cussler et al., 2000a).

Two studies have been carried out by the PEI and AGAATI, which aimed to evaluate the relevance of TAST by retrospective data analysis. The results obtained to date confirm its questionable relevance. The test design, the evaluation criteria and the requirement to be performed for batch release can no longer be justified. For several vaccines, it was shown that the TAST could be omitted, at least for routine batch control purposes (Bruckner et al., 2000; Pössnecker and Cussler, 1998; Cussler and Pössnecker, 2000). The final reports of the studies will be available shortly. At that time, consideration should be given to contacting the European regulatory authorities, asking for modification of their guidelines and monographs and specifying the deletion of the TAST on grounds that it is no longer relevant. However, if the TAST is still necessary for specific products or groups of products, the test design and requirements should be revised and clearly defined in order to obtain reliable results.

Two recent cases show that a passed TAST does not guarantee a safe vaccine. As Falcone et al. (1999) report, hundreds of cattle died after vaccination because the infectious bovine rhinotracheitis (IBR) vaccine used had been contaminated with bovine viral diarrhoea (BVD) virus. The IBR vaccine had been tested according the *Ph. Eur.* monograph and had been released. Since the serological status of the two calves used in the TAST had not been BVD negative, the contamination with BVD could not be detected. In France, the marketing authorisation for a dog vaccine was withdrawn, when several puppies died of distemper after vaccination (Anon., 2000).

4.2.2.2 Bacterial vaccines

Anthrax spore vaccine

Potency

The *Ph.* Eur. monograph on anthrax spore vaccine stipulates a vaccination-challenge test in 13 guinea pigs or rabbits or 8 sheep for the potency testing. Weisser and Hechler (1997) suggested the revision of the monograph and proposed

Tab. 5: Alternatives to the test for pyrogens in rabbits

In vitro models	Parameter	Reference
Leukocyte cell lines		
MonoMac cell line	IL-6, TNF-α	Taktak et al., 1991
THP-1; RAW 264.7	Neopterin, NO release	Peterbauer et al., 1999; 2000
Sub clones of THP-1 and MonoMAC cells	TNF-α	Eperon and Jungi, 1996; Eperon et al., 1997
Isolated primary leukocytes	IL-1, IL-6, TNF-α	Duff and Atkins, 1982; Dinarello et al., 1984; Poole et al., 1988a&b; Chia and McManua, 1990; Wind Hansen and Dencker Christensen, 1990; Morin et al., 1991
Whole blood models	IL-1, IL-6	Hartung and Wendel, 1995, 1996; Pool et al., 1998; Hartung et al., 2000

that the potency should be determined *in vivo* only during licensing. After establishment of a correlation between the number of live spores and the protection achieved in the target species, it would suffice for batch potency testing to determine the number of live spores *in vitro*, which is state-of-the-art for other live bacterial vaccines.

Brucellosis vaccine

Fifty percent persistence time

The test should show that the vaccine strain persists for a certain period in the organism. The bacteria localise intracellularly and induce the cellular immune response. 32 mice are subcutaneously injected with the vaccine to be tested and their spleens are examined for brucellosis bacteria after given periods. Alternatives to this test have not been developed. Weisser and Hechler (1997) recommend that it should only be performed during licensing and not on the finished product.

Fish vaccines

The *Ph. Eur.* includes three monographs on fish vaccines, which are furunculosis vaccine, vibriosis (cold-water) vaccine and vibriosis vaccine. It is striking that these monographs stipulate the use of far more animals, i.e. 10 fish for the safety testing and 60 for the vaccination-challenge or 35 fish for vaccination-serology, than monographs on mammalian vaccines. The EU guideline on *Specific Requirements for the Production and Control of Live and Inactivated Vaccines Intended for Fish* stipulates even 30 fish for the safety testing (EU, 1999). It should be investigated whether these numbers could be reduced.

Alternatives to the lethal vaccination-challenge test for potency testing of furunculosis vaccines are reported by Wagner et al. (1998) who used a sandwich-ELISA for the detection of potentially protective antibodies, and Pund et al. (1998), who compared the reaction of isolated lymphocytes of vaccinated and non-vaccinated fish to *Aeromonas salmonicida* isolates.

Tetanus vaccine for veterinary use

Potency

An inter-laboratory validation study was carried out with financial support of the European Commission (allocated ECVAM budget; Contract No B4-3081/91/7945). Based on the results, the Group of Experts 15V of the European Pharmacopoeia Commission agreed in 1996 to modify the monograph (Council of Europe, 1996b) on tetanus toxoid vaccines for veterinary use according to the recommendations of the study (Hendriksen et al., 1994). The currently stipulated TNT with mice will be replaced with an *in vitro* immunoassay (for example, the ToBI test). However, new homologous reference sera of rabbit and guinea pig origin were necessary to establish the methods for routine batch control. After calibration of the reference sera in an international collaborative study organised by EDQM (Lensing et al., 2000), biological reference preparations are now available and the monograph will probably come into force shortly.

Other clostridial vaccines

Safety - Residual toxicity

The *Ph. Eur.* monographs on *C. botulinum, C. novyi, C. perfringens,* and *C. septicum* vaccines require the test for residual toxicity, which involves subcutaneous injection of a given vaccine dose into 5 mice. In principle, cell culture methods can be used for the detection of residual toxicity of several toxoids, e.g. *C. novyi* and *C. perfringens* (Borrmann et al., 2001). However, in practice their use is mostly hindered by excipients such as formaldehyde.

Potency

For a long time, potency testing of clostridial vaccines and immunosera involved large numbers of animals and inflicted severe suffering on the animals. In 1997, a symposium on clostridial vaccines and immunosera organised by the EDQM, the PEI and the RIVM was held in Strasbourg (Council of Europe, 1997b). In the same year, the revision of four monographs on clostridial vaccines was initiated which already incorporated the proposals of the symposium, for example the permission to use validated immunochemical methods or tests based on toxin neutralisation in cell cultures for the potency testing of *C. novyi* (Type B), *C. perfringens,* and *C. septicum* vaccines, which would replace the mouse neutralisation test. The revised monographs came in force with the *Ph. Eur.* Supplement 2001. A number of alternative methods have been developed for this purpose (examples of which are listed in Table 6); however, none of them has been validated. The International Veterinary Industry Test Replacement Organisation (In-VITRO) and the EDQM initiated a large-scale collaborative study for establishing a multicomponent reference serum, which would facilitate to standardise and validate these methods (Redhead et al., 1999). This new European Reference Preparation has recently become available (Lucken et al., 2000).

With regard to *C. chauvoei*, the stringent pharmacopoeial requirements for the vaccination-challenge test involving guinea pigs and stipulating a 100% protection rate were changed in 1998 and came into force with *Ph. Eur.* Supplement 2001. In practice, potency testing now requires significantly fewer animals (Redhead et al., 1999). Efforts have been undertaken to identify the protective antigens for *C. chauvoei* and develop alternative methods for the potency testing of these vaccines (Roth and Seifert, 1997; Hauer, 1997; Kijima-Tanaka, 1998; Hauer and Clough, 1999).

US control authorities allow the use of antigen quantification for the potency testing of inactivated vaccines; however, the regulations have not yet been changed for clostridial vaccines. Hauer and Clough (1999) report the development of hybridoma cell lines which produce monoclonal antibodies directed against *C. perfringens* alpha, beta and epsilon toxin, *C. sordelli* lethal toxin and the flagella of *C. chauvoei*. The antibodies are internationally available and can be used in antigen quantification assays or other alternative methods.

Erysipelas vaccine

Potency

The *Ph. Eur.* monograph *Inactivated Swine Erysipelas Vaccines* has recently been changed and came into force with *Ph. Eur.* Supplement 2001. It currently stipulates a single-dilution vaccination-challenge test in 10 mice instead of a multi-dilution-assay, which needed over 100 mice for batch potency. Several promising serological models have been developed in recent years (Beckmann and Cussler, 1994; Rosskopf-Streicher et al., 1998, 1999; Redhead, 1998), which

Tab. 6: Alternative methods for the potency testing of clostridial vaccines (excl. Tetanus)

Vaccine	Method	Status	Reference
C. perfringens Type D epsilon and beta	Cell cultures (serology)	Available	Knight et al., 1990a; Payne et al., 1994; Borrmann and Schulze, 1995, 1997
	ELISA (serology)	Used by industry for batch release	Wood, 1991
		Validation in progress	Lucken, 1997
		Prevalidated	Ebert et al., 1999a
(and alpha toxin)	ELISA (antigen quantification)	Available	Hauer and Clough, 1999
C. novyi Type B	Cell cultures (serology)		Borrmann et al., 1999
	ELISA	Used by industry for batch release	Wood, 1991
		Validation in progress	Lucken, 1997
C. septicum	Cell cultures (serology)	Available	Knight et al., 1990b; Roth et al., 1999
	ELISA (serology)	Used by industry for batch release	Wood, 1991
		Validation in progress	Lucken, 1997
C. chauvoei	ELISA (cellular antigens)		Roth and Seifert, 1997
	ELISA (flagella antigen; immunochemical and antigen quantification)	Validation in progress	Hauer, 1997; Lucken, 1997; Kijima-Tanaka, 1998; Hauer and Clough, 1999
C. sordelli	ELISA (antigen quantification)	Available	Hauer and Clough, 1999

could replace the challenge. The PEI has successfully validated a direct ELISA procedure (Rosskopf-Streicher et al., 2001): 10 mice are vaccinated with the test vaccine and after a given immunisation period, the mice are bled and the antibodies to erysipelas are estimated with the ELISA. EDQM has asked the PEI to produce a reference coating antigen. It is hoped that the ELISA method will be accepted soon and incorporated into the monograph.

In the USA, work is in progress on the development of a sandwich ELISA for the antigen quantification of erysipelas bacterins, which does not involve the use of animals (Hauer, 1998; Coe-Clough, 1998).

Leptospira vaccines

The current *Ph. Eur.* monograph *Leptospiral vaccines for veterinary use* covers vaccines containing *Leptospira (L.) interrogans serovar canicola* and *serovar icterohaemorrhagiae*. A hamster challenge test is prescribed for the batch potency testing: a group of five hamsters is immunised with the test vaccine. After a given immunisation period, the immunised hamster and a non-immunised group of five hamsters are challenged with the corresponding challenge strain. At least 80% of the non-protected hamsters die due to the infection, which causes severe distress and suffering.

The hamster challenge model is also used for the potency testing of *L. hardjo* vaccines for cattle; however, the challenge procedure is regarded as less severe, since the animals often survive the infection. In this case, the re-isolation rate of bacteria from the kidneys is used to determine the degree of protection.

Some major disadvantages of this model are evident: large numbers of animals are used for the potency test itself, but also for the maintenance of the virulence of the challenge strains. Since humans are susceptible to infection with leptospiral bacteria, the test involves a high risk for infection of the laboratory personnel (Weisser and Hechler, 1997; Marbehant, 1999). At the workshop on *Alternatives to Animal Challenge Tests in the Batch Control of Leptospiral Vaccines for Veterinary Use* (Council of Europe, 1999a) a number of promising alternative methods were presented, which are based either on serology (involving the immunisation of animals) or antigen quantification (animals are not involved) (Tab. 7). However, none of these methods has yet been validated in an international collaborative study. Recently, USDA presented antigen quantification assays for several leptospira vaccines (including serovars canicola and icterohaemorrhagiae) suitable for *in vitro* potency testing. Those approved but pending Standard Requirements are listed under http://www.aphis.usda.gov/vs/cvb/lab/newsams.html.

4.2.2.3 Viral vaccines

Avian viral vaccines

The *Ph. Eur.* contains two general texts and 13 monographs on poultry vaccines (Tab. 8). Tests in animals are prescribed for extraneous agents, safety and potency testing. In particular, large numbers of animals are needed for batch control. Re-

Tab. 7: Current alternative methods for the potency testing of leptospira vaccines

Method	Serovars	Status	Reference
a) Serology			
MAT*	*L. hardjo*	Standardised	Ebert et al., 1999b
ELISA	*L. hardjo*	Under development	Ebert et al., 1999b
ELISA	*L. icterohaemorrhagiae and canicola*	Under development	Schalling, 1999
ELISA	*L. icterohaemorrhagiae and canicola*	Under development	Varney, 1999
b) Antigen quantification			
ELISA	*L. copenhagi and canicola*	Standardised	Hartskeel, 1999
ELISA	*L. icterohaemorrhagiae and canicola*	Standardised	Hickey, 1999
ELISA	*L. icterohaemorrhagiae and canicola*	Standardised	Martinon, 1999
ELISA	*L. icterohaemorrhagiae, canicola, pomona, grippotyphosa*	Standardised	Ruby, 1999

* modified O.I.E. method (2nd Edition of O.I.E. Manual; Goddard et al., 1986, 1991)

presentatives of vaccine manufacturers, which participated in the joint AGAATI/ECVAM workshop on *Three Rs Approaches in the Production and Quality Control of Avian Vaccines*, estimated that about 51.000 chickens and birds are used each year for the quality control of 1.800 batches of avian vaccines (Bruckner et al., 2000).

The following evaluation will focus on the tests stipulated for batch quality control since they offer more possibilities for replacement and reduction. However, the report of the above workshop includes a number of recommendations for the implementation of the Three Rs in the production of avian vaccines.

Batch testing for extraneous agents

The *Ph. Eur.* text *2.6.4 Avian Live Virus Vaccines: Tests for Extraneous Agents in Batches of Finished Products* stipulates tests in eggs, cell cultures and chicks in order to detect a given list of possible contaminants in avian live vaccines. The test in chicks (ten two-week-old chicks/test) is regarded as less sensitive than the tests in eggs and cell cultures (Bruckner et al., 2000). In addition, PCR methods that are at least as sensitive as the animal test are now available (Tab. 9). Some of them have already be validated and accepted, others have recently been developed and could be validated within the short-term.

Tab. 8: European pharmacopoeia texts and monographs on avian vaccines

General texts

2.6.3. Avian Viral Vaccines: Tests for Extraneous Agents Seed Lots

2.6.4. Avian Live Virus Vaccines: Tests for Extraneous Agents in Batches of Finished Product

Individual monographs

Avian Infectious Bronchitis Vaccine (live)

Avian Infectious Bronchitis Vaccine (inactivated)

Avian Infectious Bursal Disease Vaccine (live)

Avian Infectious Bursal Disease Vaccine (inactivated)

Avian Infectious Encephalomyelitis Vaccine (live)

Avian Infectious Laryngotracheitis Vaccine (live)

Avian Viral Tenosynovitis Vaccine (live)

Duck Viral Hepatitis Vaccine (live)

Egg Drop Syndrome '76 Vaccine (inactivated)

Fowl-pox Vaccine (live)

Newcastle Disease Vaccine (live)

Newcastle Disease Vaccine (inactivated)

Marek's Disease Vaccine (live)

Batch safety testing

The arguments against the TAST given in the general section on the TAST also apply to avian vaccines. However, at least ten chickens are required for avian vaccines in contrary to two for mammalian vaccines, without any scientific or statistical rationale for this difference.

Two studies are currently being performed by a working group at the PEI and by AGAATI (with the financial support of the European Commission via ECVAM). Data on the target animal safety test are being collected from vaccine manufacturers and control authorities in Europe and analysed retrospectively. The results on avian vaccines show that all batches tested in the last few years passed the test (personal communication). Passing the test had no influence on the number of the reports on vaccinovigilance collected from the field. The purpose and relevance of this test is therefore questionable, also since recent experience showed that the test is not capable of detecting major problems with vaccine batches. Deletion of the TAST from the individual monographs on avian vaccines is therefore recommended. It should be considered whether the TAST should be carried out on a limited number of batches for demonstration of consistency of production or after relevant changes in the production process (Bruckner et al., 2000).

Batch potency testing

In contrast to live avian vaccines, where potency testing of a representative batch is sufficient, the potency of inactivated avian vaccines has to be tested on each vaccine batch. This requires a large number of animals and involves suffering to the animals if, under certain circumstances, challenge with the infectious agent is required. In most cases, sero-

Tab. 9: Availability and status of non-animal methods for extraneous agents testing of batches of the finished product according to *Ph. Eur.* text 2.6.4

Agents	Alternative methods	Status	References
Infectious avian encephalomyelitis	Eggs (intravitelline injection)	Accepted	Paragraph 2.6.3.1 in Council of Europe, 1999b
Avian leucosis viruses	Cell culture PCR	Accepted Accepted	Covered by paragraph 2.6.3.3 in Council of Europe, 1999b; Häuptli et al., 1997
Avian nephritis virus	Kidney cell culture	Accepted	Paragraph 2.6.3.2 of Council of Europe, 1999b
Avian reticuloendotheliosis virus	Chick (or duck) embryo fibroblasts + immunofluorescence; PCR	Accepted Available	Paragraph 2.6.3.4 of Council of Europe, 1999b Tagaki et al., 1996
Egg drop syndrome virus	Production substrate (fibroblasts, duck eggs) or other sensitive cells	Available for substrates	Paragraph 2.6.3.1 and 2.6.3.2 in Council of Europe, 1999b
Chicken anaemia virus	Cells; PCR	Accepted Available	Paragraph 2.6.3.5 in Council of Europe, 1999b; Falcone et al., 2000
Marek`s disease virus	Chick embryo fibroblasts	Accepted	Paragraph 2.6.3.2 in Council of Europe, 1999b
Newcastle disease virus	Eggs (intra-allantoic inoculation) PCR	Accepted Available	Paragraph 2.6.3.1 in Council of Europe, 1999b Stäuber et al., 1995
Infectious bursal disease virus	Eggs (chorio-allantoic membrane and intra-allantoic inoculation) PCR	Accepted Under development	Paragraph 2.6.3.1 in Council of Europe, 1999b
Infectious bronchitis virus	Eggs (intra-allantoic inoculation) PCR	Accepted Available	Paragraph 2.6.3.1 in Council of Europe, 1999b Falcone et al., 1997
Infectious laryngotracheitis virus	Eggs (chorio-allantoic membrane inoculation) PCR	Accepted Available	Paragraph 2.6.3.1 in Council of Europe, 1999 Vögtlin et al., 1999
Salmonella pullorum	Culture	Accepted	Covered by the sterility test stipulated in (Council of Europe, 1997a)
Turkey rhinotracheitis virus	Vero cells, chick embryo fibroblasts	Accepted	Paragraph 2.6.3.2 in Council of Europe, 1999b
Chlamydia spp	Eggs (intravitelline injection)	Accepted	Paragraph 2.6.3.1 in Council of Europe, 1999b
Avian infectious haemorrhagic enteritis virus	PCR	Available	Hess et al., 1999
Avian paramyxovirus-3	Eggs	Accepted	Paragraph 2.6.3.1 in Council of Europe, 1999b
Duck/goose parvoviruses	Duck eggs/ duck embryo fibroblasts	Accepted	Paragraphs 2.6.3.1 and 2.6.3.2 in Council of Europe, 1999b
Duck enteritis virus	Duck embryo fibroblasts	Accepted	Paragraphs 2.6.3.1 and 2.6.3.2 in Council of Europe, 1999b
Duck hepatitis viruses I and II	Eggs	Accepted	Paragraph 2.6.3.1 in Council of Europe, 1999b

logical methods are used for antibody estimation after immunisation.

In recent years, efforts have been undertaken to develop antigen-estimation based *in vitro* methods, which do not require animals. Most progress has been achieved with models for the potency testing of inactivated Newcastle disease (ND) vaccines (Maas et al., 1998; Maas et al., 2000). The approach used is based on the quantification of the NDV antigens, the HN-protein and F-protein, which induce the production of neutralising antibodies after vaccination. Two ELISA procedures have been developed for this purpose. Preliminary results show that the method is sensitive, specific and reproducible. There is a good correlation between the amount of virus estimated in the vaccine and the antibodies detected in the serum after immunisation (Maas et al., 2000).

Whether antigen quantification can be used for the potency testing of inactivated bursal disease vaccine is also now under investigation.

Foot-and-mouth disease vaccine (FMD)

Safety

The safety test in the *Ph. Eur.* monograph can be regarded as a test for sufficient inactivation of the virus strain. Cell cultures are more suitable for this

purpose since they are more sensitive to infectious virus and allow the testing of large volumes of test vaccine (Anderson et al., 1970; Barteling and Vreeswijk, 1991).

Potency

Potency testing of foot-and-mouth disease vaccines is carried out as a multi-dilution vaccination-challenge test in 17 cattle. In order to reduce the numbers of animals, a single-dilution vaccination-challenge could be used. Alternatives to this highly distressing test have been developed and have been reviewed by Weisser and Hechler (1997); however, they have not yet been validated. The most suitable alternatives are based on either antigen quantification (Rweyemamu et al., 1984; Black et al., 1984; Pay and Hingley; 1987) or estimation of FMD antibodies in the serum of vaccinated animals with a sandwich ELISA procedure (Hamblin et al., 1986) or the plaque reduction test in BHK21-CT cells (Ahl et al., 1987). The latter method has been accepted for batch potency testing in the former GDR (Thalmann et al., 1987).

Rabies vaccine for veterinary use

Inactivated rabies vaccine

According to the *Ph. Eur.* monograph on rabies vaccine for veterinary use, the complete inactivation of rabies virus is tested by intracerebral injection of 10 mice. The sensitivity and relevance of this test were questioned (Hendriksen, 1988; Weisser and Hechler, 1997). The manufacturers are now allowed to carry out the test for inactivation in process immediately before addition of the adjuvant. Methods for *in vitro* detection of active rabies virus should then be used (for example, methods described by Ullrich, 1993; Blum, 1999) and the test on the finished product can be omitted.

Potency

The *Ph. Eur.* monograph on rabies vaccine for veterinary use allows estimation of rabies antibodies (for example RFFIT) and *in vitro* antigen quantification (for example, with an ELISA procedure); however, it is required that the NIH test is performed in parallel (see also 4.2.3 Humane endpoints).

Live rabies vaccines

Potency

Live rabies vaccines are used in foxes. For potency testing, a group of at least 25 foxes is immunised with the test vaccine, after a given period the vaccines and at least 10 non-vaccinated control animals are challenged with the rabies virus. Alternatives to this challenge test have not been developed. There is a need to determine whether the above-mentioned methods would be suitable to replace the challenge.

Based on statistical methods, Weisser and Hechler (1997) showed that the number of animals required could be reduced to 15 vaccines and 5 control animals.

The *Ph. Eur.* monograph allows skipping of the batch potency test provided that consistency of production has been proven. Since the TAST is not stipulated for this vaccine, it is the first veterinary vaccine, which does not require tests in animals for routine batch release purposes.

Classical swine fever vaccine

Potency

According to the *Ph. Eur.* monograph, the potency of classical swine fever vaccines is demonstrated with a vaccination-challenge test, which involves 12 piglets. Alternatives based on serum antibody estimation (Wensvoort et al., 1988) or antigen quantification (Popa et al., 1987) are available; however, they have not yet been validated.

Other viral vaccines

For a number of vaccines (Tab. 13 in Annex 2), the *Ph. Eur.* prescribes a serological test for potency testing: animals are immunised and the response to the vaccine is estimated with immunochemical methods. Efforts should be made to develop and validate *in vitro* methods (e.g. based on antigen quantification), which do not require animals.

4.2.2.4 Immunosera and antitoxins

Tetanus antiserum for veterinary use

With regard to *Tetanus Antiserum for Veterinary Use* (equine origin) see *Tetanus immunoglobulins and antisera for human use*.

Other clostridial antitoxins

The *Ph. Eur.* includes three monographs on clostridial antitoxins (*C. novyi* alpha antitoxin, *C. perfringens* beta antitoxin and *C. perfringens* epsilon antitoxin) for veterinary use. At the symposium on *Veterinary Clostridial Vaccines*, it was stated that "EDQM will enquire which sera are still manufactured and if appropriate reconsider the need for the present monographs" (Council of Europe, 1997b). In principle, serological assays (e.g. EIAs) being suitable for the potency testing of clostridial vaccines could be adapted for the testing of clostridial antitoxins (Ebert et al., 1999c).

Erysipelas immunoserum

Potency

The *Ph. Eur.* monograph *Swine Erysipelas Immunoserum* also stipulates a multiple-dilution vaccination-challenge test in mice for the estimation of potency. ELISA procedures have been developed, which can be used for *in vitro* estimation of protective erysipelas antibodies in immunosera (Dahms et al., 1991; Beckmann and Cussler, 1993; Cussler et al., 1995), but have not yet been validated.

The TAST is stipulated in this monograph, which is in contrast to the other monographs for immunosera. Retrospective analysis of TAST data released on the German market show no irregularities (Pössnecker and Cussler, 1998). Deletion of the TAST from these monographs is therefore recommended.

4.2.3 Humane endpoints

The potency testing of a number of human (whole-cell pertussis, diphtheria, tetanus, inactivated rabies) and veterinary vaccines (clostridial, leptospirosis, erysipelas) is based on a vaccination-challenge test, i.e. the capacity of the vaccine to protect against infection or against toxins is measured by challenging immunised animals with the relevant agent. Insufficiently protected animals develop signs of the disease or eventually die of the disease (up to 50%). Most of the monographs name death of the animal or severe clinical signs as endpoints, which usually involves considerable suffering and severe distress to the animals. Recently, a study has been carried out which aimed to evaluate and validate the use of humane endpoints as an alternative to severe clinical endpoints in potency tests on immunobiologicals and to promote the implementation and use of humane

endpoints in guidelines and in the animal laboratory setting.

The working group established for this study (Coenraad Hendriksen, RIVM, Bilthoven, The Netherlands; Klaus Cussler, PEI, Langen, Germany; David Morton, University of Birmingham, UK) decided to restrict the study to three vaccines: erysipelas, rabies, and pertussis vaccines, and set up a list of the parameters to be evaluated (body weight, body temperature and clinical signs, for example, ruffled fur, hunched back, changes in the movements, paresis, paralysis, prostration, agony) with animals monitored twice a day. The data obtained for pertussis showed that decrease in body temperature to 34.5°C and loss of muscular co-ordination are suitable humane endpoints, which could be used for the potency testing of whole pertussis vaccines. The duration of severe suffering of the animals could be shortened by 1-3 days (Hendriksen et al., 1999).

Rabies-infected mice did not show any increase or decrease in body temperature. Clinical signs and (loss of body weight) were more specific, and it was concluded that a 15% decrease of body weight and clinical signs of neurological disorder used as a combined humane endpoint would shorten the test period by 3 days (Cussler et al., 1998). Guidance on the use of humane endpoints in batch potency testing of rabies vaccines is available on video (HELP, 2000).

For the potency testing of erysipelas vaccines, it was not possible to identify early predictors of lethality in mice. However, to minimise pain and distress, it was recommended that terminally ill animals characterised by lethargy and hypothermia (<34°C) should be killed humanely.

The results of this study have been presented at the International Conference on *The use of Humane Endpoints in Animal Experiments for Biomedical Research* (22 - 25 November, 1998, Zeist, The Netherlands; Hendriksen and Morton, 1999) and at the *3rd World Congress on Alternatives and Animal Use in the Life Sciences* (29 August - 2 September, 1999, Bologna, Italy; Cussler et al., 2000b).

An example for the use of humane endpoints in vaccination-challenge studies in large animals was recently presented by Johannes et al. (1999) for the testing of swine erysipelas vaccine.

The general idea of the study, the application of humane endpoints, has been considered by the European Pharmacopoeia. The draft revision of the general monograph for veterinary vaccines now reads: Animal tests.

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 the light of this. For example, if it is indicated that an animal is considered to show positive, infected etc. when typical signs or death occur then as soon as sufficient indication of a positive result is obtained the animal in question shall be either humanely killed or given suitable treatment to prevent unnecessary suffering (Council of Europe, 2001).

5 Progress and criticism

5.1 Progress

5.1.1 General aspects

The Three Rs principle has become an important issue in the development and quality control of immunobiologicals. In recent years a number of workshops, seminars and conferences have been dedicated to general and special aspects of this issue. The Three Rs concept has been implemented into general guidelines/monographs. Thus, the general notices in the 3rd Edition of *Ph. Eur.* allow the replacement of any test method mentioned in a monograph: *The tests and assay 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* (Council of Europe, 1997a).

Already in 1995, the WHO has published the *Manual of Laboratory Methods for Potency Testing of Vaccines Used in the WHO Expanded Programme on Immunisation* (WHO, 1995), which includes *in vivo* and alternative methods for the potency testing of various human vaccines (e.g. ToBI test for tetanus vaccines, VERO cell test for diphtheria vaccines), which can be used provided that correlation can be demonstrated between antibody titres and protection.

5.1.2 Specific aspects

Abnormal toxicity test (ATT)

Based on the outcome of an enquiry of the *Ph. Eur.* Secretariat (Council of Europe, 1994) and the outcome of a study of the PEI (Duchow et al., 1995), the *Ph. Eur.* Commission decided to delete the ATT from all *Ph. Eur.* monographs for veterinary vaccines, veterinary and human immunosera and immunoglobulins and from most of the monographs for human vaccines. Due to the introduction of the principles of GMP and GLP, the relevance of the ATT had been questioned for a number of years. The ATT is still required by some *Ph. Eur.* monographs on human vaccines, however, it has been shifted upstream, i.e. it is no longer performed on the finished product but *in process*. The ATT can also be omitted for these vaccines, provided that the manufacturer could demonstrate that a sufficient number of batches gave negative results (Schwanig et al., 1997). After deletion of the ATT, there are now several *Ph. Eur.* monographs on human vaccines, which do not stipulate tests in animals for routine batch release (Tab. 10). The PEI estimates that these modifications of *Ph. Eur.* monographs will reduce the total number of animals needed in Germany for vaccine quality control by 10 - 20.000 mice and guinea pigs per year. Nevertheless, the FDA and WHO still stipulate the ATT; however, the WHO Expert Committee on Biological Standardization recently stated that the collection of global data on the value of the ATT would be initiated (WHO, 1999b).

Upstream testing

As already mentioned above, several tests, which used to be carried out on the

Tab. 10: Human vaccines, which do not require animal testing for batch release

Influenza vaccine (split virion)
Influenza vaccine (surface antigen)
Influenza vaccine (whole virion)
Measles vaccine
Mumps vaccine
Rubella vaccine
Typhoid polysaccharide vaccine
Typhoid vaccine (live), (oral, strain Ty 21a)
Varicella vaccine

finished product have been shifted upstream in the production process and are carried out on the bulk stage or even earlier. This principle has been introduced for safety testing, for example, ATT, residual toxicity testing of clostridial vaccines for veterinary use, toxicity testing of tetanus and diphtheria vaccines for human use, and for the potency testing of several human vaccines (for example, *Haemophilus influenza* b, Hepatitis A and B; see also Tab. 11 Annex 1).

The *Ph. Eur.* generally accepts the principle of upstream testing for residual live virus in inactivated vaccines for veterinary use. This normally allows the use of *in vitro* methods instead of tests (with questionable sensitivity) in animals.

Introduction of single-dilution tests

Many potency tests have been or are classical multi-dilution tests, which require large number of animals. In recent years, more and more guidelines/monographs allow the manufacturer to replace the multi-dilution test with single-dilution test (e.g. WHO for diphtheria and tetanus, *Ph. Eur.* diphtheria, tetanus, acellular pertussis, erysipelas).

The potency test for swine erysipelas vaccine had to be changed due to the detoriation of the International Standard. This included the introduction of a single-dilution assay, which reduced the number of animals used by more than 70%.

Replacement of animal tests with alternative methods

Introduction of *in vitro* methods (based on antigen quantification) to replace an animal based model (immunisation-challenge or serological model) has been achieved for several vaccines, e.g. hepatitis A and B, inactivated rabies vaccine for veterinary use. Replacement of the challenge procedure during potency testing of inactivated vaccines with *in vitro* methods (serum antibody titration on cell cultures, immunochemical methods, etc) looks promising for a number of vaccines; in particular, for tetanus vaccine for human and veterinary use (ToBI test and ELISA procedure) and erysipelas vaccines (ELISA procedure).

With regard to live poliomyelitis vaccine, the WHO has revised its recommendations for quality control and introduced the MAPREC test and a transgenic mouse model (TgPVR21 mice) for neurovirulence testing of poliovirus type 3 to replace the test in monkeys (Wood et al., 2000).

5.2 Criticism

Acceptance and implementation of alternative methods

The participants of the ECVAM Workshop 31 (Hendriksen et al., 1998) estimated that 9.5-11.5 years are needed from the development of a test to its implementation in monographs and guidelines. In particular, the period between successful validation and the implementation appears to be far too long (3.5 years). Reasons for this could be the slow process of multinational agreement to revise pharmacopoeial monographs and guidelines, and the time-consuming and expensive production of sufficient reference material (antigen, sera etc) for the new test systems. In the light of this, it is hardly understandable why the revised monograph on erysipelas vaccine and the currently revised monograph on human tetanus vaccine do not include a statement that validated alternative methods could be used for batch potency testing. By the time of the revision, validation of alternative methods for the potency testing of human tetanus vaccine and erysipelas vaccine were well on their way and *Ph. Eur.* had been kept informed on the progress. In the case of several clostridial vaccines, this approach of speeding up the implementation and promoting the use of alternative methods has been taken although none of the alternatives had been validated by that time.

There should be an (better) information system to raise the awareness for accepted alternatives and to ensure that the legal obligation of implementing them is followed. There is evidence that some OMCLs are rather reluctant in accepting the use of Three Rs methods or the deletion of animal tests (for example, one OMCL was still requiring the ATT although it had been deleted). If there is a need for training, then measures should be taken to offer this to OMCLs and manufacturers.

Variation process

Changes in the production of a vaccine and the quality control have to be licensed by the control authorities. If a product is not centrally licensed, the manufacturer has to apply for variation to each national control authority. This applies also to the introduction of an alternative method. There should be quick and effective mechanisms for manufacturers to reach a harmonised decision after initiating a variation to introduce an alternative for a product, which has not undergone a centralised procedure. Until now, this has not been achieved and it is most likely that a manufacturer would not receive a harmonised reply from the competent authorities (Communication of Dow in Council of Europe, 2000h; p. 281).

In addition, the EMEA and the national control authorities should consider the possibility of waiving or at least reducing the fees for variations, which are in accordance with the requirement of Directive 86/609/EEC and replace, reduce or refine animal tests.

Retesting of vaccine for batch release

OMCLs have the possibility to retest vaccine batches after the manufacturer has submitted the dossier for batch release. However, different policies are used in OMCLs in Europe: some repeat all tests, others do not retest. It is clear that retesting uses a substantial number of animals. Since the manufacturer has already tested these batches it is questionable whether this approach can still be justified in the age of GMP and quality assurance.

Monographs/guidelines for new immunobiologicals

Animal tests are still easily introduced into monographs without a rationale on necessity, animal numbers or distress – a justification could be given e.g. in the

preface, when monographs are published for comments in *Pharmeuropa*.

International harmonisation

Harmonisation at a European level will be or has already been overtaken by globalisation. Most of the manufacturers produce for the world market, so harmonisation of the requirements or mutual recognition of tests would help to reduce the use of animals. However, little progress has been achieved. For example, there is no common international approach for potency testing of diphtheria and tetanus vaccines, which are amongst the most used vaccines worldwide. Vaccine batches, which have already been tested according to the e.g. WHO requirements, have to be re-tested according to the e.g. *Ph. Eur.* requirements, due to the lack of a universally accepted potency test.

European industry reports that the additional testing in the EU for those manufacturers who bring in their products from offshore increases animal testing in such situations (communication of Vose in Council of Europe, 2000h, p. 270-272).

Another example is the ATT, which has been deleted in *Ph. Eur.* monographs (see above) but is still required by FDA, USDA and WHO. This problem has recently been highlighted at an international conference where representatives of the two organisations stressed the need to remove this test (Communication of Egan in Council of Europe, 2000h, p. 282; Communication of Griffith in Council of Europe, 2000h, p. 283; WHO, 1999b).

With regard to veterinary vaccines, different approaches are evident in replacing batch potency testing of inactivated vaccines. In Europe, the focus is on serological models, which replace the challenge procedure but still require animals whereas the USA rely on antigen quantification, which does not require animals (Hauer and Clough, 1999).

The ICH and the VICH should undertake efforts to harmonise requirements and promote mutual recognition.

Target animal safety test

As already outlined in the paragraph on the TAST, the relevance of this test has been discussed for years without any evident progress. There is hope that the new initiative of the VICH will be more successful.

6 Future aspects

6.1 Consistency of production

As mentioned at the beginning of this report, a new concept of quality control is already in place for the new well-defined vaccines. In most cases, non-animal methods are used for monitoring consistency at critical steps in the production and testing of a vaccine. These methods can already be developed during the development of the product. Therefore, manufacturers should be encouraged to develop such methods and control authorities should be encouraged to release batches, which have been tested with these methods.

Whether the concept of consistency of production could be also applied to the conventional, less-defined products, should be investigated. In recent years, not only has the knowledge on these conventional products increased but also highly sophisticated methods such as immunochemical, physico-chemical, *in vitro* functional methods became available which could be used for an accurate fingerprinting of a vaccine (Leenaars et al., 2001). A battery of *in vitro* tests could be used to monitor the stability, conformation and integrity of antigens and to define parameters, which correlate antigenicity and immunogenicity (Hendriksen and Gupta, 2000).

Until now, the concept of consistency of production is mainly applied to human vaccines but not for veterinary vaccines.

6.2 Antigen quantitation versus serological methods

In the introduction, it is stated that there are two main approaches to replace *in vivo* potency testing of inactivated vaccines: antigen quantitation and replacement of the challenge procedure with a *in vitro* serological method. The latter approach should be considered as an interim step, which has a high impact on the reduction of animal numbers, and even more important, the suffering due to infection or intoxication is avoided. The goal, however, is the complete replacement of the immunisation-challenge model for batch testing purposes.

6.3 Novel vaccine production technologies and new vaccines

In the last decade, a number of developments have taken place that might have an impact on the use of laboratory animals for the production and quality control of immunobiologicals. GMP and quality assurance systems have been established and the concept of consistency of production has been introduced. Conventional vaccines are more defined since the production technology has continuously been improved. Biotechnological methods became available which allow a better understanding of diseases and mechanism of immune response and thus the improvement of existing immunobiologicals and the development and production of new, well-defined products.

Regarding the vaccines listed in Annex 1 & 2, it is evident that the future has already started: there are several vaccines, so-called subunit (e.g. influenza, meningococcal, pneumococcal, HIB, *Escherichia coli*, FeLV vaccines) or synthetic (e.g. hepatitis B) vaccines, which are well-defined and released without using animals for batch potency testing. In contrast to attenuated live vaccines, whole cell bacterial vaccines or inactivated vaccines, subunit vaccines contain only fragments of the infectious agent; whereas synthetic vaccines are produced chemically or with recombinant methods (review article Liljeqvist and Ståhl, 1999). The so-called "Third Revolution on Vaccines" started about 10 years ago with the use of genetic immunisation or DNA vaccination. Instead of injecting the antigen into the target organism, bacterial plasmid DNA encoding bacterial, viral or parasitic protein antigens are inoculated by various routes (intramuscular, intradermal, intranasal, oral) and techniques (needle inoculation, gene gun, air gun, food uptake). Depending on the administration route, the DNA is taken up by e.g. muscle cells or skin Langerhans cells. This *in vivo* transfection of the target cells results in the expression of antigens. The organism recognises the antigen(s) and generates humoral (antibody)- and/or cell-mediated immune response. The correlates of immunity can be manipulated e.g. by the method of vaccine delivery, presence of genetic adjuvants or vaccine regimen (reviewed articles: Cichutek, 2000; Shedlock and Weiner, 2000).

DNA vaccines have been used to stimulate protective immunity against many infectious pathogens, malignancies and autoimmune disorders in animal models (review articles: Donnelly et al., 1997; Donnelly and Ulmer, 1999; Cichutek, 2000; Shedlock and Weiner, 2000; Heppell and Davis, 2000; Krishan, 2000; Liu and Ulmer, 2000) and entered into the first clinical trials about five years ago. There are ongoing clinical trials in B cell lymphoma, HIV, influenza, malaria, herpes simplex, hepatitis B, FIV, etc (more information see www.dnavaccine.com).

The ease of production, their stability and possibilities of transport are considered to be the main advantages of DNA vaccines over conventional vaccines. In addition, cultivation of dangerous infectious agents is no longer necessary and they provide the possibility to induce long-term immunity against one or multiple pathogens after a single shot.

Numerous articles on DNA vaccines indicate that large numbers of animals (rodents, cats, primates) are used for the development of such vaccines. Since new pathogens are emerging, or well-known pathogens re-emerging, even more animals might be used for the development of new vaccines.

Concerns about the safety and efficacy of DNA vaccines have been considered by EMEA, the WHO and FDA, all of whom issued guidelines on their production and quality control (FDA, 1996; EMEA, 1998; EMEA, 1999 (draft); WHO; 1998). According to the CVMP and CPMP, animals will still be needed during production in order to demonstrate safety and efficacy; however, fully validated *in vitro* expression assay would be considered sufficient for establishing batch potency; whereas the TAST is still required for batch safety testing of veterinary DNA vaccines.

There is agreement that for the time being animals will still be needed for the development of vaccines in order to gain best knowledge on the disease, the pathogen and the specific immune response, including: pathogenesis, identification of the protective antigens, the way the antigen is processed, the dynamics of the immune response, the induction of memory, and the selection of the best adjuvant (Hendriksen and Gupta, 2000).

With regard to routine batch release of conventional products, a number of Three Rs approaches are already available and should further be developed and validated. Whereas routine batch release of new products should be based on *in vitro* methods already established during their development.

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List of abbreviations

Organisations/Institutions/etc.

AGAATI	Advisory Group on Alternatives to Animal Testing in Immunobiologicals
BMBF	German Federal Ministry of Education and Research
CPMP	Committee for Proprietary Medicinal Products (EMEA)
CVMP	Committee for Veterinary Medicinal Products (EMEA)
DZ	Doerenkamp-Zbinden Foundation
EC	European Commission
ECBS	WHO Expert Committee on Biological Standardization
ECVAM	European Centre for the Validation of Alternative Methods, Ispra, I
EDQM	European Directorate for the Quality of Medicine, Strasbourg, F
EFPIA	European Federation of Pharmaceutical Industries' Associations
EMEA	European Medicines Evaluation Agency, London, UK
EPC	European Pharmacopoeia Commission
EU	European Union
FDA	Food and Drug Administration,
FFVFF	Fonds für Versuchstierfreie Forschung, Zürich, CH
IABS	International Association for Biologicals, Geneva, CH
ICH	International conference of Harmonisation
IWP	Immunological Veterinary Medicinal Working Party (EMEA)
IFMPA	International Federation of Pharmaceutical Manufacturers Association
In-VITRO	The International Veterinary Industry Test Replacement Organisation
JRC	Joint Research Centre (European Commission) Ispra, I
MS	Member States of the European Union
NIH	US National Institute of Health
O.I.E.	Offices internationales des épizooties, Paris, F
PEI	Paul-Ehrlich-Institut, Langen, D
Ph. Eur.	Pharmacopoea Europaea (European Pharmacopoeia), Strasbourg, F
RIVM	National Institute of Public Health and the Environment, Bilthoven, NL
set	Foundation for the Promotion of Research on Replacement and Complementary Methods to Reduce Animal Testing
USDA	United States Department of Agriculture,
VICH	Veterinary International Cooperation on Harmonisation
WHO	World Health Organization, Geneva, CH
ZON	Zorg Onderzoek Nederland,

Terms related to immunobiologicals/methods/production etc.

ACPV	Acellular pertussis vaccine
ATT	Abnormal toxicity test
BCG	Bacillus of Calmette and Guérin (Mycobacterium bovis BCG)
BVD	Bovine viral diarrhoea
BSP	Biological Standardisation Programme
CHO	Chinese hamster ovary cells
DPT	Diphtheria-Pertussis-Tetanus vaccine
ELISA	Enzyme linked immunosorbent assay
FeLV	Feline leucosis virus
FMD	Foot and mouth disease
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
HS	Histamine sensitisation test
IBR	Infectious bovine rhinotracheitis
IBRP	International Biological Reference Preparation
IL-1	Interleukin 1
IL-6	Interleukin 6
IPV	Inactivated poliovirus vaccine
LAL	*Limulus* amebocyte lysate
LP	Leukocytosis promotion test
LPS	Lipopolysaccharides
MAT	Micro-agglutination test
MAPREC	Molecular analysis by PCR and restriction enzyme cleavage
MDCK	Madin-Darby-Canine-Kidney cell line
MNVT	Monkey neurovirulence test
MWG	Mouse weight gain test
ND	Newcastle disease
OPV	Oral poliovirus vaccine
PCR	Polymerase chain reaction
RFFIT	Rapid fluorescent focus inhibition test
SOP	Standard operating procedure
TAST	Target animal safety test
TNF-α	Tumour necrosis factor α
TNT	Toxin neutralisation test
ToBI	Toxin binding inhibition test
WCPV	Whole cell pertussis vaccine

ANNEX 1

Tab. 11: Animal tests stipulated for the batch testing of human vaccines and immunosera (based on *Ph. Eur.* Monographs, Council of Europe, 2000g)

Immunobiological	Tests in Animals Carried out on the Final Bulk/Lot	Numbers/Species	Distress*	Comments
Bacterial vaccines				
BCG vaccine	Virulent mycobacteria Excessive dermal reactivity	6 guinea pigs 6 guinea pigs	1 4	May be omitted on the final lot May be omitted on the final lot
Cholera vaccine Cholera vaccine, freeze-dried	Antibody production Antibody production	6 rabbits or guinea pigs or mice 6 rabbits or guinea pigs or mice	1	
Diphtheria vaccine (and combined vaccines cont. diphtheria component)	Specific toxicity (only final lot) Absence of toxin Irreversibility of the toxoid in guinea pigs or cell cultures Potency (challenge)	5 guinea pigs/lot 5 guinea pigs/bulk guinea pigs guinea pigs, number not defined; 20 control animals might be required	1 1 1 4	Deletion foreseen *In vitro* should be preferred Validation of serological test with Vero cells foreseen
Haemophilus type B conjugate vaccine	Pyrogens Potency (serology; final bulk)	3 rabbits 16 mice	1 1	Table 5
Meningococcal polysaccharide vaccine	Pyrogens	3 rabbits	1	Table 5
Pertussis vaccine (and combined vaccines cont. pertussis component)	Specific toxicity Potency (challenge)	10 mice 136 mice	1 4	Table 2 Serological model available
Pertussis acellular vaccine	Absence of pertussis toxin Irreversibility of toxoid Potency (serology; multi-dilution)	5 mice 5 mice mice (6 groups of appropriate number)	1 1 1	
Pneumococcal poly-saccharide vaccine	Pyrogens	3 rabbits	1	Table 5
Tetanus vaccine	Specific toxicity (final lot) Absence of toxin Irreversibility of the toxoid Potency (challenge)	5 guinea pigs 5 guinea pigs mice or guinea pigs guinea pigs or mice; number not defined	1 1 1 4	Deletion foreseen Deletion as final bulk test forseen Deletion as final bulk test forseen Single-dilution serological test foreseen
Typhoid vaccine Typhoid vaccine, freeze-dried	Antigenic power (estimation of antibodies)	Susceptible laboratory animals	1	No longer relevant
Viral vaccines				
Hepatitis A vaccine (inactivated)	Potency (serology or antigen content)	Mice; number suitable to meet statistical requirements -	1	*In vitro* method recommended
Hepatitis B vaccine (rDNA)	Pyrogens Potency (serology or antigen content)	3 rabbits Mice or guinea pigs; number suitable to meet the requirements for a valid test -	1 1	*In vitro* method recommended
Poliomyelitis vaccine (inactivated)	Potency (serology and D-antigen content)	60 chickens/guinea pigs or rats -	1	Test in rats recommended in *Ph. Eur.* monograph
Poliomyelitis vaccine (oral)	Neurovirulence (monovalent harvest)	> 80 monkeys	5	Alternatives available for polivirus type 3; under investigation for type 1 and 2
Rabies vaccine for human use prepared in cell cultures	Pyrogens Potency (challenge)	3 rabbits 6 groups of mice of a suitable size to meet the requirements for a valid test	1 4	Table 5 Single-dilution assay allowed Table 3

Immunobiological	Tests in Animals Carried out on the Final Bulk/Lot	Numbers/Species	Distress*	Comments
Tick-borne encephalitis vaccine	Pyrogens Potency (challenge)	3 rabbits 6 groups of mice of a suitable size to meet the requirements for a valid test	1 4	Table 5
Yellow fever vaccine	Potency (determination of mouse LD50; challenge)	6 mice per dilution	4	
Immunosera/Antitoxin/Immunoglobulins				
Botulinum antitoxin	Potency (*in vivo* toxin neutralisation)	4 mice per dilution	4	Serological model available
Diphtheria antitoxin	Potency (intradermal toxin neutralisation)	2 guinea pigs or rabbits per dilution	4	Vero cell test
European viper venom antiserum	Potency (for each venom, *in vivo* toxin neutralisation)	6 mice per dilution	4	No alternatives available
Mixed gas-gangrene antitoxin	Potency (for each toxin, *in vivo* toxin neutralisation)	6 mice per dilution	4	Table 6
Human tetanus Ig	Potency (*in vivo* toxin neutralisation or serology)	6 mice per dilution	4	Serological methods (ToBI test) prevalidated
Tetanus antitoxin	Potency (*in vivo* toxin neutralisation)	6 mice per dilution	4	Serological methods (ToBI test) prevalidated
Human normal immunoglobulin	Pyrogens	3 rabbits	1	Table 5
Human normal immuno-globulin for intravenous use	Pyrogens	3 rabbits	1	Table 5
Tuberculins				
Tuberculin purified protein derivative for human use (and Old tuberculin)	Identification (combined with potency) Toxicity Sensitisation Potency	 2 guinea pigs 6 guinea pigs 6 guinea pigs	 1 4 1	 Deletion recommended Alternative available

* Distress categories: 1 – slight; 2 – moderate; 3 – severe, duration <1 day; 4 – severe, duration 1-7 days; 5 – severe, duration 7-30 days; 6 – severe, duration > 30 days

ANNEX 2

Animal tests stipulated for the batch testing of veterinary vaccines and immunosera (based on *Ph. Eur.* Monographs, Council of Europe, 2000g)

Tab. 12: Batch potency testing with immunisation-challenge model

Immunobiological	Tests in Animals	Numbers/Species	Distress*	Comments
Bacterial vaccines				
Anthrax spore vaccine	Safety Potency (challenge)	2 susceptible animals 13 guinea pigs (or rabbits) or 8 sheep	1 4	*In vitro* quantification of live anthrax spores
Brucellosis vaccine	Safety 50% persistence time	2 sheep 64 mice	1 2	
Clostridium botulinum vaccine	Safety Residual toxicity Potency (challenge)	2 susceptible animals 5 mice 30 mice	1 1 4	

Tab. 12: continued

Immunobiological	Tests in Animals	Numbers/Species	Distress*	Comments
Clostridium chauvoei vaccine	Safety Potency (challenge)	2 susceptible animals 15 guinea pigs	1 4	 Table 6
Clostridium novyi (type B) vaccine	Safety Residual toxicity Potency (*in vivo* neutralisation or serology)	2 sheep 5 mice 10 rabbits at least 2 animals/dilution at least one repetition 10 rabbits	1 1 4 1	 May be omitted by manufacturer Table 6 Validated immunochemical methods are allowed for routine batch control
Clostridium perfringens vaccine	Safety Residual toxicity Potency (*in vivo* neutralisation or serology)	2 susceptible animals 5 mice 10 rabbits at least 2 animals/dilution/toxin at least one repetition 10 rabbits	1 1 4 1	 May be omitted by manufacturer Table 6 Validated immunochemical methods are allowed for routine batch control
Clostridium septicum vaccine	Safety Residual toxicity Potency (*in vivo* neutralisation or serology)	2 susceptible animals 5 mice 10 rabbits at least 2 animals/dilution/toxin at least one repetition 10 rabbits	1 1 4 1	 May be omitted by manufacturer Table 6 Validated immunochemical methods are allowed for routine batch control
Furunculosis vaccine	Safety Potency (challenge or serology)	10 fish 60 fish 35 fish	1 4 1	 Reduction of animal numbers should be investigated
Leptospira vaccine	Safety Potency (challenge)	2 target animals 10 hamsters	1 4	 Table 8
Swine erysipelas vaccine	Safety Potency (challenge)	2 piglets 30 mice	1 4	 Validated alternative available
Tetanus vaccine	Toxicity Potency (vaccination and *in vivo* neutralisation)	5 guinea pigs at least 2 mice per dilution at least one repetition	1 4	 Replacement of animal test with ELISA or ToBI is expected soon
Vibriosis (cold-water) vaccine	Safety Potency (challenge or serology)	10 fish 60 fish 35 fish	1 4 1	Reduction of animal numbers should be investigated
Vibriosis vaccine	Safety Potency (challenge or serology)	10 fish 60 fish 35 fish	1 4 1	Reduction of animal numbers should be investigated
Viral vaccines				
Aujeszky's disease vaccine (inactivated)	Safety Potency (challenge or suitable validated method)	3 piglets 10 pigs	1 4	
Avian infectious bronchitis vaccine (inactivated)	Safety Extraneous agents Potency (suitable validated method)	} combined; 10 chickens	1	Table 9
Avian infectious bronchitis vaccine (live)	Safety Extraneous agents	10 chickens 10 chickens	1 1	Table 9

Immunobiological	Tests in Animals	Numbers/Species	Distress*	Comments
Avian infectious bursal disease vaccine (inactivated)	Safety Extraneous agents Inactivation (only for certain strains) Potency (serology)	}combined; 10 chickens 10 chickens 20 chickens	1 1 1 1	Table 9
Avian infectious encephalomyelitis vaccine (live)	Safety Extraneous agents	10 chickens 10 chickens	1 1	Table 9
Avian infectious laryngotracheitis vaccine (live)	Safety Extraneous agents	10 chickens 10 chickens	1 1	Table 9
Avian paramyxovirus 3 (inactivated)	Safety Extraneous agents Potency (suitable validated method)	10 turkeys 10 chickens	1 1	Table 9
Duck viral hepatitis vaccine	Safety Extraneous agents	10 ducklings 10 ducklings	1 1	Table 9
Foot-and-mouth disease (ruminants) vaccine (inactivated)	Safety Potency (challenge)	3 cattle 17 cattle	1 3	
Fowl-pox vaccine (live)	Safety Extraneous agents	10 chickens 10 chickens	1 1	
Newcastle disease vaccine (inactivated)	Safety Extraneous agents Potency (serology or challenge)	10 chickens (or 10 other birds) 10 chickens (or 10 other birds) 20 chickens or 70 chickens (or 30 other birds)	1 1 1 4	Table 9
Newcastle disease vaccine (live)	Safety Extraneous agents	10 chickens 10 chickens	1 1	Table 9
Rabies vaccine (inactivated) for veterinary use	Safety Inactivation (adjuvanted vaccine) Potency (serology)	2 dogs 10 mice 5 mice	1 1 1	
Swine fever vaccine (live), classical	Safety Extraneous agents Potency (challenge)	3 piglets 10 mice 12 piglets	1 1 4	
Immunosera/Antitoxins				
Clostridium novyi alpha antitoxin	Potency (*in vivo* neutralisation)	At least two mice per dilution step; At least one repetition	4	
Clostridium perfringens beta antitoxin	Potency (*in vivo* neutralisation)	At least two mice per dilution step; At least one repetition	4	
Clostridium perfringens epsilon antitoxin	Potency (*in vivo* neutralisation)	At least two mice per dilution	4	
Swine erysipelas immunoserum	Safety Potency (challenge)	2 pigs 70 mice	1 4	
Tetanus antitoxin for veterinary use	Potency (*in vivo* neutralisation)	At least two mice per dilution step; At least one repetition	4	

* Distress categories: 1 – slight; 2 – moderate; 3 – severe, duration <1 day; 4 – severe, duration 1-7 days; 5 – severe, duration 7-30 days; 6 – severe, duration > 30 days

Tab. 13: Batch potency testing is based on serological model

Immunobiological	Batch tests involving animals	Number of Animals/Species	Distress*
Neonatal piglet colibacillosis vaccine (inactivated)	Safety Potency (serology)	2 piglets 7 pigs or 7 rabbits, guinea pigs or mice	1 1 1
Neonatal ruminant colibacillosis vaccine (inactivated)	Safety Potency (serology)	2 calves 7 rabbits, guinea pigs or mice	1 1
Porcine actinobacillosis vaccine	Safety Potency (serology)	2 pigs 5 mice	1 1
Porcine progressive atrophic rhinitis vaccine	Safety Potency (serology)	2 pigs 7 pigs or 7 laboratory animals	1
Canine parvovirus vaccine (inactivated)	Safety Potency (serology)	2 dogs 2 dogs or 5 guinea pigs	1 1
Egg drop syndrome '76 vaccine (inactivated)	Safety Potency (serology)	10 chickens 10 chickens	1 1
Equine influenza vaccine (inactivated)	Safety Potency (serology)	2 horses 5 guinea pigs	1
Feline calicivirosis vaccine (inactivated)	Safety Potency (serology)	2 cats 15 mice	1
Feline infectious enteritis vaccine (inactivated)	Safety Potency (serology)	2 cats 2 or 4 cats or guinea pigs for routine control	1 1
Feline viral rhinotracheitis vaccine (inactivated)	Safety Potency (serology)	2 cats 15 mice	1 1
Porcine influenza vaccine (inactivated)	Safety Potency (serology)	2 pigs 5 guinea pigs	1
Porcine parvovirus vaccine (inactivated)	Safety Potency (serology)	2 pigs 5 guinea pigs	1

* Distress categories: 1 – slight; 2 – moderate; 3 – severe, duration <1 day; 4 – severe, duration 1-7 days; 5 – severe, duration 7-30 days; 6 – severe, duration > 30 days

Tab. 14: The only routine batch tests involving animals is the target animal safety test

Immunobiological	Batch tests involving animals	Numbers of Animals/Species	Distress*
Aujeszky's disease vaccine (live)	Safety	3 piglets	1
Bovine parainfluenza virus vaccine (live)	Safety	2 calves	1
Bovine respiratory syncytical virus vaccine (live)	Safety	2 calves	1
Canine adenovirus vaccine (inactivated)	Safety	2 dogs	1
Canine contagious hepatitis vaccine (live)	Safety	2 dogs	1
Canine distemper vaccine (live)	Safety	2 dogs	1
Canine parvovirus vaccine (live)	Safety	2 dogs	1
Distemper vaccine (live) for mustelids	Safety	2 ferrets	1
Feline calicivirosis vaccine (live)	Safety	2 cats	1
Feline infectious enteritis vaccine (live)	Safety	2 cats	1
Feline leukaemia vaccine (inactivated)	Safety	2 cats	1
Feline viral rhinotracheitis vaccine (live)	Safety	2 cats	1
Infectious bovine rhinotracheitis vaccine (live)	Safety	2 calves	1

* Distress categories: 1 – slight; 2 – moderate; 3 – severe, duration <1 day; 4 – severe, duration 1-7 days; 5 – severe, duration 7-30 days; 6 – severe, duration > 30 days