

# Refinement, Reduction, and Replacement of Animal Use for Regulatory Testing: Current Best Scientific Practices for the Evaluation of Safety and Potency of Biologicals

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## Abstract

Biologicals, such as vaccines, require batch-related quality control to ensure their safety and potency. Part of quality control is based on animal models; consequently, the use of laboratory animals is extensive. In this paper, the characteristics of animal testing and its regulatory framework are described. Current trends in the development and implementation of alternatives to animal use are discussed. These trends include *in vitro* antigen quantification tests, serological approaches, the use of humane endpoints, and application of the principles of good laboratory animal practice. Emphasis is also given to a change of regulatory policies on licensing authorization. It is concluded that 3Rs' progress is frustratingly slow and that a real breakthrough in terms of animal reduction will be possible only after adoption of the "consistency approach," a new concept of vaccine quality control. This consistency approach has become state-of-the-art for the new generations of vaccines. Full implementation of the rules of good manufacturing practice and quality assurance would also allow the application of a consistency approach to quality control of conventionally produced vaccines.

**Key Words:** 3Rs; best practice; consistency; laboratory animals; potency; safety; vaccine

Biologicals, such as vaccines, may be roughly defined as products that are derived from or produced by a living organism in a batch-wise procedure. This definition implies that their characteristics can vary from batch to batch. Consequently, extensive batch-related quality control is required to ensure that these products are both safe and potent. The production and particularly the quality control of biologicals are closely intertwined with laboratory animal use. A substantial number of animals are needed for the category of vaccines.

This discussion provides an overview of current best scientific practices in the field of vaccine quality control. Characteristics of animal testing and their regulatory framework are presented. As explained, these characteristics ne-

cessitate the 3Rs' approach. Fortunately, this need is now also recognized at the regulatory level, and increased efforts are devoted to the development and acceptance of alternatives to animal testing. The current trends in 3Rs research are illustrated by the recent successes described below; however, a real breakthrough in terms of 3Rs depends on the acceptance of a new strategy in vaccine quality control, the consistency approach. This approach is based on the principle that the extent of vaccine batch release testing should reflect the level of consistency in production obtained with that vaccine.

## Biological Products

Biologicals include a wide variety of products, such as hormones, immunoglobulins, blood products and vaccines. Their beneficial role in preventive and curative health care programs is indisputable. The worldwide immunization campaigns against a number of infectious diseases (e.g., tetanus, diphtheria, and polio) have led to substantial decreases in mortality and morbidity rates and, in the case of polio, to almost a complete eradication.

Most biologicals are complex mixtures, which might differ from batch to batch. This can be best illustrated by looking at the vaccines. The conventionally produced vaccines are purified and detoxified components of micro-organisms (e.g., tetanus vaccine) and inactivated or attenuated whole micro-organisms (e.g., the whole cell pertussis vaccine and the polio vaccine). Furthermore, vaccine antigens are often combined to reduce the number of injections. It is well known that some vaccine antigens, such as the whole cell pertussis, enhance the potency of other vaccine antigens. Finally, most vaccines include a number of substances, such as an adjuvant and a preservative, which may or may not affect the quality of the product. This unknown quality makes each batch of vaccine a unique product, and strict controls must be in place to ensure the safety and potency of each batch. A major part of the tests being used for quality control are based on animal models, and consequently the use of laboratory animals is extensive, particularly for vaccine quality control.

## Vaccine Quality Control

### Characteristics of the Regulatory Framework

Three levels of responsibilities exist within the regulatory framework of vaccine quality control: the (inter)national

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regulatory (or competent) authority, the vaccine manufacturer, and the control authority. The regulatory authority approves procedures to ensure that vaccines intended for use are of adequate safety and potency. The vaccine manufacturer is responsible for demonstrating that the vaccine batch produced meets the requirements, based on the test specifications given by the competent authority. Finally, the regulatory authority is responsible both for the official vaccine batch release process, based on the data and information provided by the manufacturer and, eventually, for confirmatory testing. The leading regulatory bodies are the following: in the United States, the Food and Drug Administration and the US Department of Agriculture; in the Member States of the Council of Europe, the European Pharmacopoeia; and (although not legally binding) the World Health Organization (WHO<sup>1</sup>) in the developing countries. The US Food and Drug Administration and the Department of Agriculture also act as the control authorities in the United States, whereas the respective Control Authority Official Medicines Control Laboratory in each of the member states is responsible for batch release in Europe.

Test guidelines established by the international and national regulatory authorities are product specific. They might differ substantially between the regulatory bodies, as can be seen in Table 1. As a consequence, manufacturers must perform quality control according to all relevant test specifications when exporting a vaccine batch to various countries. The International Conference on Harmonization has been established to promote harmonization of guidelines; however, progress is very slow, and the effect on the use of animals is expected to be limited in the near future. Another approach, which requires less time and is more effective, is the mutual recognition of test guidelines. Unfortunately, there is no political support for such an approach at the time of this writing.

Another issue that merits discussion in the context of the

<sup>1</sup>Abbreviations used in this presentation: ELISA, enzyme-linked immunosorbent assay; NIH, National Institutes of Health; WHO, World Health Organization.

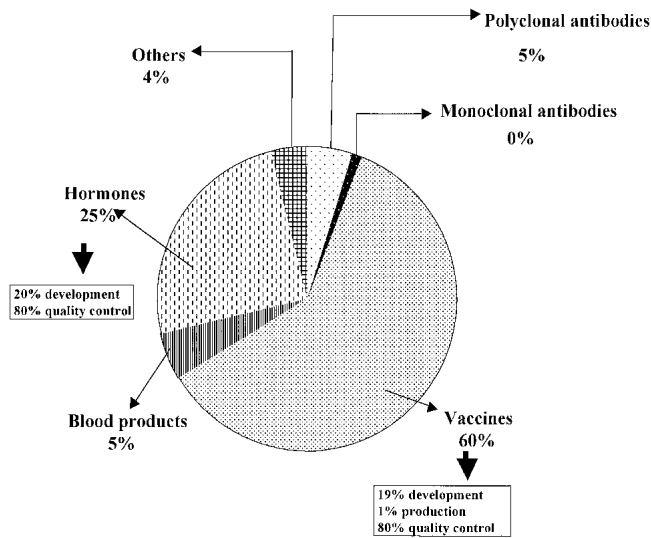
3Rs is the official retesting policy of regulatory authorities. Generally, vaccine batches are retested for potency and/or safety by the Official Medicines Control Laboratory to confirm manufacturer's data based on specific characteristics that make each batch unique. This retesting might make sense in cases in which there is no assurance regarding consistency in production or when the data submitted show invalidity. However, most vaccines are produced in a consistent way, and no additional information is obtained by retesting. Savings in the number of animals will be enormous if regulatory authorities ground their licensing authorization on site visits and data monitoring rather than on performing animal confirmation tests.

### Characteristics of the Use of Laboratory Animals

There is a traditional link between laboratory animals and biologicals (vaccines). As far back as the end of the 19th century, vaccine research provided a major impetus for the development of laboratory animal models. Some of the animal models currently used in routine quality control, such as the toxin neutralization test, are in fact slight modifications of the tests developed by Emile von Behring or Paul Ehrlich. The close association between laboratory animals and biologicals still exists and is reflected in the extent of animal use. In the Netherlands, for example, it accounts for about 15% of the total number of laboratory animals used annually (Keuringsdienst 1999), and although data are not available for other countries, it may be assumed that animal usage for this purpose is also extensive elsewhere. Figure 1 provides a rough breakdown in percentages of animals used for the various categories of biological products. As can be seen, most animals are required for the category of vaccines, which accounts for 60% (of which 80% is quality control). In terms of animal usage, it is noteworthy that 60% of all vaccines are produced in Third World Countries. In particular, these countries face major problems with regard to animal testing due to the high costs and difficulties of test standardization.

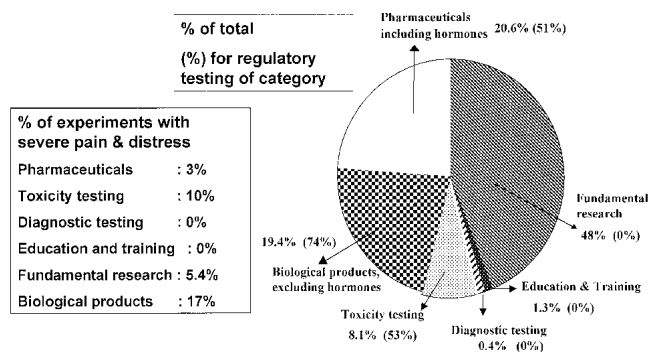
**Table 1 Specifications of existing guidelines for potency testing of tetanus vaccine for human use**

Specification	European Pharmacopoeia	US Food and Drug Administration
Animal species	Guinea pig/mouse	Guinea pig
No. of animal groups	3	1
No. of animals/group	Not specified, but generally 16	4
Toxin challenge	Yes	No
Bleeding	No	Yes
Criterion	Death/paralysis after challenge	Titration of pooled serum sample in 6 mice
Use of reference preparation	Yes	No



**Figure 1** Estimates for the use of animals for the production and quality control of biologicals in the Netherlands. Data from the Dutch statistics on the use of laboratory animals in 1999 (information in the public domain of the Netherlands by inspection of the Ministry of Public Health, Welfare, and Sports).

Apart from the large number of animals involved, the level of pain and distress inflicted on the animals also is high for the category of biologicals. As shown in Figure 2, the total number of animals (including mice and rats) used in the Netherlands in 1999, as well as the percentage of animals for each category of biomedical research and testing that suffer from severe pain and distress, comprise almost 17% for the category of biological products. The reason for this high level is the fact that vaccine quality control includes a number of animal models that use lethality or severe clinical signs as the endpoint. Vaccine quality control broadly consists of two types of tests involving animals: the safety test and the potency, or efficacy, test. Safety denotes freedom from extraneous agents, absence of toxicity, and, in the case of live attenuated vaccines, absence of residual virulence. Generally, these tests induce only minor



**Figure 2** Use of laboratory animals in the Netherlands: regulatory testing, purposes, and level of pain and distress (n = 720,000).

suffering inasmuch as most batches are free from adverse effects. However, in a few tests, positive control groups are used, as in the case of the histamine sensitization test for the safety testing of acellular pertussis vaccine.

The aim of the potency test is to ensure that the vaccine induces protective immunity after its administration. Although few examples exist, the potency of live vaccines is determined by germ count or virus titration, that is, entirely in vitro. However, potency testing of inactivated vaccines is often based on a direct or indirect protection test in animals. Examples are immunization challenge procedures and toxin neutralization procedures. Within the entire process of quality control, most experimental animals are required for potency testing, and these tests in particular cause severe distress to the animals involved.

Indeed, the percentage of animals used for regulatory purposes is high, as shown in Figure 2. It should be noted that although experts in vaccine quality control usually draft protocols for statutory required tests, these experts often lack a laboratory animal science background. As a consequence, procedures might be specified that do not meet the requirements of best laboratory animal practice. Some examples from the European Pharmacopoeia monographs are given in Table 2. Why is it, one might wonder, that non-regulatory test protocols submitted for approval can be modified by an animal care and use committee, whereas this is not the case for protocols in regulatory testing? We should at least recommend the involvement of laboratory animal science experts in drafting new in vivo test requirements.

**Table 2** Examples of questionable techniques in laboratory animals

Technique specified	European Pharmacopoeia Specified volume (mL)	GLAP <sup>a,b</sup> Maximum provided volume (mL)
Subcutaneous injection of guinea pigs for specific toxicity; test diphtheria and tetanus vaccine	5	1–2
Intramuscular injection of piglets for safety testing	2	0.2
Intravenous injection of mice for histamine sensitization test acellular pertussis vaccine	0.5	0.2

<sup>a</sup>GLAP, good laboratory animal practice.

<sup>b</sup>Van Zutphen LFM, Baumans V, Beynen AC, eds. 2001. Principles of Laboratory Animal Science. Rev ed. Amsterdam: Elsevier.

## General Trends in Best Scientific Practices

Best scientific practice means that procedures using laboratory animals are in line with best laboratory animal practice. As stated above, this consistency does not always result. Nevertheless, it should be acknowledged that much effort is currently devoted to the development of 3Rs' methods in the quality control of vaccines, including that of regulatory authorities. The reasons for this increased interest are diverse and only partly driven by animal welfare considerations. Economical factors, safety aspects, and scientific relevance are equally important motives. Interest might also be driven by existing legislation regarding the use of laboratory animals. Thus, the European Convention on the Protection of Animals for Experimental and Scientific Purposes and the corresponding European Union Directive have played an important role in stimulating action in Europe.

An in-depth and complete overview of 3Rs achievements and activities in the field of vaccine quality control is beyond the scope of this discussion. Interested readers are referred to Weisser and Hechler's (1997) excellent detailed overview. The discussion below addresses general trends.

### *Vaccine Potency Testing*

It might be clear that potency testing has been a priority in 3Rs research. Several approaches are being studied. In terms of animal reduction, progress in the replacement of in vivo potency testing by in vitro methods has produced the best, albeit most difficult, result. These studies have focused on vaccine antigen quantification by the use of an enzyme-linked immunosorbent assay (ELISA<sup>1</sup>) procedure, based on specific monoclonal antibodies. In vitro antigen quantification has the scientific and psychological barrier that it is only a measure of antigen quantity, and not necessarily of biological activity. In addition, other factors that influence immunogenicity, such as adjuvant activity, are not determined. Table 3 provides a summary of some of the products

**Table 3 Summary of 3Rs<sup>a</sup> methods as an alternative to the challenge/toxin neutralization potency test in vaccine quality control**

3R Alternative	Vaccine product
In vitro alternatives: antigen quantification test (ELISA) <sup>a</sup>	Rabies vaccine, hepatitis B vaccine, leptospirosis vaccine
Serological alternatives	Tetanus vaccine, diphtheria vaccine, various clostridial vacines, erysipelas vaccine, leptospirosis vaccine
Humane endpoints	Rabies vaccine, whole cell pertussis vaccine

<sup>a</sup>3Rs, reduction, refinement, and replacement of animals used in research and testing; ELISA, enzyme-linked immunosorbent assay.

for which the in vitro approach has been studied. Until now, in vitro tests have been accepted for only a few products, such as rabies vaccine and hepatitis B vaccine. In the case of rabies vaccine, the European Pharmacopoeia specifies the in vitro ELISA test as an alternative to the official National Institutes of Health (NIH<sup>1</sup>) lethal challenge test in mice, on the condition of demonstrated validity. Current practice, however, is that manufacturers continue to perform the NIH test for at least two reasons: (1) statistically sound validation data are difficult to obtain due to the high intrinsic variance of NIH test; and (2) lack of harmonization between the regulatory bodies—acceptance of in vitro data by one regulatory body does not necessarily mean acceptance by another regulatory body.

### *Serological Testing*

Another approach that still relies on the use of animals is the replacement of the challenge procedure by serology. Animals are immunized and, after a specified number of weeks, are bled. Protective antibody responses are estimated by an in vitro method, such as the toxin neutralization in cultures of vero cells (in the cases of diphtheria vaccine) or in ELISA or the Toxin Binding Inhibition test (in the case of tetanus vaccine). Although many in vitro serological methods have been developed (Table 3), few have been validated in a collaborative study. One of the exceptions is the validation of the ELISA and toxin binding inhibition test for potency testing of tetanus vaccines for veterinary use (Hendriksen et al. 1994) and human use (Winsnes and Hendriksen 2000), which the European Centre for the Validation of Alternative Methods, the European Union, and the European Pharmacopoeia have commissioned. A number of advantages are associated with in vitro serological methods (Table 4). Unfortunately, in vitro serological methods are not feasible alternatives in vaccine-induced protection cases, based on both humoral (antibody) and cellular responses.

In cases in which no alternative approaches for challenge-based potency tests exist, humane endpoints should be implemented. Although most requirements that include a challenge procedure still specify lethality or severe clinical

**Table 4 Advantages of in vitro serological test methods**

- No challenge or toxin neutralization procedure (less pain and distress)
- Quantitative data (antibody titre) versus qualitative data (death/survival in case of challenge)
- Storage of serum samples (good manufacturing practice)
- Monitoring for consistency is easier than in case of challenge test
- Exchange of serum samples
- Combined potency testing in case of combined vaccines

signs as the endpoint, regulatory bodies are now willing to accept humane endpoints on the condition of proven validity; the humane endpoints should correctly predict eminent death. The studies on humane endpoints in vaccine potency testing that have been published (Cussler et al., 1999; Hendriksen and Steen, 2000) have evaluated the use of clinical signs or pathophysiological processes (e.g., decreased body temperature or body weight) as an alternative endpoint in these tests. Although animals still suffer, it was concluded that using test-specific humane endpoints could reduce the pain and distress of individual animals by 1 to 3 days.

### *Vaccine Safety Testing*

3Rs' activities have also taken place in vaccine safety testing. A few developments are cited below as examples.

The European Pharmacopoeia has deleted the abnormal toxicity test for the quality control of human vaccines if consistency in production is demonstrated and vaccines are produced according to the principles of good manufacturing practice and quality assurance. A proposal to delete the safety test for the quality control of veterinary vaccines is now under discussion. A polymerase chain reaction method (the MAPREC test) and the use of a transgenic mouse model are now being validated by WHO to replace the neurovirulence test in monkeys for the safety testing of oral polio vaccine. This test is probably the world's largest consumer of nonhuman primates in biomedical research.

Although there is a strong commitment toward alternatives both at the manufacturing and regulatory levels, 3Rs' progress is frustratingly slow. Many obstacles, as in other fields of regulatory testing, stand between dream and reality; and it is not always easy to bridge this gap. More validation studies and financial resources are needed for such studies, and these studies should be realistic and less bureaucratic to reduce the time between development and acceptance of an alternative model, which is now more than 10 yr.

### **Consistency Approach**

There is a need for more realism with regard to the concept of quality control. Because the current concept is based on the uniqueness of each batch of vaccine produced, each vaccine batch must be tested extensively. In this respect, vaccines differ from drugs or chemicals, which are tested only once for registration purposes. Since the 1970s, a number of developments in vaccine production have taken place that might ultimately influence the quality control of conventionally produced vaccines. The key issue of "consistency" has emerged with the new generation of vaccines (e.g., glycoconjugate vaccines, rDNA products, or synthetic vaccines). These vaccines, which are based on new technologies, are produced in a consistent way; and the stress of quality control is on in-process monitoring rather than on

final batch testing. In-process control is almost exclusively based on in vitro biochemical and physicochemical tests.

The consistency concept has become state-of-the-art for the new generations of vaccines. However, also in the field of conventionally produced vaccines, continued advances in production technology (e.g., optimization of culture conditions and improvement of purification procedures) have resulted in more defined and thus less variable products. In addition, vaccine production currently applies to the strict rules of good manufacturing practice and is monitored by a system of quality assurance. Therefore, some people argue that for a conventionally produced vaccine, the extent of batch release testing should reflect the level of consistency in production and testing obtained with that vaccine. Thus, a vaccine manufacturer should perform extensive testing, including animal tests, on the first few batches of a new product to characterize the vaccine thoroughly. However, if consistency in production is demonstrated, then testing should rely on a battery of easy-to-use in vitro assays, used in process and on the final batch to characterize (fingerprint) the vaccine and to confirm consistency. This in vitro approach might include physicochemical/biochemical techniques (e.g., electrophoresis, chromatography, and spectroscopy), immunochemical techniques (e.g., ELISA), using monoclonal antibodies or biosensor analysis and in vitro functional bioassays (e.g., cytokine induction assays or B/T cell proliferation assays) (Leenaars et al. 2001). By applying this new approach, the number of animals currently used for quality control of conventional vaccines could be reduced to an absolute minimum.

### **Summary**

Biologicals and conventionally produced vaccines, in particular, are the subjects of great animal welfare concern, both with regard to the number of animals being used and to the pain and distress caused. Vaccine manufacturers, regulatory authorities, and society as a whole therefore have the obligation to support the development and implementation of 3Rs alternatives, in accordance with the recommendations of Russell and Burch (1959). With 60% of all vaccines being produced (and tested) in Third World countries, transfer of 3Rs in technology and animal husbandry should be an essential aspect of this support. In this respect, organizations like the International Council for Laboratory Animal Sciences and WHO should take the lead by, for example, organizing training courses and making materials available.

Several recommendations for implementation of 3Rs methods are provided above. It should be stressed that these recommendations include not only bench-related activities but also, and perhaps even more important, the following activities at the regulatory level: full implementation of good laboratory animal science principles in test guidelines, the harmonization of test guidelines, and a critical analysis of the retesting policy. The investment in time and human resources might be minimal compared with the develop-

ment and validation of 3Rs methods, and the effect might be many-fold. However, the real breakthrough in terms of animal reduction will be possible only after adoption of the consistency approach. This quality control approach makes us less dependent on the use of animals and therefore paves the way for a complete replacement.

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