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Replacement, reduction and refinement of the use of animals in the quality control of vaccines

OPENING REMARKS

Prof. Dr C. Hendriksen, RIVM, (NL)

The conference "Replacement, reduction and refinement of the use of animals in the quality control of vaccines" held at the EDQM, November 7-8, 2002, is the first of a series of VACTRAIN activities. VACTRAIN is a project sponsored by the European Commission (5th Framework Programme, accompanying measures). Project participants are the National Institute for Public Health and the Environment (RIVM, NL), Paul-Ehrlich-Institut (PEI, D) and the Institute of Virology and Immunoprophylaxis (IVI, CH).

The goals of the project are as follows:

- to organise and to hold workshop/training courses on the theoretical background and practical training of Three Rs methods for the quality control of vaccines;
- to discuss new approaches and concepts for the quality control of vaccines;
- to stimulate the use of Three Rs methods;
- to produce manuals on Three Rs methods;
- to produce a final report.

The project is open to all relevant parties within the European Union, and the candidate countries, but particularly to those coming from vaccine quality control laboratories. The conference at EDQM can be seen as the kick-off of the VACTRAIN activities, followed by 5 training courses on 3Rs methods in vaccine quality control. These training courses will provide an opportunity for technology transfer on new approaches in vaccine quality control that aim to replace, reduce and/or refine the use of laboratory animals. Each training course will include theoretical background information, presented by recognised experts, as well as hands-on training. Additionally, a platform will be offered for networking and exchange of information between participants and regulatory authorities. Each training course has a length of 3-4 days, with a maximum of about 10 participants per course.

The following training courses will be given:

- Training Course 1 - *Avian Vaccines -Extraneous Agents Testing with PCR* .Venue: IVI (CH).
- Training course 2 - *Bacterial Vaccines for Veterinary Use*. Venue: PEI (D).
- Training Course 3 - *DPT Vaccines I*. Venue: RIVM (NL).
- Training Course 4 - *Rabies Vaccines for Human and Veterinary Use*. Venue: IVI (CH).
- Training Course 5 - *DPT Vaccines II*. Venue: RIVM (NL).

We would like to invite those who are interested to participate in these training courses to apply. We particularly focus on participants from industry, regulatory authorities and academia. The fee will be low and we will be able to financially support a limited number of participants coming from the candidate EU countries.

Additional information on the VACTRAIN project can be found on home page: www.vaccinetraining.com or can be obtained from one of the co-ordinators of the project:

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OPENING REMARKS

Dr A. Artiges, (EDQM), Council of Europe

It is my pleasure to open this international symposium that the EDQM has co-organised with the RIVM and the Paul-Ehrlich-Institut with the support of European Union funding for the implementation of the 3Rs in the use of animals in the quality control of vaccines.

As you know, the Council of Europe with its convention for the protection of vertebrate animals used for scientific purposes and the European Pharmacopoeia's Commission have developed a strong policy for promoting animal welfare.

Since the adoption of the European Convention on animal protection the European Pharmacopoeia Commission has conducted a programme of work over the last decade directed at application of the 3Rs: replacement, reduction and refinement of the use of animals in the specifications of its monographs.

The first state of the work can be seen as phasing out animal tests which had become superfluous in current production conditions (for example, deletion of the abnormal toxicity test for vaccines and sera) and replacement of animal tests by *in vitro* methods (for example, replacement of bioassays of hormones by physicochemical assays). Where deletion or replacement of tests is not possible, the scope for reduction of the number of animals used and the possibilities of refining the tests in ways that cause less suffering have to be considered.

All these changes require extensive validation work and collaborative trials in a number of control laboratories to ensure that the quality standards are maintained with the alternative methods. From 1992 onwards, an extensive programme of biological standardisation has been carried out by the EDQM and is still continuing. For the progress made in pharmacopoeial standards to have its full effect, co-operation with the other interested parties (on one hand, licensing and control authorities) and on the other hand, animal welfare organisations, manufacturers) is essential. This should be seen by all concerned as an areas for future efforts.

This symposium which brought together participants of 16 countries from all over Europe, USA and Canada should be the occasion of extensive exchanges of view points on past experiences and future orientations.

As you know the aim of the VACTRAIN project is to provide first hand education and training methods. In this 1st opening conference, prior to the training courses, we have especially foreseen 8 workshops where participants can have a lively debate on specific and define action plan for continuing improvements on varied aspects.

I hope that you will have a fruitful meeting and am happy to give the floor for more details to Dr Klaus Cussler from the Paul-Ehrlich-Institut, one of our co-organisers. Dr Hendriksen from the RIVM and our partner on this project is not able to attend this meeting and we wish him a rapid recovery.

SESSION I: CURRENT REGULATORY AND SCIENTIFIC BACKGROUND

Introduction to the Three Rs concept: Replacement, Reduction, Refinement

Prof. Dr C. Hendriksen, RIVM (NL)

The European legislation on the use of experimental animals: current status and future developments

Mrs L. Lwoff, Directorate General I – Legal Affairs, Council of Europe

Refinement of animal testing - same gain less pain

Prof. Dr D. Morton, University of Birmingham (UK)

Discussions

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Session I: Current regulatory and scientific background

INTRODUCTION TO THE THREE RS CONCEPT:

REPLACEMENT, REDUCTION, REFINEMENT

Prof. Dr C. Hendriksen, RIVM (NL) and Dr K. Cussler Paul-Ehrlich-Institut (D)

Recently, the Vactrain project was approved for financial support by the European Commission within the framework of the 5th Framework programme. The project aims in organising 5 training courses and 1 workshop/symposium. For the symposium, co-operation was received by the EDQM.

The reasons in organising these series of training courses are diverse:

- a) in making the vaccine quality control community familiar with the 3Rs concept,
- b) to improve the implementation of 3Rs methods in routine quality control practice and
- c) to offer a platform for networking.

The history of vaccine development dates back to the 18th century. In 1798, Jenner introduced the small pox vaccine for a disease that we have successfully eradicated but which is back on the agenda since the 11th of September 2001. Many vaccines have been developed since then, such as against many contagious human and veterinary infectious diseases. The success of vaccination is well recognised and several of the infectious diseases with a high morbidity and mortality are now eradicated, at least in the industrialised countries.

The technique of vaccine development has evolved since Jenner, particularly the last few decades. Modern vaccine development is based on sophisticated isolation and purification or even expression of the relevant epitopes. However, many of the 1st generation vaccines are still produced using the technique of inactivation/detoxification or attenuation of the virulent micro-organism. Due to the way the traditional vaccines are produced, extensive quality control is a necessity and specifications for quality control are laid down in product specific monographs. Many of the monographs mention one or more animal tests and consequently the use of laboratory animals for vaccine quality control is extensive. According to the Dutch statistics on the use of laboratory animals, about 17% of the total number of animals is required for quality control of biologicals, the vaccines being the major type of products.

There is an ethical dilemma concerning vaccines and laboratory animals. In one hand we have the moral obligation to produce vaccines and to guarantee safety and efficacy for the welfare of humans (often children) and also animals. On the other hand, however, we use large numbers of laboratory animals, often in procedures that inflict serious pain and distress to the animals involved. Although society as such accepts the use of animals for these purposes, there is a strong public incentive that this situation is only acceptable in case no alternatives are available. Therefore, there is a strong pressure to combining product safety and efficacy and animal welfare.

The 3Rs concepts relates to the book “The Principles of Humane Experimental Technique” published by Russell and Burch in 1959. They defined a strategy to uphold the quality of research and testing while at the same time finding a way to replace the use of animals by *in vitro* methods or non-vertebrates, to reduce the numbers of animals or to refine procedures in such a way that less pain and distress would be inflicted. The 3Rs concept has become the red-line in many regulations of the use of laboratory animals, such as the Council Directive 86/609/EEC or the Convention of the Council of Europe. Slowly, the 3Rs concept is now also finding its way into the guidelines and policies of regulatory bodies such as the European

Pharmacopoeia and of scientific organisations (e.g. the European Science Foundation). In Council Directive 86/609/EEC, art.23 stipulates that “the Commission and Member States should encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals but which involve fewer animals or which entail less painful procedures, and shall take steps such as they consider appropriate to encourage research in this field”.

As indicated above, substantial numbers of animals are used for vaccine quality control purposes. Taking into account article 23 of the Council Directive 86/609/EEC, we, as being involved in vaccine quality control, can ask ourselves the following questions:

- What information is a vaccine quality control test expected to provide?
- Is the information still essential for evaluating the quality of each batch of the product?
- Have developments in the mode of production made it unlikely that impurities detectable in an animal safety test are present?
- If the information provided is still essential, can it be provided now by another model not involving the use of whole animals?
- If the animal model is still essential for providing the information, can it then be provided by a method involving fewer animals (e.g. by reducing test variation), or is it possible to reduce the level of pain and distress?

The 3Rs concept is now a legitimate activity in vaccine quality control. Several examples can be given of successful acceptance of 3Rs approaches by the *Ph.Eur*:

Replacement: the use of immunochemical tests instead of the *in vivo* potency test of tetanus IgG; the use of the Vero cell test instead of the guinea-pig test for residual toxicity of batches of D toxoid.

Reduction: deletion of the specification of numbers of animals in vaccine potency tests; the use of serological-type assays for potency testing instead of the challenge-type models.

***Refinement*: the use of humane endpoints.**

However, although progress has been made, it is clear that we are only at the beginning of the process leading to a complete elimination of the use of animals. In this context it might be good to mention some of the major problems that have to be solved:

- The lack of a scientific basis for 3Rs development in some cases, such as for finding *in vitro* alternatives for safety tests of veterinary vaccines.
- The lack of priority given to 3Rs development, even in case a scientific basis is available.
- The lack of validation that is needed for acceptance of a 3Rs method by regulatory authorities. This might be because of the high costs of validation studies or because of the time needed. Also the unwillingness of laboratories to be involved in such studies might be a reason.
- Inconclusive results of validation studies due to improper design of such a study or due to the high intrinsic variability of the animal test.
- Even in case a 3Rs method has been validated, acceptance and implementation might be frustrated for various reasons.

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

We believe that an optimal incorporation of the 3Rs concept in vaccine quality control requires an adaptation of the 3Cs principle – Common sense, Communication and Commitment.

First, we should be realistic about the possibilities and limitations of 3Rs methods. On the other hand, we should also be realistic about the relevance of current quality control and of the animal models we use. It might be so that 3Rs methods have their limitations, this is also true for some of the animal methods we currently use for routine quality control.

Communication is vital too. We need to cultivate the dialogue between vaccine manufacturers, regulatory bodies and the academic community in order to optimise the exchange of information and to combine our 3Rs efforts. The 3Rs approach is not a stand-alone animal welfare issue but it is beneficial for the scientific basis of quality control as well.

Finally, commitment is essential. 3Rs activities are too much dependent of individuals. Institut's policies on 3Rs development and implementation are often lacking. This is not a good basis for continuation. Therefore, we plea for an integrated approach: regulatory bodies that put emphasis on the 3Rs, vaccine manufacturers and regulatory authorities that invest in development and implementation and funding agencies that support studies in these areas.

Conclusion

Vaccine quality control is based on the attitude of respect for life. If this is true, than this moral principle should also be applied to the use of laboratory animals.

Session I: Current regulatory and scientific background

**THE EUROPEAN LEGISLATION ON THE USE OF EXPERIMENTAL ANIMALS:
CURRENT STATUS AND FUTURE DEVELOPMENTS**

Mrs L. Lwoff, Directorate General I, Legal Affairs, Council of Europe

There are two legally binding instruments at the European level covering the use of animals for experimental purposes: the Council of Europe's Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123) and the Council Directive of the European Communities on the approximation of laws, regulations and administrative provisions of the member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC).

If both organisations are very different in their geographical membership and their objectives, the two instruments are very similar in their wording.

Indeed, the Convention, which was the first international legal instrument in this field, was used as a basis for the elaboration of the Directive. There are, however, some differences between these two instruments in particular in their respective scopes. The Directive, in contrast to the Convention, does not cover the use of animals in fundamental research, as well as - due to its legal basis - in forensic inquiries, education and training. Another difference concerns the tables to be used for the presentation of the statistical information on the use of animals. Whereas statistical information is required by both instruments, the Convention is the only one to provide tables for its presentation.

The Convention and the Directive

These instruments have three main objectives, which are the three R's rule defined by Russel and Burch (1959). Firstly, they intend to reduce the number of animals used in experiments by reducing the number of the latter (Reduction). Secondly, they aim at guaranteeing as far as possible the welfare of the animals before and after the experiments and ensuring that they suffer as little as possible during them (Refinement). Finally, they encourage research into the development of alternative methods (Replacement).

The provisions of the Convention and the Directive relate to the use of animals in procedures. Their articles include requirement relating to the procedures themselves but also concerning breeding and user establishments, the persons involved in the care and the animals (see presentations of the provisions of the Convention in Appendix).

The Convention provides also for a follow up of its implementation through regular meetings (Multilateral Consultations) of the Parties¹.

The implementation of the Directive is followed up by the European Commission, however, the Directive provides for the establishment of "a consultative committee within which the member States [are] represented." This committee assists the Commission in "the questions raised by the application of the Directive."

¹ **Parties**: country having ratified the Convention, i.e. having agreed, under international law, to be bound by the Convention.

Council of Europe: Multilateral Consultations

Since the entry into force of the Convention, work at the Council of Europe has been focusing on its implementation. Regular meetings (Multilateral Consultations) of the Parties are organised to monitor the implementation of the provisions, to adapt the Convention to changing circumstances or new scientific evidence or to develop co-ordinated programmes. These Consultations are extremely important to maintain the technical and political value of the text. Delegates from non-governmental organisations representing all the fields concerned (veterinarians, representatives of the pharmaceutical industry, scientists, animal welfare organisations, etc.) take part in these Consultations, which also allow for extensive exchange of information. Furthermore, non-member States, such as the United States and Japan, have expressed great interest in the work carried out under the Convention and are invited to participate in the meetings as observers.

Close collaboration between the EU and the Council of Europe

Close collaboration has developed between the Council of Europe and the European Union (European Community) in this field. The European Community, represented at the level of the Commission, has been participating in the Multilateral Consultations, first as Observer and as Party since May 1998.

Independently of this work, the two organisations collaborated on specific issues such as the elaboration of tables for the collection of statistical information under the EU Directive. The tables included in the Convention were used as model for the elaboration of EU tables, with a view to harmonising the two systems of tables which will be used to implement, on the one hand the Directive 86/609/EC and, on the other hand, the Convention ETS 123.

The close relation between the two organisations will continue to intensify now that the Community is bound by the Convention.

Current work and initiatives

Council of Europe

The Convention was finalised in 1985. Since then, research has been carried out, in particular on animal behaviour, providing new scientific evidence. Furthermore, practical experience has been acquired. Finally, scientific research and its requirements in the field of animal experiments have evolved. In this context, consensus was reached on the need for a revision of Appendix A presenting guidelines for accommodation and care of animals. The revision is already well advanced and its finalisation is foreseen for the end of 2003. It will incorporate the latest available scientific knowledge.

In parallel, a proposal for a conference on the ethical review process and committees is being considered with a view to help identifying a common minimum basis for the functioning of the ethical review process.

European Union

At the EU level, a revision of the Directive is foreseen. A first step should be to introduce a “regulatory committee procedure” to simplify the modification of Appendix II (corresponding to Appendix A the Convention) and to ensure “timely implementation of Community obligation under the Convention”.

The second step should be an in-depth revision of the Directive. It is proposed that this revision look into certain issues such as the existing legal base to cover animals used in education and training, the protection of the welfare of animals killed for tissues and organ

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

removal, the control and welfare of certain species such as non-human primates, how genetic modification and cloning is taken into account and the role of ethical reviews.

Conclusion

The Convention and the Directive ensure a harmonised minimum basis for the use of animals for experimental purposes. Since 1986, work has been focusing on the improvement of their implementation.

In accordance with the evolution of the field and the knowledge and experience acquired, their update has now been undertaken or is considered.

This process will benefit from the close collaboration between the two organisations concerned i.e. the Council of Europe and the European Union and from their complementary work and geographical representation.

Appendices - European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123) (see page 107)

Session I: Current regulatory and scientific background

REFINEMENT OF ANIMAL TESTING - SAME GAIN LESS PAIN

Prof. Dr D. B. Morton, Biomedical Services Unit, University of Birmingham (UK),

Dr K. Cussler, Paul-Ehrlich-Institute (D) and Dr C. Hendriksen, RIVM (NL)

Refinement can be defined as those methods that aim to alleviate or minimise the potential pain, distress and other adverse effects suffered by the animals involved in an experiment, or that enhance their well being (Smaje *et al.* 1998).

Refinement of animal experiments is mandated in European law (Council of Europe 1986, European Directive 1986) for all those carrying out research on animals including regulatory testing; there is no specific exemption for this area of animal use. Furthermore, refinement should be applied to the husbandry and routine care of animals as well as to their use in science as animals can suffer from poor husbandry as well as from the scientific procedures being carried out on them.

One or two other concepts are important when discussing refinement. First, what do we mean by 'welfare/well being' and 'suffering'.

It can be said that "an animal's welfare is compromised when its physiological health and psychological well being, in relation to its cognitive capacity, are affected negatively."

Suffering, similarly takes into account these concepts, and can be defined as "a negative emotional state which derives from adverse physical, physiological and psychological circumstances in accordance with the cognitive capacity of the species and of the individual being and its life's experience."

What is ethically, legally, and scientifically important is that only the minimum suffering should be caused to an animal, i.e. only that necessary to achieve the scientific objective. More than this cannot be justified, is a breach of the law, and may well confound some areas of science as it adds uncontrolled variables to the experiment. This latter suffering can be termed 'avoidable suffering' and is usually to do with how the research is conducted and not to do with the overall aim of the work (see Figure 1).

Hence refinement can be seen as achieving the same scientific gain with less pain. Finally it needs to be recognised that animals in a coma are not suffering, but that they almost certainly have been before that time judged by their behaviour. While this suffering may not always be pain it certainly is at a level of feeling extremely unwell and that in itself will cause distress (Morton, 1999).

Defining early or the earliest endpoints at which experiments can be stopped, so causing less animal suffering, is a humane thing to do, and hence has been termed 'implementing humane endpoints' (Morton 1995, 2000, Mellor and Morton 1997, Hendricksen and Morton 1999). The most fundamental endpoint is stopping the experiment when the scientific objective has been achieved, as there is surely no point in going on with it. These endpoints can also occur when an animal is no longer going to produce good scientific results because it is so physiologically or psychologically perturbed. Another time is when the level of suffering is simply too high in relation to the scientific benefits, i.e. when there is a loss of ethical proportionality, or it can be when the level of suffering is simply unacceptable.

In the present context of vaccine safety and potency testing the endpoint can be when the results from a test batch of vaccine show that the vaccine poses an unacceptable risk to humans or other animals. Or when, in a potency test, we know the animal has not been

adequately protected, and this is usually obvious as the first clinical signs of disease are observed. Thus in vaccine testing a humane endpoint is the earliest indicator in an animal experiment that the test result sought has been achieved. The OECD has also been concerned about the level of suffering in acute toxicity tests particularly when death of the animal may occur and for these reasons they have defined ‘moribund’, and ‘predictable and impending death’, given some practical guidance on these states, and how to recognise and how to avoid them (OECD 2001).

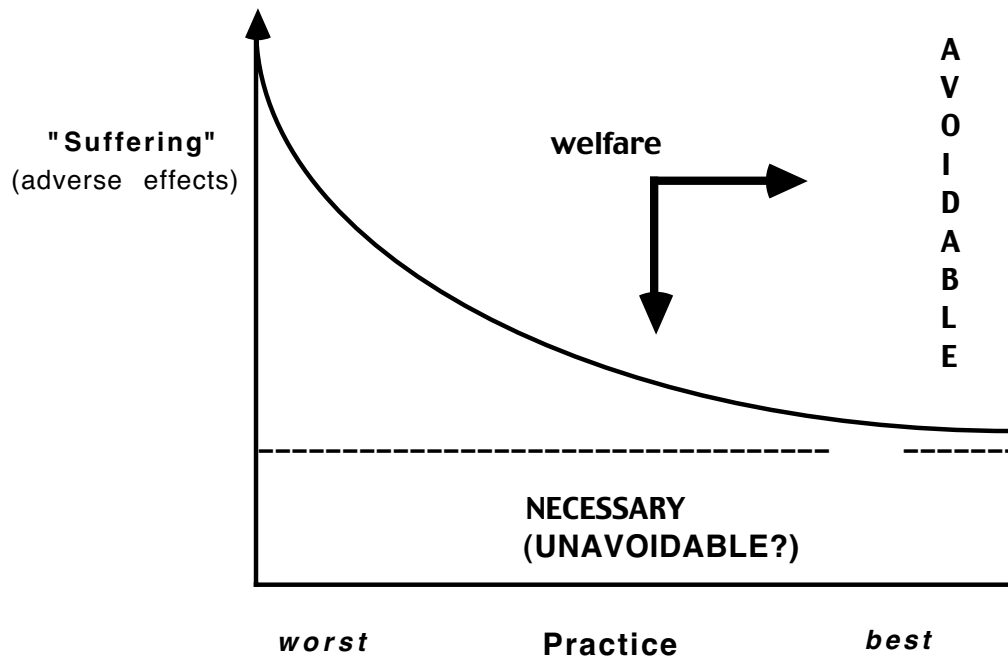


Figure 1: A theoretical relationship between avoidable suffering relating to good practice and that suffering that is unavoidable and necessary to achieve the scientific objective.

In this context it is interesting to consider why animals actually die. It stems from either the heart stopping or from severe brain damage particularly to the vital centres. From some of our observations in mice several factors emerge. First that mice reach a stage when they are no longer able to eat and drink e.g. they are unable to reach up to the water bottle and food hopper, and so they become dehydrated. This leads to haemoconcentration and the resulting thickening of the blood to a point where the heart cannot cope. The viscosity of the blood is so high that heart failure ensues and the animal dies. This is an indirect cause of death and not strictly due to the action of the virus or bacteria being tested. In other circumstances the micro-organism may affect the neurones in the brain stem and that causes a failure to maintain normal homeostasis, with a consequent progression to death.

Humane endpoints can be validated by following animals through to the traditional endpoints (often death), as well as noting the clinical signs and other scientific measures that can be made. A retrospective analysis of the results can then be carried out to determine the usefulness or otherwise of those signs. In such studies, one outcome measure of ‘humanity’ or the effect of an ‘early intervention’ can be based on the days that an animal would have lived had the early endpoint not been implemented. From a scientific viewpoint, validity can

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

be interpreted from the number of false positives and false negatives that an early endpoint would give in the test, and then to see whether they are acceptable or unacceptable when interpreting the data in terms of risk assessment.

Some practical examples

Husbandry

There are now several standard texts looking at how the husbandry of animals can be improved through increased space and the provision of objects that an animal can manipulate or use to meet its behavioural needs within a caged environment or in a pen (e.g. JWGR 1993, 1998, 2001, Council of Europe 1997, Bayne *et al.* 2002; Jensen 2002). Increasingly there is a move away from barren caging of single animals such as rabbits and primates (rodents were normally group housed except for male mice) to the provision of group housing in enriched areas such as pens or large cages with perches and shelves, so meeting the social and behavioural needs of the animals. There is an added bonus to this in that it provides not only better environments for the animals but also the human staff caring for them feel enriched through this care.

Safety, potency and efficacy testing

In a test of phage activity against *Staphylococcus aureus* Soothill and colleagues (1992) used body temperature as an early indicator of effectiveness of treatment compared with untreated animals. They showed that when the temperature of mice dropped below 35°C then those animals would die, whereas the body temperature of survivors did not drop below that temperature. Similar results have been found by others (Acred *et al.* 1994).

In another experiment on rabies potency testing Cussler and colleagues (1999, 2000) showed that when animals started to show neurological signs of infection that they almost all went on to die. As a consequence they have recommended to the EU Pharmacopoeia that the monograph be rewritten so that when mice showed slowing of movement together with circling, or later signs such as convulsions, then those animals are counted as having developed the disease. (Moreover, they have recommended that the traditional endpoint of death be expressly prohibited as a validated alternative exists that causes less pain and suffering and so the old lethality test would be illegal under nearly all legislation concerning animal experimentation in the world).

In another study by Hendricksen and colleagues (1999), on pertussis vaccine potency testing, they showed that clinical signs of disease could satisfactorily be used as alternatives to death. In their papers they have looked at the incidence of false positives that arise when various clinical signs are considered as the endpoint. Thus with varying temperature, a cut off point of 35.5°C gave 47.8% false positives, <35°C gave 4.3%, and less than 34.5°C gave 0%. Bodyweight was not so clear cut or useful (one mouse lost nearly 40% of its bodyweight and survived the observation period), but a judgement made on overall clinical signs resulted in just 2.3% false positive results. When 'days of suffering' saved for the animals was evaluated, a cut-off of 34.5°C led to animals being culled a mean of 2.17 days earlier (range was 1-7 days).

In conclusion, the implementation of early endpoint will lead to less animal suffering, fewer animals suffering, better science, and possibly financial savings. Above all, whilst we are wait and hope for the development of alternatives not involving the use of animals, we can reduce the suffering of animals and improve their welfare. If we should to claim that our present use of animals in vaccine testing is both humane and responsible and complies with both the spirit and letter of the law, then this is the least we can all do.

References

- Acred, P., Hennessey, T. D., MacArthur-Clarke, J. A., Merrikin, D. J., Ryan, D. M., Smulders, H. C., Troke, P. F., Wilson, R. G. and Straughan, D. W. Guidelines for the welfare of animals in rodent protection tests. A report from the Rodent Protection Test Working Party. *Laboratory Animals* **28**, 1-8 (1994).
- Bayne, K. A. L., Mench, J. A., Beaver, B. V., and Morton, D. B. Laboratory Animal Behavior. In: *Laboratory Animal Science ACLAM series 2nd Ed.* Publishers Elsevier Sci. USA. 1240-1264 (2002).
- Council of Europe, (1986). European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific purposes. European Treaty Series No 123 (ETS123).
- Council of Europe, Multilateral Consultation of the Council of Europe. Resolution on the accommodation and care of laboratory animals. (1997).
- Cussler, K., Morton, D. B. and Hendriksen, C. F. M. Humane endpoints in vaccine research and quality control (1999). In: *Humane Endpoints in Animal Experiments for Biomedical Research. Proceedings of the Intl Conference, 22-25 Nov Zeist, The Netherlands.* Eds. C. F. M. Hendriksen and D. B. Morton. 95-101 (1998). ISBN 1-85315-429-6 Publrs Royal Soc. Med. London WIM 8AE.
- Cussler K. Morton D. B. and Hendriksen C. F. M. Possibilities for the use of humane endpoints in vaccine potency tests (2000). In: Balls M., Zeller van A. M. and Halder M. E. (ed) *Progress in the Reduction, Refinement and Replacement of Animals Experimentation.* Elsevier Science B.V **31 B**:915-927.
- European Union Council Directive, Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (1996). *Official Journal of the European Communities L.358.* **29** 1-29 (1986).
- Hendriksen C. F. M., Steen B., Visser J., Cussler K., Morton, D. B. and Streijger, F. The evaluation of humane endpoints in pertussis vaccine potency testing (1999). In: *Humane Endpoints in Animal Experiments for Biomedical Research. Proceedings of the Intl Conference, Zeist, The Netherlands (1998).* Eds. C. F. M. Hendriksen and D. B. Morton. 106-113. ISBN 1-85315-429-6 Publrs Royal Soc Med. London WIM 8AE.
- Hendriksen, C. F. M. and Morton D. B. Eds *Humane Endpoints in Animal Experiments for Biomedical Research (1999).* Proceedings of the Intl Conference, Zeist, The Netherlands. 150. ISBN 1-85315-429-6 Publrs Royal Soc Med. London WIM 8AE (1999).
- Jensen, Per *The Ethology of Domestic Animals – An Introductory Text Ed.* ISBN 0-85199-602-7 Publrs CABI Wallingford Oxford (2002).
- JWGR: Joint Working Group on Refinement Morton, D. B., Jennings M., Batchelor G. R., Bell D., Birke L., Davies K., Eveleigh J. R., Gunn D., Heath M., Howard B., Koder P., Phillips J., Poole T., Sainsbury A.W., Sales G. D., Smith D. J. A. , Stauffacher M. and Turner R. J., Refinements in rabbit husbandry, *Laboratory Animals*, **27**: 301-329 (1993).
- JWGR: Joint Working Group on Refinement Jennings M., Batchelor G. R., Brain P. F., Dick A., Elliott H., Francis R. J., Hubrecht R. C., Hurst J. L., Morton D.B, Peters A. G., Raymond R, Sales G. D., Sherwin C. M. and West C., Refining Rodent Husbandry: the mouse, *Laboratory Animals*, **32**, No. **3**, 260-269.

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

JWGR: Joint Working Group on Refinement Eds Hawkins, P. Morton D. B. *et al* Laboratory birds: refinements in husbandry and procedures (2001). 5th Report of the BVAAWF/FRAME/RSPCA/UFAW. *Laboratory Animals* **35**: Supplement 1.

Mellor, D. J. and Morton, D. B. Humane Endpoints in research and testing Synopsis of the workshop. In 'Animal Alternatives, Welfare and Ethics.' Eds. LFM van Zutphen and M. Balls. Publr. Elsevier Science BV, Amsterdam, The Netherlands. ISBN 0-444-82424-3 297-299 (1997).

Morton, D. B. Practical ideas for refinement in animal experiments. Proceedings of Animals in Science Conference Perspectives on their Use, Care and Welfare. 157 - 167. Ed. N.E. Johnston. Monash University. Melbourne, Australia (1997).

Morton, D. B. Ethical aspects of the use of Animal Models of Infection. In: Handbook of Animal Models of Infection. Eds. Otto Zak, 1998 ISBN 0-12-775390-7 Publishers: Academic Press. 29-48 (1999).

Morton, D. B. Humane end points in animal experimentation for biomedical research: Ethical, legal and practical aspects. In: Humane Endpoints in Animal Experiments for Biomedical Research. Proceedings of the Intl Conference, 22-25 Nov 1998 Zeist, The Netherlands. Eds. C. F. M. Hendriksen and D. B. Morton. 5-12. ISBN 1-85315-429-6 Publr Royal Soc Med. London WIM 8AE (100%) (1999).

Morton, D. B. A Systematic Approach for Establishing Humane Endpoints. *ILAR Journal* **41**: No 2. 80-86 (2000).

OECD, Environmental Health and Safety Publications Series on Testing and Assessment **19** (2001). Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation Environment Directorate. Organisation for Economic Co-operation and Economic Development, Paris (November 2000.).

Smaje, L., Smith, J. A., Combes, R. D., Ewbank, R., Gregory, J., Jennings, M. and Morton, D. B. Advancing refinement of laboratory animal use. A report prepared on behalf of the Boyd Group. *Laboratory Animals* **32**, 137 – 142 (1998).

Soothill, J., Morton, D. B. and Ahmed A. The HID50 (hypothermia-inducing dose 50): an alternative to the LD50 for measurement of bacterial virulence. *Int. J. Experimental Pathology* **73**, 95-98 (1992).

Session I: Current regulatory and scientific background

DICUSSIONS

Question from the floor to Mrs L. Lwoff: Could you explain the system behind the European legislation which verifies that the member States implement the instrument which you have mentioned during your presentation.?

Mrs L. Lwoff: In fact, there are several systems and which I did develop at the beginning of my presentation, is that the Council of Europe and the European Commission are very different. In the Council of Europe we work on co-operation between States which means that the Convention is an agreement between states. The States undertake to apply and implement the provision with regard to the other countries who have ratified the Convention. The Council of Europe is not a competent authority as such who is able to ensure implementation, it is the moral obligation under international law which makes this system work. The way that the implementation is followed-up is by reports given by the member States during this multi-national consultation on how they are implementing the Convention and the difficulties that they have met or are having. The only requirement is to provide the Council of Europe with statistics on the use of animals and this works well. There are some people who do indeed criticize this approach as there is no policy behind the implementation and it even works also in countries which are not yet party to the Convention. An example of this is when we elaborated guidelines for training and education of the different category of persons involved in the use of animals – those guidelines normally concerned the parties to the Convention however, just after the adoption of these guidelines, Slovenia who is not a party of the Convention, announced that in their new law these guidelines were already introduced. There are some countries which have not accepted this Convention as it requires a procedure at national level, through the parliament and which takes time, however the instruments which we develop are practical in the sense that the wording is easy in order to make them applicable.

At European Union level, the Commission is the competent Authority and is responsible for the enforcement of the directive. As you know, some countries have already had problems with the Commission in respect to directive 86/609. The Commission is presently making inquiries as to how this is being implemented.

These two systems are different but the results seem similar.

Session I: Current regulatory and scientific background

WORKSHOP I: REFINEMENT OF EXPERIMENTAL PROCEDURES

CONCLUSIONS AND RECOMMENDATIONS

Prof. Dr D. B. Morton, University of Birmingham (UK) and
Dr E. Hanenberg, Intervet International (NL)

Refinement of experimental procedures

Discrepancy between legislative requirements and animal welfare e.g. dosing volumes

Awareness, Training and Competence

- All involved
- Worldwide

Research and development of refinements:

- Better sharing of existing data ?Databases?
- Better chance of multicentre trials and validation

Refinement of experimental procedures

Score sheets to identify and implement endpoints

clinical signs reduced to being present or absent;

they should be spelled out clearly in the Ph Eur.

with guidance on how to use them in terms of terminating an experiment.

Recommendations

An international authority, like OECD, ICH, VICH, OIE or WHO, should be set up to oversee and harmonise testing requirements so that mutual acceptance of data becomes possible, and detail endpoints

International fora should be established to share best practice, especially from internal studies, within the regulatory and scientific community.

There should be some form of World Standard for the care and husbandry of animals.

Pharmacopoeial monographs detailing death or any suffering as a requirement, when it is more severe than a validated alternative method, should be deleted.

To promote mutual acceptance of data, training and competence of those carrying procedures on animals, should meet internationally agreed SOPs.

More resources should be directed at e.g. validation of earlier endpoints, providing training, ensuring competence, developing alternatives.

Session I: Current regulatory and scientific background

WORKSHOP II: REFINEMENT OF CHALLENGE TESTS BY SEROLOGY

CONCLUSIONS AND RECOMMENDATIONS

Dr K. Cussler, Paul-Ehrlich- Institut (PEI), (D) and
Dr K.-H. Buchheit, (EDQM), Council of Europe

Refinement of Challenge Tests by Serology (For Batch Potency Test)

When is Serology Acceptable? (Acceptance by authorities?)

Differences: vaccines for veterinary and human use

Veterinary vaccines: Protective antibodies response, defined by challenge

Carefully designed collaborative studies

Obtain serological data in field studies for support

For new vaccines (in absence of monographs)

Need for more information exchange & guidance

Use of Serology Impaired by Lack of Reference Vaccines (Veterinary Field)?

Impossible for all vaccines

Different approach to human Vaccines

Challenge test during licensing --> no need for reference vaccine ?

but,

some examples where ref, vaccine useful (e.g. avian influenza)

Evaluation where reference vaccines are useful

Availability of Reference Vaccines, - Anti-sera, ELISA kits, reagents

Essential to consider necessities, sources at beginning of project

Source: Commercial?, manufacturers? (product-specific?), EDQM, WHO ? (no conclusion reached)

Information exchange between competent centres (publish web-sites in proceedings)

SESSION II: IMPLEMENTATION OF THE 3RS: REGULATORY ASPECTS

Applying the three Rs within the regulatory system

Mr Peter Castle, EDQM, Council of Europe

The role of E.U. guidelines in reducing animal usage in the testing of veterinary vaccines

Dr D. Mackay, Veterinary Medicines Directorate (VMD), (UK)

The role of the OMCL Network in the implementation of three Rs methods – special case of vaccines

Dr R. Dobbelaer, Institute of Public Health, Louis Pasteur (SIPH) (B)

The three Rs concept in candidate countries:

Human biologicals:

Dr E. Vitkova, Head of Biological Control, State Institute for Drug Control (CZ)

Veterinary biologicals:

Dr G. Kulcsar, PhD, Institute for Veterinary Medicinal Products (H)

Existing Three Rs methods: are they really used?

Dr L. Bruckner, Institute for Virology and Immunoprophylaxis (IVI), (CH)

Discussions

Conclusions Workshop III: Implementation of Three Rs methods: human vaccines

Dr R. Dobbelaer, (SIPH) (B) and Dr A. Sabouraud, Aventis Pasteur (F)

Conclusions Workshop IV: Implementation of Three Rs methods: veterinary vaccines

Dr K. Cussler, PEI (D) and Dr L. Elsken, UDSA (USA)

Session II: Implementation of the 3rs: regulatory aspects

APPLYING THE THREE RS WITHIN THE REGULATORY SYSTEM

Mr Peter Castle, (EDQM), Council of Europe

It has often been said that the Pharmacopoeia is a brake on the introduction of improved tests from the point of view of animal welfare. People say that we would like to use tests which are preferable from point of view of the animal welfare, but where a test is included in the Pharmacopoeia it hinders progress.

If there is something I would wish you to remember from today, it is the following message:

Animal welfare is a part of the regulatory system, not the same regulatory system as for medicinal products, nevertheless, a part of the regulatory system and the Pharmacopoeia is very committed to promoting this. While there is of course an obligation, whenever we remove an animal test, for you to follow the lead, there are also some opportunities for you to make improvements in animal welfare, within the framework of the Pharmacopoeia tests, even if the basic Pharmacopoeial tests have not changed.

The real starting point for this campaign was the signature of the European Convention in 1986. The European Pharmacopoeia Commission made a commitment to making improvements wherever possible in animal welfare and published this declaration in the introduction to the Pharmacopoeia. It has been the subject of continuous attention since 1986.

There has been progress for all of the 3Rs:

Replacement, where we have been able to introduce some *in vitro* tests and eliminate unnecessary tests.

Reduction, by transferring tests up-stream, deleting requirements for minimum number of animals in assays and specifically mentioning one dilution assays in many cases.

Refinement, for example by the introduction of serological assays.

There are also some 'grey zones' and it is really on the grey zones that I should like to concentrate: some of the monographs do permit replacement, reduction and refinement even though the official test remains the same.

Reduction: the abnormal toxicity test has been moved up-stream but as Alain Sabouraud has mentioned, although we have eliminated the test for routine application, manufacturers cannot drop the test completely because it remains in regulations elsewhere in the world, and the vaccine business is a worldwide business. It would seem that there is some harmonisation to be done to resolve this current problem.

The requirements for minimum number of animals in the diphtheria and tetanus assays have been removed; the requirement for statistical validity in the test is a more satisfactory, scientific approach. We have also specifically mentioned the use of one-dilution assays. We need to encourage people to use them. The basic assay remains the three-dilution test but by specifically mentioning one-dilution assays, we hope that people will put in a variation to the regulatory authorities.

Some examples of refinement: serological assays for clostridial vaccines and some others are being introduced, for example, tetanus vaccine, diphtheria vaccine and swine erysipelas vaccine. These are some examples of the grey zones where monographs explicitly or implicitly would allow you to apply the 3Rs. You may have to validate the test for each product which is one of the reasons we cannot make an across-the-board change within the

Pharmacopoeia. Use of an alternative method needs approval from the competent authority. This means within the Pharmacopoeia Member States approval from 30 competent authorities may be needed and we have to recognise that this can be a barrier to progress.

We have to remember a statement from the General Notices which is the very first section of the Pharmacopoeia:

“... does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia.... The manufacturer may [use] data derived, for example, from validation studies of the manufacturing process and from in-process controls ...”

The essential requirement of the Pharmacopoeia is compliance. A validated alternative can always be used if it gives an assurance that the product, if tested by the official method, would comply with the requirements of the monograph. This opens a door to many 3Rs methods – for a given product. To have one single method, which can be applied for all products, is a more difficult thing to do but this is an opportunity for everyone to apply 3Rs methods.

Another example is for monographs on veterinary vaccines: we continually have the comment that people want to use *in vitro* identification tests for these vaccines and of course that is possible after validation, but, the validation is usually product specific and in order to counter this and hopefully not to receive the same comment again and again, we have put a statement into the general monograph on *Vaccines for Veterinary Use*:

“... The identification test can often be conveniently combined with the batch potency test to avoid unnecessary use of animals. For a given vaccine, a validation *in vitro* test can be used to avoid the unnecessary use of animals”

This is one of the means we have to encourage people to use better methods by putting these statements into the general monograph.

Some years ago, ECVAM organised a workshop in Zeist on the use of humane end-points. It was clear that there was scope for action in monographs. As a first step a positive statement has been included in the European Pharmacopoeia, in the general monographs on human and veterinary vaccines.

“... 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.”

In the workshop on refinement and humane endpoints we saw that there is a lot of scope for refining the tests in the Pharmacopoeia – without having to make any essential change to the monographs. The door is already open for humane endpoints. It may be that we should be giving more guidance on these humane endpoints and this was one of the conclusions of the workshop.

The use of humane endpoints is part of the application of the 3Rs and refinement is in many ways the poor relation of animal welfare. Certainly, it is the one to which least attention has been paid. It should now be clear that it has to be considered by anyone carrying out testing according to the European Pharmacopoeia. We still do have one or two tests in the Pharmacopoeia where alternative methods are allowed for example with different endpoints and we can only say that people should make every effort to apply the more humane one. This may mean extra work for introduction of a new method but this should not be allowed to hinder the introduction of better methods.

There has been another change recently in monographs on veterinary vaccines regarding the target animal safety test, which has so far been required for each batch. There is now a

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

provision in the general monograph for waiving of the test after a sufficient number of consecutive batches have been tested with satisfactory results. Of course, waiving the test does depend on the decision of the competent authority – data on batches tested will have to be submitted and there may be some cases where the test cannot be discontinued. To try to avoid having problems with mutual recognition, we have proposed to CVMP that a guideline should be drafted to back-up the statement which we have now put into the general monograph to try to have everybody moving in the same way and taking decisions when the manufacturers do ask for this test to be waived.

Having looked at the grey zones we can now look at what is clearer, in black and white.

Residual toxin and irreversibility of diphtheria toxoid: the guinea-pig test has been removed from the monograph and people will now change to the *in vitro* method.

Immunochemical assay of tetanus immunoglobulin: the mouse neutralisation test has not been completely removed from the monograph; satisfactory correlation of the immunochemical assay has to be shown, since an ELISA test only determines binding, it does not demonstrate neutralisation. However, it is the routine method included in the monograph so people do have to do that validation and we hope that the regulatory authorities are aware of this.

There are similar schemes for other clostridial veterinary sera in the Pharmacopoeia.

The abnormal toxicity test: this has now been moved up-stream. It was on the basis of a historical study carried out Paul-Ehrlich-Institut with a large amount of data collected. To get rid of a test you have to submit data on a certain number of batches to the licensing authorities, but in most cases the manufacturers had this data when the test was moved up-stream since they had been doing the test routinely for some years. For new vaccines, the tests will be carried out on the consistency batches during licensing.

It is also possible for the licensing authorities simply to waive the application of a test. Wherever there is a test in the monograph, a vaccine has to comply, but there are cases where the licensing authority will be satisfied that once the manufacturer has demonstrated consistency of production, then the test does not need to be carried out any longer and there could be good scientific reasons for doing that. This is entirely compatible with the requirements of the European Pharmacopoeia. For some producers the test for residual pertussis toxin is no longer carried out for the acellular vaccine. For inactivated poliomyelitis vaccine, the *in vivo* assay has been waived for some manufacturers too so that now they are now carrying out an antigen determination for that vaccine.

I hope that this will convince everybody that you must be reviewing your procedures continuously to see whether within the frame-work of the Pharmacopoeia you could be making some improvements from the point of view of animal welfare.

Session II: Implementation of the 3rs: regulatory aspects

THE ROLE OF EU GUIDELINES IN REDUCING ANIMAL USAGE IN THE TESTING OF VETERINARY VACCINES

Dr D. Mackay, VMD (UK)

The legislative requirements for immunological veterinary medicinal products (IVMPs) within the EU are laid down in Directive 2001/82/EC. Amongst other provisions, this directive stipulates that the minimum requirements that a product must meet are those laid down in the relevant European Pharmacopoeia (Ph. Eur.) monograph, where one exists. In addition, guidelines on general and specific requirements for IVMPs are developed by the Committee for Veterinary Medicinal Products (CVMP) through its immunologicals Working Party (IWP). The intention of these guidelines is not to repeat requirements that are already described in the Ph. Eur., but to interpret the legislative requirements of the Directive in such a way as to promote harmonisation between Member States. The guidelines clarify and elucidate the text of the directive and, where appropriate, of the relevant Ph. Eur. monograph. Unlike Ph. Eur. monographs, the guidelines are essentially advisory in nature and have no formal legal status. The guidelines can therefore propose and support a reduction in animal testing but cannot actually enforce it. Where the guidelines are particularly useful is in providing an explicit description of the requirements that must be met for replacement of an *in vivo* test with one conducted *in vitro*, thus ensuring that, if met, the results obtained from *in vitro* testing will generally be acceptable to all Member States. There should therefore be no need for a manufacturer to perform additional testing, perhaps in animals, to satisfy individual national authorities. In addition, there should be less redundancy in terms of testing as, provided that the guidelines are followed, the tests performed should be acceptable throughout the EU.

The guidelines developed by the CVMP are intended to address harmonisation of requirements under EU pharmaceutical legislation. Although other countries frequently refer to these guidelines, they have no formal status outside the EU. The pharmaceutical industry however is becoming increasingly international due to mergers and acquisitions creating fewer, but larger, pharmaceutical companies. These companies now pursue global research and development strategies with the result that the testing performed during development must be acceptable to a range of different regulatory bodies. In response to this, and to reduce the amount of testing required to meet different national requirements, the International Conference on Harmonisation of Technical Requirements For the Registration of Veterinary Pharmaceutical Products (VICH) was established. This seeks to establish harmonised requirements between participants, principally the EU, USA and Japan, with several other countries, such as Australia and New Zealand, acting as observers. Harmonisation is a slow process but the ultimate result is that harmonised guidelines are implemented in the legal framework of participating countries, replacing any existing national requirements and thus ultimately ensuring that tests performed in any one VICH participant country will be acceptable in any other. To date, none of the harmonised guidelines that have reached the final stage of acceptance have involved *in vivo* tests, but it is hoped that the process of harmonisation will eventually encompass such testing.

Examples of replacement (e.g. specifying the requirements that must be met to replace an *in vivo* potency test for an inactivated vaccine such as equine influenza vaccine with an *in vitro* antigen quantification test); reduction (e.g. specifying the use of a batch safety test involving only two animals during authorisation of a change of strain for an equine influenza vaccine in

place of a full safety test involving ten); and refinement (e.g. specifying the requirements that must be met for the use of serology in place of challenge as an indicator of the efficacy of a booster vaccination) can all be found in recent CVMP guidelines. Work in this area is ongoing (e.g. developing guidance on the requirements that must be met for Licensing Authorities to waive the need for routine target animal safety tests on every batch) and moving into new areas (e.g. specifying the requirements to use a serological test for potency of FMD vaccines in place of challenge).

A new initiative is the development of harmonised guidelines for the re-testing of batches of IVMPs by Official Medicines Control Laboratories (OMCL) within the EU as part of Official Batch Release. This initiative has the potential to reduce animal testing by reducing duplication of re-testing of the same batch of an IVMP by different Member States through mutual recognition of a single Official Batch Release. A similar process of harmonisation has been very successful in relation to human vaccines and has reduced the number of animals used for Official Batch Release. The system has also improved the quality and consistency of batches of human vaccines released onto the EU market. However, there are significant differences between the markets for human and veterinary vaccines in that there are many more veterinary than human vaccines and, for a large number of vaccines, the market, and therefore the batch size, is small. Currently, only very few types of vaccine are systematically re-tested by OMCLs in animals and these tend to be for the most significant diseases such as rabies, tuberculosis and Newcastle Disease. The introduction of compulsory re-testing by OMCLs as a condition of Official Batch Release for a wider range of veterinary vaccines therefore has the potential to substantially increase the amount of re-testing in animals carried out by OMCLs. In adopting a harmonised Official Batch Release scheme that is intended specifically for veterinary vaccines, consideration must also be given to putting in place systems of official control that ensure that batches are adequately tested by manufacturers before release by their Qualified Person without the need for additional tests, particularly tests in animals, by OMCLs. In the development of new guidelines, it is therefore important that a full assessment is made of the impact in terms of animal usage. New guidelines and new requirements should only be introduced where this assessment clearly shows that the benefits in terms of added safety clearly outweigh the costs in terms of additional animal testing.

Incorporation of the 3 R's into new and existing regulatory guidelines tends to be a slow process due to a different "3 R's" – reluctance, revalidation and regulations. In terms of reluctance, both manufacturers and regulators can be reluctant to accept new tests, particularly *in vitro* tests, as they represent a move away from established tests with which they are familiar and 'comfortable'. Any new test requires extensive re-validation which can be expensive, and it can be difficult to persuade finance directors that the cost:benefit analysis is worthwhile, particularly when the benefits appear to be more in terms of animal welfare than profit. The corollary to this is that it is often a worthwhile investment to replace animal tests with alternatives, which normally work out cheaper in the long run. Finally, there is a 'Catch 22' situation in terms of regulations in that there is no incentive to develop a new test, unless a company is sure that it will be acceptable to a regulatory authority. Conversely, there is no incentive for a regulatory authority to change a regulatory requirement, until there is a validated alternative to the existing test. All of these disincentives to change can be addressed by meetings and projects such as VACTRAIN which bring together all of the interested parties to build confidence and promote adoption of validated alternatives to animal tests.

The views expressed in this paper are the personal views of the author and should not be taken as those of the Veterinary Medicines Directorate or the Immunologicals Working Party of the CVMP.

Session II: Implementation of the 3rs: regulatory aspects

**THE ROLE OF THE OMCL NETWORK IN THE IMPLEMENTATION OF THREE
RS METHODS – SPECIAL CASE OF VACCINES**

Dr R. Dobbelaer, Louis Pasteur (SIPH) (B)

In vivo tests may be used at all stages of the life span of any medicinal product. Biologicals are a special case as physico-chemical testing is often insufficient and has to be complemented by biological assays, including *in vivo* assays on each production batch. In addition, mainly in view of to their important role in public health, vaccines and plasma-derivatives are submitted to the OMCL Batch Release procedure including a producer-independent, pre-marketing re-testing of each batch to verify and monitor conformity with the marketing authorisation dossier and the European Pharmacopoeia and the World Health Organization requirements. This experience of OMCLs is also used by the European Union and national licensing authorities and authorities involved in establishing regulations such as the CPMP and the European Pharmacopoeia. As a result, OMCLs have played and will continue to play an important role in implementing the 3Rs principles. This is illustrated by the nature of the testing selected for European Union batch release as well as by numerous initiatives taken or planned by EDQM and OMCLs.

Session II: Implementation of the 3rs: regulatory aspects
THE THREE RS CONCEPT IN CANDIDATE COUNTRIES:

Human biologicals:

Dr E. Vitkova, State Institute for Drug Control (CZ)

The 3Rs are known, studied, applied in the institute and step by step introduced in the laboratory control of biologicals. The main activity is dedicated to refinement and reduction, replacement is up to now more on the theoretical basis than practically applied and used. The department personnel had possibility to gain knowledge in RIVM, Bilthoven and took part on several meetings with the possibility to exchange experience on that field.

Due to many changes in the country and in the institute too, implementation is developing quite slowly and is expected in the future years.

In Reduction there were new trends accepted when Pharmacopoeial basis was introduced and covers the test for abnormal toxicity which is omitted in most cases of the control or specific toxicity tests which are provided on the reduced groups of tested animals.

Reduction of laboratory tests is closely connected with the batch release system applied in EU countries and kindly offered to be introduced also outside. The system has been adopted for blood products and now is created and expected to be used for vaccines next year.

Refinement of animal tests is regulated with the law requirements for the correct animal housing, there are prescribed conditions for animal housing and handling with the animals during the tests (application in anaesthesia, use of recommended application methods), and all the staff undergo regular training and exams.

Replacement on general basis can present introduction of LAL testing of endotoxin and use of the method when available. The laboratory cooperates with laboratories outside the institute as consultant and reference unit and takes part in proficiency testing studies (PTS) when organised. The test is also used officially or experimentally in the endotoxin control of vaccines.

Except that the use of tissue cultures was fully adopted in the testing of vaccines and other tests routinely used are ELISA, and proficiency testing studies.

The vaccines used for mass vaccination in the country are the main target of the future control and adoption of 3Rs methods. The routine use of ELISA for tetanus, diphtheria and hepatitis vaccines, the use of Vero neutralisation test which has already been introduced, the further development of LAL testing and pyrogen testing in general, adoption of tests for specific toxicity.

The laboratory team appreciates very much high support from EDQM and other European OMCLs and hopes to reach success when starting its work in fully reconstructed laboratory department.

Session II: Implementation of the 3rs: regulatory aspects
THE THREE RS CONCEPT IN CANDIDATE COUNTRIES:

Veterinary biologicals:

Dr G. Kulcsar, PhD, Institute for Veterinary Medicinal Products (H) and
Dr T. Soos, Institute for Veterinary Medicinal Products (H)

Animal experiments traditionally play an important role in the development, production and control of vaccines. As early as at the end of the 18th century Jenner infected calves deliberately with cowpox to maintain the infectious material for vaccination. The 3Rs concept of Russel and Burch to replace, reduce and refine animal tests was introduced in 1959 (1). More than forty years have passed, but today we still have a lot of question marks on this field.

Veterinary vaccines in the candidate countries

In this presentation we would like to show the current situation of veterinary vaccine control in the candidate countries, focusing on the role of the animal experiments. Our data are not complete, as we do not have any information from certain countries.

The market and the control system of the veterinary vaccines in the candidate countries are not uniform. There are huge differences between the states according to the number of the vaccines and the work of the competent authorities. On this relatively small market there are 19 local companies. In some countries they produce a remarkable percent (about 50%) of the total vaccine volume, while in other one's the home production is almost negligible (2.6%). The additional part of the vaccines is mostly imported from the European Union, but there is also import from the USA and other countries.

Control of the veterinary vaccines in the candidate countries

Each competent authority requires registration samples for testing during the marketing authorisation procedure. On the other hand the control of the vaccines after granting the marketing authorisation is very different. Lithuania is the only country where the state control does not exist. The system of the state control in the different countries is shown on Fig. 1.

Fig.1: System of state vaccine control in the candidate countries

	batch to batch	random	control of certificate of manufacturer
Bulgaria	yes	yes	yes
Czech Republic		yes	yes
Estonia			yes
Latvia		yes	yes
Lithuania	no state control		
Poland	yes		
Romania	home products	imported products	all products
Slovakia		yes	yes
Hungary	rabies, ND vaccines	yes	yes

In Bulgaria and Poland there are batch-to-batch control for all the vaccines, while the Romanian authority apply batch-to-batch control only in the case of home products. In Hungary only the rabies and the Newcastle disease vaccines are controlled batch-to-batch by the competent authority, the other vaccines are controlled randomly. It means that in Hungary about 400 batches are tested in every year for quality and potency. All the candidate countries follow the requirements of the European Pharmacopeia and the methods written in the manufacturers' registration dossiers. Some of them also accept the methods of Office International des Epizooties (OIE) and the United States Code of Federal Regulations (CFR). All these methods require a lot of target and laboratory animals.

Fig. 2 shows the number of target animals used for vaccine control in three candidate countries last year.

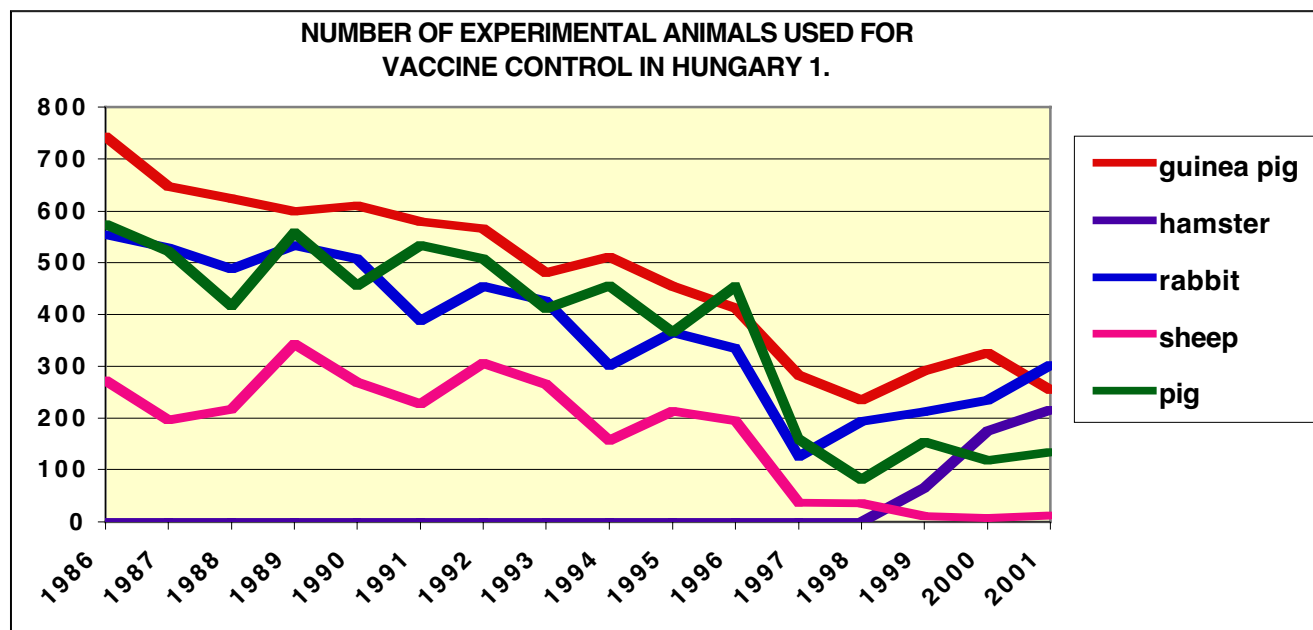
Fig. 2.: Number of target animals used for vaccine control in three candidate countries in 2001

	Czech Republic	Slovakia	Bulgaria
dog	64	13	5
cat	11	0	5
cattle	46	0	0
horse	98	0	0
pig	1701	0	15
sheep	0	0	30
poultry	110148	715	1000

References: Russel W. M. S., Burch R. L.: The principles of Human Experimental Technique. London, Methuen, 1959

In Hungary a significant decrease can be observed in the number of animals used for vaccine control in the last 15 years. In spite of this tendency about 10.000 mice are still used in Hungary in every year besides the large number of other laboratory and target species.

Fig. 3.:



What can the national authorities do to change this situation? The answer is not easy, as we have to follow the abovementioned requirements. Therefore harmonisation is necessary between competent authorities, international organisations and manufacturers to achieve the goal.

We have to accept, that there are still no suitable non-animal models in a lot of cases. An effort should be made to look for refined procedures that minimize pain and distress of our experimental animals. It means that the animals are kept under good circumstances and the painful actions (e.g.: blood sampling, intracerebral infection) are made in anaesthesia. In each country it has a legal background as an animal protection law is in force everywhere. They have paragraphs for experimental animals.

It is very important to reduce the number of target animals in the control. We can reach this goal with evaluation of laboratory animal models. There are a lot of examples for this among the inactivated vaccines: equine influenza, Aujeszky-disease or swine erysipelas vaccines. The next step is the replacement of laboratory animals with *in vitro* methods. For example instead of intracerebral injection of mice, extraneous viruses in the vaccines can be detected by inoculation of tissue cultures. To use the novel methods of molecular virology in the control is inevitable, as it can reduce the number of experimental animals. A PCR method was developed in our institute to identify the virus strains in the Newcastle disease vaccines. We found that some vaccines contained an additional strain, besides the declared one. This result would have required numerous chickens with the classical method.

Animal experiments still have a high importance in the vaccine control, but they have to be restricted to those areas where no true alternatives are available. As the safety and also the potency of the vaccines are priorities, the 4th R is very important: the *Reliability* of the new methods. The correlation between the classical and the new methods must be scientifically established.

Session II: Implementation of the 3rs: regulatory aspects

EXISTING THREE RS METHODS: ARE THEY REALLY USED?

Dr L. Bruckner, Institute for Virology and Immunoprophylaxis (IVI), (CH)

Existing 3Rs methods, Are they really used? A provocative title, but a question which has to be asked while looking at present testing for the quality control of vaccines.

Most of the examples presented, originate from testing of veterinary vaccines; but also in human medicines there is still a lot to do, to further encourage the use of 3 Rs methods.

Examples are presented from all fields of the 3Rs and a forth R is added: Remove.

Remove

Thanks to extensive review of data it could be demonstrated by the Paul-Ehrlich-Institute in Germany that results obtained from the abnormal toxicity test are of limited value in the case of products for human use and cannot produce conclusive results if products for veterinary use are tested.

As a consequence monographs in the European Pharmacopoeia on human and veterinary vaccines were adapted. The test was waived for products for veterinary use and replaced by the safety test in the target species.

What happened in reality. The vast majority of the manufacturers continued the performance of the test for each batch. Upon request manufacturers explained that the test has to be continued, because it is part of the licensing files. In addition it was necessary to continue the test because the authorities of some countries continued requesting to test every batch in the abnormal toxicity test.

For vaccines for human use the abnormal toxicity test was deleted in the European Pharmacopoeia on the final product. The test was kept in the production section. Moving the test upstream in production would lead to considerable reduction of animal usage.

In the case of vaccines for use in humans, too, no real break-through has been achieved. Manufacturers continue with the test on the majority of final batches because Ph.Eur. requirements are not the only requirements which have to be fulfilled. In developing countries, where large numbers of human vaccines are used, usually WHO recommendations are considered as the golden standard. According to WHO guidelines the abnormal toxicity test has still to be performed on each final batch. Experts of WHO are negotiating the necessity to continue to test for abnormal toxicity on each batch, but no consensus to change the testing requirements has been achieved, yet.

The same applies also to U.S. regulations and thus manufacturers are normally continuing the performance of the test on each batch.

Refine

In potency tests usually half of the animals become sick after virulent challenge and may die subsequently. Experienced animal caretakers can often foresee the death of sick animals. Humane killing of these animals can considerably reduce distress. The concept of humane killing of severely sick animals has led to a paragraph in the general monograph of vaccines for human use in the European Pharmacopoeia. It is stated that animals shall be humanely killed, as soon as sufficient indication of a positive result is obtained. A similar statement is included in European Pharmacopoeia general monograph for vaccines for veterinary use.

Recently a video on humane endpoints of rabies vaccine has been published by the Paul Ehrlich Institute; such material can be used for training of animal caretakers.

It is difficult to evaluate to which extent killing of sick animals is applied in routine testing. Usually manufacturers do not distinguish in batch test protocols between animals, which have been humanely killed and those which died from infection. Target animals may eventually profit of a better observation during the test and thus more often be redeemed from unnecessary distress.

Reduce

The monograph on swine erysipelas vaccine may serve as an example for the reduction of animal usage. The former monograph of the European Pharmacopoeia outlined the potency test as follows. 4 groups of mice were immunized with different doses of vaccine. Another 4 groups of mice were vaccinated with a standard vaccine. 3 weeks after immunization the mice were challenged with virulent *Erysipelothrix rhusiopathiae*. The animals were observed for 10 days after challenge. The potency of the vaccine was calculated by comparing the protective activity of the vaccine to be tested against the activity of the standard preparation. To pass the test the vaccine had to show a fixed minimum activity. In order to demonstrate that the vaccine under test is not less potent than the requested minimum activity, it is not necessary to test a whole series of dilutions; it is sufficient to test the vaccine in one single dilution. By careful selection of the dilution to be tested, it can be concluded from the survival rate within this single dilution, if the potency of the vaccine is superior to the minimum requirement. To demonstrate the validity of the test, the reference preparation has to be tested in a full series of dilutions.

This procedure allows to reduce effectively the number of animals to be used without a significant loss of information.

Only few manufacturers have changed to single dilution tests so far. The single dilution test would lead in addition to a reduction of costs. It is not possible to change to the single dilution test without any investments. The dilution of the vaccine to be tested has to be carefully selected, to assure with an acceptable confidence limit, that the tested vaccine has not less than the required potency.

The statistical approach can not only be applied to the monograph on swine erysipelas, but to any other potency test which compares the activity of the product under test with the activity of a reference preparation in a multi dilution test. The potency test for rabies vaccines is another example for such a test.

Replace

The last version of the monograph on rabies vaccines for veterinary use includes in the chapter batch testing a paragraph on the determination of the antigen content. This test would allow to abandon any test in animals for potency testing. The potency test involves, due to nature of rabies virus, serious distress for the animals and a considerable danger of infection for the laboratory personnel.

Nevertheless data on the determination of the antigen content of rabies vaccines are not submitted by any vaccine manufacturer to licensing authorities.

In contrast to Europe, determination of antigen content is common practice for many veterinary vaccines on the U.S. market.

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

Hints to overcome for the introduction of alternative testing

The following factors complicate the application of new methods in the quality control of vaccines:

the lack of harmonization between the different regulations the Pharmacopoeias in Europe, the U.S., Japan and elsewhere the recommendations of WHO for the products for human use the O.I.E. for veterinary products.

Harmonization of regulations is a must in a world with free trade. Every effort has to be undertaken to remove these artificial and not justified barriers.

the introduction of an new testis not free of costs, it needs financial investment, costs for the development and validation and costs for the communication of the change of test to authorities.

The introduction of an *in vitro* test does not automatically increase costs. Usually *in vitro* tests are cheaper to perform, and secondly results from these tests are available more rapidly than those from tests in animals.

Authorities should be convinced that changes in test methods which considerably reduce animal suffering should be dealt with free of charge.

the lack of knowledge about alternatives methods hamper the introduction of such testing.

The widespread dissemination of knowledge is essential for a better implementation and acceptance of alternative methods in the quality control of vaccines.

Session II: Implementation of the 3rs: regulatory aspects

DISCUSSIONS

Dr A. Sabouraud to Mr P. Castle: It is nice to see that the European Pharmacopoeia is open to register files without testing which is in the monograph and well documented and validated – this is a good step forward.

Mr P. Castle: it is true that mentalities have changed and we have managed to put this statement in the general notices which give people the basis for introducing this.

Dr A. Sabouraud: in some cases, it would seem that we still consider that challenge test will remain the reference test. However, we should keep in mind that switching to an invitro test or an alternative test would result to loosing the competencies of the challenge test which is high in some cases. It is probably difficult to keep the competencies and the know-how but keeping the test as a reference.

Mr P. Castle: It should be up to the manufacturers to ensure that if they do need to maintain the competence in-house, then they do this by doing the test at regular intervals otherwise, you could compromise your production, if you are not able to test the vaccine. In some cases, if we introduce a serological method that has been probably validated then the challenge test may go. In this case, I wonder if you do have to maintain the competence or not there are no examples yet as far as I know.

Comment from the floor: For new monograph and an example is the chicken vaccines, I noticed that the general intention is to reduce the general number of animals for the batch testing, however, for other tests the number of animals are increased. For the safety test you have to use a another group and this additional group is infected with a challenge strain and this is for a safety test of the vaccine, this infection is done to reproduce the disease however, we do not understand why this is going to be introduced into the monograph.

Mr P. Castle: The revision proposals for the Avian vaccines have replaced the chicken test in many cases, so that will mean that the tests are carried out in cell culture. Which is the vaccine that you stated?

Comment from the floor: It is a chicken vaccine monograph for live vaccine and this has a challenge group in addition to the groups which you normally have.

Mr P. Castle: This I would imagine is therefore a development safety test on the production and I think that this could only be for manix disease vaccine.

Dr R. Dobbelaer: I can only see one reason for this and that is to test the susceptibility of your animals to the non-inactivated virus. This is therefore a validation.

Comment from the floor: I think that it is necessary to define in more detail animal welfare because animal welfare is not equal to ‘absence of suffering’. Prof. Brume from Cambridge defined animal welfare as ‘the state of the animal trying to cope in its daily environment’. Therefore, I think that taking into account this definition, it is important to improve methods for welfare assessment especially concerning animals for laboratory experiments.

Comment from the floor: Prof. Brouwer is talking about farm animals and has written several books on how to measure welfare of animals and it is possible by looking at their behaviour as well as looking at their various hormonal levels and whilst it is not possible to check the hormonal levels for the work which we are doing here, I think that their behaviour is extremely indicative of whether they are suffering or not.

Any deviation from normality can be suspected as affecting their welfare.

Comment from the floor to Dr D. Mackay: I would like express my agreement with Dr Mackay – the better the guidelines are and the monographs are the easier it is for a manufacturer to carry out the testing meaning that the manufacturer does not have to do re-testing as there were no misunderstandings. I also hope that the new monographs which are due to appear will not change again in the next few years as these monographs cause a lot of trouble for the manufacturers and a lot of re-testing of old products which are on the market (since 20/30 years).

Comment from the floor: I would like to comment on the conclusion of Dr D. Mackay. I fully agree and follow your consideration. However, when I think about why the changes are so slow, you are right in saying that there is a reluctance in changing and accepting alternative methods. Habit and custom however prevents us from moving forward and obstructs sometimes the law, as we do have laws obliging us to follow alternative methods when they are available and when these are validated.

Attitudes therefore have to change as concerns responsibility to accepting new alternative methods. We cannot accept reluctance just because this is the habit or custom to do a test in a certain way.

Comment from the floor: Many thousands of animals are used to verify vaccines imported from new developing countries which we did not import from before and I think that we can adopt the recommendations to remove unnecessary experiments if that vaccine is in use already in European countries.

Dr D. Mackay: This is a good point in terms of the requirement for retesting all vaccines.. As far as I am aware there is no proposal under the 2001 community review of legislation but I think that this should be considered.

Comment from the floor: it would seem that the batch release process for a veterinary vaccine is much less developed than for human vaccines.

Mr J.M. Spieser: I have to ask for a correction: it could be misleading the comment from Dr D. Mackay saying that in an institute such as ours (EDQM) which is very careful in applying the 3Rs at any stage, that we would give through the implementation of that system an open door for increase in animal testing. What we are doing is putting order as you rightly mentioned, it is an very anarchical system at present as concerns the veterinary area and Dr D. Mackay explained very well as concerns the differences between the different member states and what we really would like to achieve is codification of this (and we are only at the beginning of the procedure). Recently, we have had very promising meetings on this in a not too far future, we will issue codified guidelines, and hopefully eliminate retests for a large number of vaccines which exist in Europe only for a certain number of products which have been traced as being problematic will retest be performed but under strict conditions and we will also hopefully arrive at a situation where there will be a periodically review of the situation, with a possibility to then diminish the retesting or modify it on the basis of practical cases and results through which it has been demonstrated that there is no added value.

This is to eliminate the impression that could be given that we would be the promoter of adding something, like increase of animal testing, however our goal is only to guarantee good quality of products through a codified system available as in the human field of vaccines.

Dr D. Mackay: I would not have wished to give this impression, however, the point which I was making was that the situation has a long way to go to be developed and it certainly has the potential to do that if it is mishandled. My point was to emphasise that we should keep as

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

our objective the need for added value when we are doing what you presented and contribute to harmonization.

Comment from the floor to Dr R. Dobbelaer: How many batches are you testing every year and what is the percentage of rejection (of the batches)?

Dr R. Dobbelaer: The percentage of rejection is extremely low, less than 1% of the batches which are either withdrawn from the batch release procedure or rejected.

This question is always put forward when a ministerial department asks us 'what are we doing if you are not rejecting batches' which might be also one of the reasons why you put the question forward. This does not give an answer as to what would happen if there was not a procedure for batch release.

The total amount of batches released, in our case, and in my own Institute it is over 1,000 per year.

Dr A. Sabouraud: There is another answer to this question – probably manufacturers produce good quality vaccines!

Comment from the floor: Based on your assay of IPV – I made a similar proposal for the veterinary part as we are getting ourselves into the position that we have serological assays namely toby testing as you have for human vaccines for diphtheria and tetanus this would reduce the animal use considerably for the OMCLs if they have the possibility in receiving the sera from the companies, and this does not mean that they skip all testing, but they are in a position to do full testing when they think it is really necessary, otherwise to continue with the samples of the manufacturer and in addition the manufacturer has an advantage of at least 3 to 4 weeks in time until the new sera is available at the OMCLs. There is a lot of reluctance however on the veterinary side therefore, how did you come to this position? Was this your position or was there an agreement with the OMCLs and how was this realized?

Dr R. Dobbelaer: This was done in the framework of the licensing of a new combined vaccine. Whenever there is a new license, at least a centrally licensed vaccine, there needs to be proposals for batch release and this is what we propose for batch release and this is what was accepted for batch release. There have been discussions about pros and cons of this approach but we mainly proposed it in the framework of the 3Rs approach and it halves the number of animals used in batch release.

Comment from the floor to Dr G. Kulcsar: Which were the main reasons for the decline of the use of animals in your country? It would seem that you are testing almost everything in your country – is there is special reason for the reduction of the use of animals related to 3Rs?

We saw this reduction in the use animals but in recent years it would seem that it has gone up again – is there a reason for this?

Dr G. Kulcsar: There is no special reason for this – the reason is that we have to follow the requirements of the international organizations, the European Pharmacopoeia and this is written in the manufacturer's dossier. This resulted in the decline of the number of animals. As you mentioned, there is an increase in the last few years of animals used for testing. This depends on the number of vaccine imported to Hungary as they are tested by our Institute – there is no special reason or programme to promote the 3Rs method in our institute.

Comment from the floor: Mr P. Castle told us this morning that there is a broad part of interpretation and own-initiative to comply to the European Pharmacopoeia requirements and also reducing animal testing. This is one point of view and perhaps, you could reduce some testing if you clarify if it is really necessary according to the European Pharmacopoeia's

requirements or not, or if you have another method you wish to use and the European Pharmacopoeia is in principle open to this.

My second point is that : If you are in position to have a member of industry, for example in group 15V, so if there are problems we should bring these to light and if there is good data, I know that the European Pharmacopoeia will consider this data, therefore please put an active input into this.

Comment from the floor to Dr L. Bruckner: Hearing both Mr Castle's and your presentation, I think that there is a need for some feedback on what implementation is based on. Especially, for example, rabies vaccine monograph and we experienced in the workshop on this issue that there are possibilities included in the monograph since 5/6 years but there have been no changes at all and we heard about the problems of this. There should be better feedback as concerns abnormal toxicity test – I do not know what the real situation is as we insisted on deleting the test and for most protocol this is not included anymore, this does not mean it is not done (at least in the protocols that we receive) I think that 99% simply do not include that abnormal toxicity test.

The costs of variations: the Paul-Ehrlich Institut has a new regulation that we can reduce costs however, this is only for human vaccines and I hope that this will be the case for veterinary vaccines also in future so that applications for variations which are targeted for 3Rs issues may be reduced or even waived.

Dr R. Dobbelaer: two comments: Abnormal toxicity test and the second, single dilution test.

I can only speak for the human vaccines sector but at least I have personally seen 5 or 6 variations applying for the deletion of the abnormal toxicity test with sufficient validation and all these have been approved. It is true that on the global level there is a dis-harmonisation. The argument which is put forward by WHO is that they have to take into account producers also from developing countries and the rationale is that the abnormal toxicity test is the very last minute safeguard against breaches in GMP. Levels of GMP are not equal in between the industrialized countries and the developing countries, this is why WHO is reluctant to accept this deletion. Manufacturers who sell vaccines also to WHO also have to, in some cases, perform the abnormality toxicity test, although it has been deleted from their marketing authorization application.

I know of at least three instances in the human vaccine field, where the single dilution approach has been submitted as a variation and accepted. This is true for acellular pertussis and for diphtheria antigens. There are some positive notes in this over all over view!

Mr J.-M. Spieser: To add to Dr R. Dobbelaer's comment: during the different visits which I have had the opportunity to do, over the recent years in different countries in OMCLs, there was an approach which could be considered, which was as long as there was not a GMP certificate and a GMP approval for a manufacturing site, there would be the continuation of this safety test for all products produced on that site but which then would be automatically reviewed once there is in place an approved and certified GMP. That could be a point that we could consider in order to begin to elucidate this issue globally. I know that a certain number of countries have began to organize this on their own national territories.

Dr T. Sesardic: There is general comment concerning the effectiveness of transfer of methodology to laboratories it would probably be most effective at the end of the validation studies, once the protocols are available. The purpose of the training should be at a specific time or the best suitable time is probably when the methods are available for purpose.

Session II: Implementation of the 3rs: Regulatory aspects
WORKSHOP III: IMPLEMENTATION OF 3RS METHODS:

Human Vaccines

CONCLUSIONS AND RECOMMENDATIONS

Dr R. Dobbelaer, (SIPH) (B) and Dr A. Sabouraud, Aventis Pasteur (F)

Rabies vaccine:

Development of guideline and database for relevant Glycoprotein antigen content ELISA (Mcl to protective epitopes)

Not yet ready for replacement of NIH challenge test

Use humane endpoints (video Dr. Morton)

Will *in vitro* = *in vivo*?

In many cases not (e.g. imprecise *in vivo* versus precise *in vitro*)

Get as « functional » as possible

« imperfection » should not inhibit acceptance for routine batch release of vaccine of demonstrated consistency

Sense and non-sense of OMCL repeat *in vivo* testing:

Repeating variable tests makes sense

Call for procedure for « waiving » (*in vivo*) testing: US system vs. EU « reduced » testing procedure

Shared serology between manufacturer/OMCL:

Pro: 50% animal use reduction

Con: loss of independent nature of OMCL re-test

Lack of harmonisation (EU, US, WHO – manufacturer, OMCL):

Causes redundant testing (also on same batch) for e.g. rabies vaccine, abnormal toxicity, aP, D and T (harmonisation efforts ongoing)

(Even subtle) differences in manufacturer/OMCL methodologies may cause unnecessary repeat testing

Session II: Implementation of the 3rs: Regulatory aspects
WORKSHOP IV: IMPLEMENTATION OF 3RS METHODS:

Veterinary Vaccines

CONCLUSIONS AND RECOMMENDATIONS

Dr K. Cussler, PEI (D) and Dr L. Elsken, UDSA (USA)

Slow / no use of 3R monographs

- Time needed for validation
- Too frequent changes?
- Loss of standards or references
- Monograph changes
- Continued acceptance of existing data/ methods?

Regulatory view

- law required?
- guide +/-
- Suggestion +/-
- Multiple approvals needed
- Multiple tests for same purpose
- If regulatory testing is only per monograph **then** product must pass per monograph?

Costs (time and money)

- Savings
- 3Rs ethics/ image
- redirect resources, people, facilities, to *in vitro*
- If test not needed/ not applicable don't do

Costs

- Validation studies
- multiple tests for one purpose
- needed? Due to formulation – assay issues
- unnecessary- multiple regulators = multiple standards
- New tests/ requirements (IBD + Immunosuppression?)

Future conclusions

- Awareness of validation costs, cost of change
- Reconsider need for (batch/ lot) host animal safety (n=2)
- Accept *in vitro* antigen quantification (batch) potency
- Reference AG, A, PREP + validation + sharing by regulators (ex. Leptospire)

How to read “Pharm”.

SESSION III: IMPLEMENTATION OF THE 3RS: NEEDS AND PRACTICAL EXPERIENCES

Efforts done and problems encountered by Industry

Dr A. Aerts, Intervet International (NL)

Experience in validation of alternative methods: case studies (Tetanus and Diphtheria)

Dr M.-E. Behr-Gross, EDQM, Council of Europe

Discussions

Conclusions Workshop V: Statistical aspects of validation studies

Mr A. Daas, EDQM, Council of Europe and Dr P. Volkers, Paul-Ehrlich-Institut (D)

Conclusions Workshop VI: In-house validation vs. inter-laboratory validation

Mr K. Redhead, Intervet International (UK) and Mr A. Akkermans, RIVM (NL)

Session III: Implementation of the 3Rs: Needs and practical experiences
EFFORTS DONE AND PROBLEMS ENCOUNTERED BY INDUSTRY

Dr A. Aerts, Intervet International (NL)

Implementation of the 3Rs has been a priority of Intervet International during many years. Being one of the major veterinary vaccine companies use of experimental animals during the development and batch control of these vaccines is of concern both from an ethical as well as from an economical point of view. In this presentation I would like to discuss with you the various aspects of the use of animals in a company like Intervet ranging from the objectives of a vaccine manufacturer to in the end suggestions for the future.

Objectives of vaccine manufacturers

The objectives of a veterinary vaccine manufacturer are very clear. We want to develop safe, efficacious vaccines, which have a constant quality and of course are cost effective. Secondly, in doing so we develop these products in compliance with regulatory requirements. We have regulatory requirements regarding the environment, regarding Good Manufacturing Practice and Good Laboratory Practice and of course all the relevant directives governing the registration of these products.

Vaccine quality

Instruments to monitor the quality of our vaccines can be divided into quality systems and quality tests.

Quality systems are: GLP and GMP, both of which have now been implemented in all the major vaccine companies and which have led to a very strong improvement of the quality and consistency in vaccine development and production within the veterinary vaccine industry.

The *in vivo* and *in vitro* tests performed during research and development and production include the studies which are required to establish the safety and efficacy profile of the vaccine in the target animal as well as all the *in vitro* and *in vivo* tests which are being applied to raw materials, intermediate products and final products. These are the instruments with which the quality and the consistency of products are currently being monitored in the veterinary vaccine industry.

And those are quite similar to those applied in human vaccine industry.

Why 3Rs in industry?

As stated in my introduction the 3Rs are very important for a company like Intervet. Mainly because Intervet is part of the society. With over 5000 staff, that is 5000 families in 53 countries, Intervet is positioned as a responsible company in the middle of the society. Also we have over 800 young, well-educated scientists who are involved in our research. These, you might say human factors, strongly regulate the principle of 3Rs in the company. In addition animal testing is of course also very costly and time consuming. You need containment facilities, SPF-flocks and long time taking animal experiments.

How is the 3R principle embedded into the organisation of the company?

First, we have during the R&D phase the Animal Experimentation Committee, which is of mixed membership with staff members from outside the company and inside the company and chaired by an external expert. It evaluates all the animal experiments which need to be done

during R&D of the product. The people who determine which studies need to be done are the R&D project members and Regulatory Affairs Staff. They look very critically which studies have to be performed to comply with the regulations and to obtain an authorisation in the European Union. The same people in R&D are responsible for the set-up of our batch testing methods. They will propose the necessary tests and indicate whether a study will be an *in vivo* or an *in vitro* test based on their interpretation and their technical know-how. In this respect there is a difference between the US and Europe. The US is much more directed toward *in vitro* potency tests and towards batch safety tests in non-target animals.

I think we could learn from developments in other parts of the world and the implementation of 3Rs as done over there.

Examples: implementation 3Rs

Let us now discuss shortly some examples of the implementation of the 3Rs in the company: To start with reduction.

By increasing batch sizes of our final products we have been able during the last 5 years to diminish the number of experimental animals of batch release with over 30%. This of course at the cost of large investments in equipment and in increased stock and risk. We have now reached the limit of this batch increase and we look for other ways for diminishing the use of experimental animals. One of that may be the combination of animal studies, both in R&D as well as in batch release. Also the elimination of studies, like the elimination of batch safety tests, after having obtained a proven track record will lead to reduction of use of animals. The use of sequential challenge or combination of one-dose, over-dose and repeated dose tests during R&D will lead to a reduction in animals used.

Refinement of animal testing has been achieved by determination of so-called protective markers, which co-relate with efficacy. By the establishment of protective markers it is no longer needed to perform challenges during subsequent efficacy studies or during field or duration of immunity trials. Also we have modified batch release challenge tests and we have implemented batch-release tests based on serology.

And finally we have been able to replace animal tests by *in vitro* tests by implementing *in vitro* potency tests and also by the replacement of *in vivo* extraneous agents tests of viral vaccines by *in vitro* extraneous agents tests.

Interactions with regulatory authorities

All this has to be discussed with and approved by regulatory authorities. This is not a strait forward process. In some cases we find opposition against the diminished use of experimental animals by regulatory authorities. One of the main problems is the lack of harmonisation of interpretation of regulatory guidelines. So it may well be, and this has happened too often, that a company has to do a specific animal test for one specific country while the other countries have already accepted, leading to unnecessary additional testing. Another point of concern is the increased requirements for data packages to be submitted when small variations to the registration dossier are being sought for. Extensive stability testing or animal experiments lead to increased costs but also unnecessary use of experimental animals in trying to have variations approved.

I would also like to mention the increased role of statistics in safety and efficacy trials, which require an increasing number of animals without often leading to an improved test result.

Then there is an increasing number of problems encountered during development of new products where the trend within regulatory authorities is that any risk has to be eliminated and

Replacement, reduction and refinement of the use of animals in the quality control of vaccines

therefore we are required to invest large number of animals and large sums of money in additional animal experiments of which the usefulness is often uncertain. One specific recent development I would like to mention, which is the proposal to introduce the OMCL system also for veterinary vaccines. For human vaccines, with only 50 vaccines on the European market the harmonised batch release by an OMCL laboratory seems to be feasible. However, within the veterinary vaccine industry we have more than 350 products on the market. Implementation of such a system will lead to a tremendous pre-validation phase with a concomitant use of large numbers of experimental animals and without any certainty that this system will lead to improved quality of products entering the market. We would hope that this system will not be used for veterinary industry and instead we concentrate on the existing QA/QC system and the existing quality systems in the industry. Of course there are also positive aspects on our interactions with regulatory authorities. The collaboration we have on the development of improved potency tests is very positive. Erysypela's, Leptospirosis and Newcastle vaccines have now improved potency tests without the need of challenge. Also some countries are sympathetic to our efforts to eliminate the routine use of batch safety testing when the vaccine has a proven track record and pharmaco vigilance data have shown there is no problem to be expected or observed in practice.

A third point is the support we have received for the replacement of *in vivo* extraneous agents testing for viral vaccines, although I must say that this will only lead to a diminished number of experimental animals when elimination of this test is combined with elimination of routine batch safety testing, because they are often combined.

This brings us to suggestions for the future in order to even better implement the 3R principle in veterinary vaccines practice.

What does Industry need for further implementation 3Rs?

Most importantly, an open discussion and positive interaction between industry and regulators is needed. Instead of using the no-risk principle as a guideline, regulatory authorities would have to consider the extend of animal testing in relation with the presence of existing knowledge and data. In this respect the acceptance of the existing QA/QC system for official batch release will prevent the introduction of unnecessary animal testing without compromising product quality.

Finally, guidance from the CVMP regarding the requirements for *in vitro* potency testing could encourage its introduction in the EU.

Session III: Implementation of the 3Rs: Needs and practical experiences

**EXPERIENCE IN VALIDATION OF ALTERNATIVE METHODS: CASE STUDIES
(TETANUS AND DIPHTHERIA)**

Dr M.-E. Behr-Gross, (EDQM), Council of Europe

Alternatives to animal experiments for the quality control of medicines should be implemented whenever possible for ethical reasons.

This approach, known as 3Rs concept, is supported at the European level by the Council of Europe Convention¹ and the European Commission Directive². The implementation and acceptance of alternatives by the competent authorities has been promoted by the adoption of the European Convention on animal protection by the European Pharmacopoeia.

In this framework monographs are revised in order to phase out animal tests which have become superfluous, to replace animal tests by *in vitro* methods whenever possible or to reduce the numbers of animals and to refine methods if deletion or replacement is not possible³.

To support this general move away from animal testing for routine quality control, collaborative studies have been initiated in the framework of the joint Biological Standardisation Programme of the Council of Europe and the European Commission⁴. Several projects involving the different stakeholders in the field i. e. manufacturers and national, regional or international official bodies are currently ongoing or have been successfully completed.

Priority has been given to validation of alternatives to methods implying severe distress of animals and to the establishment of new Biological Reference Preparations which are needed for the alternative tests^{5,6,7,8,9}. Among these studies the validation of serological assays for batch potency testing of tetanus^{5,6} (projects BSP019-BSP035) and diphtheria (project BSP034) vaccines for human use (Table 1) represent interesting case studies.

Table 1. Validation studies of serological methods for batch potency testing of tetanus and diphtheria vaccines for human use

	BSP019-BSP035	BSP034
Reference method	Assay of tetanus vaccine adsorbed (Ph. Eur. 2.7.8)	Assay of diphtheria vaccine adsorbed (Ph. Eur. 2.7.6)
Principle of reference method	Quantitative direct challenge in mice and guinea pigs (lethal or paralytic)	Intradermal or lethal challenge in guinea pigs
Alternative methods	Toxin binding inhibition test ELISA	Vero cell assay ELISA
Status	completed	ongoing
Project leaders	C. Hendriksen – D. Sesardic – R. Winsnes	
Statisticians	A. Akkermans - A. Daas - P. Rigsby	
Project monitors	M-E Behr-Gross - M Berg-Candolfi - G. Rautmann – J-M Spieser	
Participants	Vaccine manufacturers and official medicines control laboratories involved in vaccines production and testing (Europe – Americas – India – Australia)	

The acceptance of alternatives to current methods by manufacturers and regulatory authorities is depending on the availability of appropriate experimental data. Such data can be obtained through international collaborative studies, following initial development and method transfer studies.

Based on the experience gained in the Biological Standardisation Programme programme, key features for conduction of validation studies can be elaborated. Compliance with the various points listed in Table 2 have a positive impact on the quality of the validation study results, but might easily lead to study duration of several years due to the heavy work load imposed on organisers and study participants.

Table 2. Critical points for validation of alternative methods based on experience gained in Biological Standardisation Programme

1.	Enrolment of scientists (Project leaders/Scientific advisors), pilots laboratories and statisticians
2.	Evaluation of potential alternative method(s): cost, duration, availability of reagents
3.	Evaluation of data available on alternative method(s) in the context of quality control of medicines
4.	Pre-selection of methods to be evaluated, choice of animal model for serology
5.	Careful elaboration of study plan by project leaders and organisers, with a sequential organisation
6.	Identification of potential participants based on experience in the field
7.	Selection of test samples representative of marketed products and inclusion of borderline quality product(s)
8.	Sourcing of non commercially available reagents and test samples
9.	Performance of preliminary assays according to standard operating procedures available in pilot labs
10.	Determination of parameters influencing assay results to determine level of standardisation needed
11.	Careful drafting of protocols and documents, review process involving participants
12.	Provision of data reporting sheets to participants
13.	Performance of independent centralised statistical evaluation for each step of study
14.	Performance of retrospective statistical evaluation if needed
15.	Evaluation and critical analysis of the outcome of each step
16.	Performance of additional experiments if needed
17.	Adaptation of study plan, in the light of conclusions of completed steps

The length and complexity of validation studies is a draw back for the rapid implementation of the 3Rs concept. However on the other hand high quality of the data generated in a collaborative study is an important prerequisite for modifying the current European regulations on the control of biologicals.

Overcoming remaining obstacles for the implementation of alternatives, either of economical or technical nature, will nevertheless require the commitment of both the private and public sector organisations in charge of the quality control of biologicals.

References

1. European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Council of Europe, *ETS* 1237-1986.
2. Council Directive 86/60/EEC of 24 November 1986 on the Approximation of Laws, Regulations and Administrative Provisions of the Member States regarding the Protection of Animals used for Experimental and other Purposes. *Off. J. European Commission* L358, p1-29.
3. Artiges A. Alternatives to Animals in the Development and Control of Biological Products for Human and Veterinary use. The Role of the European Pharmacopoeia. *Dev. Biol. Stand.*, Kärger Basel 1999, vol **101**, p29-35.
4. The Biological Standardisation Programme. *Pharmeuropa* Special Issue BIO 96-1, p1-5.
5. Collaborative study for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use, part 1. *Pharmeuropa* Special Issue BIO 2000-1, p83-124.
6. Collaborative studies for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use, *Pharmeuropa* Special Issue BIO 2001-2.
7. Collaborative study for the establishment of *Clostridium tetani* guinea pig antiserum (human) Ph. Eur. BRP Batch No. 1. *Pharmeuropa* Special Issue BIO 2001-1, p5-11.
8. Collaborative study for the establishment of *Clostridium tetani* antiserum Ph. Eur. BRPs for serological potency testing of tetanus vaccines for veterinary use. *Pharmeuropa* Special Issue BIO 2000-2, p43-54.
9. Collaborative study for the establishment of *Clostridia* antiserum Ph. Eur. BRP for serological potency testing of *Clostridia* vaccines for veterinary use. *Pharmeuropa* Special Issue BIO 2000-2, p65-87.

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DISCUSSIONS

Dr D. Mackay (following Dr R. Aerts' presentation): One point which could illustrate the problem on both sides of the fence is the issue of variations which could well be of interest to the 3Rs and yet, companies have to end up paying for them. There is a real problem in that essentially quite often the changes which are being introduced may potentially impact on the quality of the product and the issue is to be able to have the data in order to ensure that they do not. That data then needs to be assessed. Essentially, the question is who pays for the assessment, and certainly in most member States now, it is perceived that it is the industry which should pay for those kind of changes. If the suggestion, and this is good suggestion, that there is an impediment to companies seeking to introduce these changes which are of little benefit to them directly, but certainly do benefit the 3Rs, if one of the impediment is the fact that they have to pay and generate all the necessary data. This should be one of the points which should figure in the conclusion of the meeting is to look at how can a system be found to pay for this? Ultimately, this means that it will be the tax payers have to pay for it and are they in agreement to pay?

Comment from the floor: I do not think that the money is the most important factor – timing is much more important. When are you able to introduce this in all the countries? If there is real concern about the change of a product then I would agree with you because it means major investment for the companies to introduce these. We would of course then be very pleased to receive support in doing that.

Comment from the floor: I think that the two spoke about one problem however, in the industry we are having to change components all the time. We have an example in our company concerning production media to avoid the BSE problems and to give better quality to the antigen, we made a huge effort to make a semi-synthetic media. I agree with you that after this we also have to do some trials in order to justify that these changes are improving the quality of the vaccines.

My question is that knowing that you will be meeting with the regulators responsible for this, I would like to know the feedback of these discussions and if the tests will be reduced for the manufacturers if an ingredient should be changed in the media.

Reply: this depends on what kind of change you have and what kind of product you have. I agree that it makes a difference if you change something for chicken vaccine or cattle vaccine (BSE) and also if you have to repeat all you cattle studies, it will take four years but if you have to do some studies on chickens, it will only take you a few months. Therefore, this is a case-by-case basis.

Dr M. Tollis: Dr Aerts mentioned a very important point, where reduction refinement replacement of tests is very important and you mentioned minor species. We are discussing this point in several expert groups at present. In your opinion, what is the scientific basis in avoiding some specific studies on minor species or minor indications. You mentioned the canary pox vaccine but this is not a simple vaccine which could have disastrous effects, this has happened in the past where the problems were related to processing the virus. However, what is the scientific basis in allowing minor studies for minor indications or minor species.

Dr R. Aerts: the scientific basis is not clear cut – this depends on whether you develop an ND vaccine for pigeon, all the ND vaccines or other species already licensed and they use the same strain, then I would say that there is a scientific basis to have various studies in pigeons

as you have a lot of knowledge about the strain and the disease in other poultry species. This is an easy example. If however, it is a totally dedicated strain for a minor species, then the scientific arguments are not so strong. If you put the levels of studies at the same level as the others, nothing will be developed. We are split into two different directions, if we do what is scientifically justified, no products will come and if we alleviated products may come and you may have some, and the company can make a risk assessment for that, some risk may be accepted.

For ND there is no problem. For something very specific the risk assessment has to be made but we will not develop anything other for canaries at this stage.

Comment from the floor: For influenza I compared the human vaccine testing or potency testing and the veterinary potency testing. For the human vaccine you have an *in vitro* test where you only have to determine the haemagglutinin content. For the veterinary testing, there is a potency testing which is necessary – why is this?

Dr M. Tollis: The problem is related to influenza virus – it the reference preparation, what you have for human vaccines, you do not have for veterinary vaccines. This is the main problems. You can compare the quantity of haemagglutinin present in human vaccines but you cannot do this easily for all the haemagglutinin present in a veterinary vaccine. We faced this problem when we wanted to make reference preparation for influenza. You need at least your in-house preparation to compare and this is not easy.

Comment from the floor: There is also another couple of issues related to equine and human vaccine for influenza. The first is that most of the equine vaccine are adjuvanted and therefore antigen quantification on the final product is quite difficult. The whole issue of the efficacy estimation for equine flu vaccine is based on challenge systems in the target species, which it is not in the human system and because horses are traded internationally and there is legislation concerning the requirement for horses to be vaccinated on a regular basis, the jockey club is extremely insistent that there is every possible assurance that those vaccines are of maximal efficacy and it is felt that you get a better assurance of efficacy testing by doing the testing on a batch basis than you would on mere antigen quantification.

Comment from the floor to Dr M.-E. Behr-Gross's: concerning TOBI testing for tetanus. Thank you for this work which will enable us to replace the TNT test for our vaccines concerning tetanus valence. My concern is for one of the reagents which is the toxin at 300 Lf per ml. It was confirmed when I spoke with my colleague, that it is dangerous to work with this toxin and my proposal is that if we can replace this toxin by toxoid of the same value – 300 Lf? There is a Bresilian team which did this with toxoid – can we do this work without validation or is this too risky that without a good validation we will not have the same results than with the toxin?

Dr M.-E. Behr-Gross: You will have to make the validation internally. If you want to implement TOBI, you need to show in your laboratory to show that you are able to reproduce the levels of repeatability which were observed in our collaborative study (BSP035) and that for your vaccines, TOBI is an efficient. Of course, if you wish to switch to toxoid rather than toxin for TOBI it is a good time too as the in-house validation still remains to be done. In the tetanus study some experiments were run during the pre-validation and apparently the toxoid was equivalent to the toxin. The main reason for leaving toxin in was because it was mimicking the toxin neutralisation *in vivo*. In the end, if we use TOBI for batch potency control we might have to be less stringent as regards to the use of toxin and/or toxoid. The demonstration that you are able to assess potency of vaccine with TOBI, either with toxin or with toxoid, is submitted to validation.

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Dr T. Sesardic: All the data up until present, shows that there is no need to use toxin in any of these tests for *in vitro*.

Comment from the floor: Could you explain the meaning of animal welfare in the statement: "animal welfare aspects for immunobiologicals are crucial".

Dr M.-E. Behr-Gross: It is a permanent feature of immunobiologicals that *in vivo* tests are required. This has to be addressed and considered as a main target for 3Rs when drafting or revising monographs for immunobiologicals. Animal welfare is an important aspect and wherever possible it should be avoided to perform *in vivo* testing, and if this is not possible an alternative like refinement should be used.

Comment from the floor: It was shown for human vaccines that a guinea-pig serological model is more suitable than the mouse. For veterinary vaccines, have you looked at this or do you know if guinea-pig is more suitable than mouse or if it is mice that is more suitable?

Dr M.-E. Behr-Gross: For tetanus veterinary vaccines, I am not sure that there has been work performed on mice because the models which have been chosen were rabbit and guinea-pigs so there are still two models allowed according to the European Pharmacopoeia monographs. I do not know if one has demonstrated superiority.

Mr J.-M. Spieser: For the tetanus, the studies were anterior to the human ones and in the monograph you have the choice between guinea-pig and rabbit anti-sera and it was the tetanus which induced then the whole study on the human vaccines.

Comment from the floor: There is no data to show if the rabbit is a suitable animal that mimics antibody responses in sheep or in cattle.

Dr M.-E. Behr-Gross: there is data on which the experts have based the decision that there will be two models.

Dr T. Sesardic: There has been quite a lot work years ago comparing immune responses of vaccines in different species and of all the animals guinea-pigs were chosen on the basis of providing consistent data cross-fold of all the antigens that are used in vaccines. Guinea-pigs were chosen for the ability to be able to demonstrate the dose response to different antigens. Mice tend to have a wider variability and therefore do not have the ability to provide a dose response for many different antigens.

Dr R. Dobbelaer: When you discussed about the movement concerning the single dilution test it may have been understood that you do not need a reference vaccine which is not the intention. This will still be needed – a comparison, or a reference, will still be needed even for that approach.

Session III: Implementation of the 3Rs: Needs and practical experiences
WORKSHOP V: STATISTICAL ASPECTS OF VALIDATION STUDIES

Mr A. Daas, and Dr P. Volkers, Paul-Ehrlich-Institut (D)

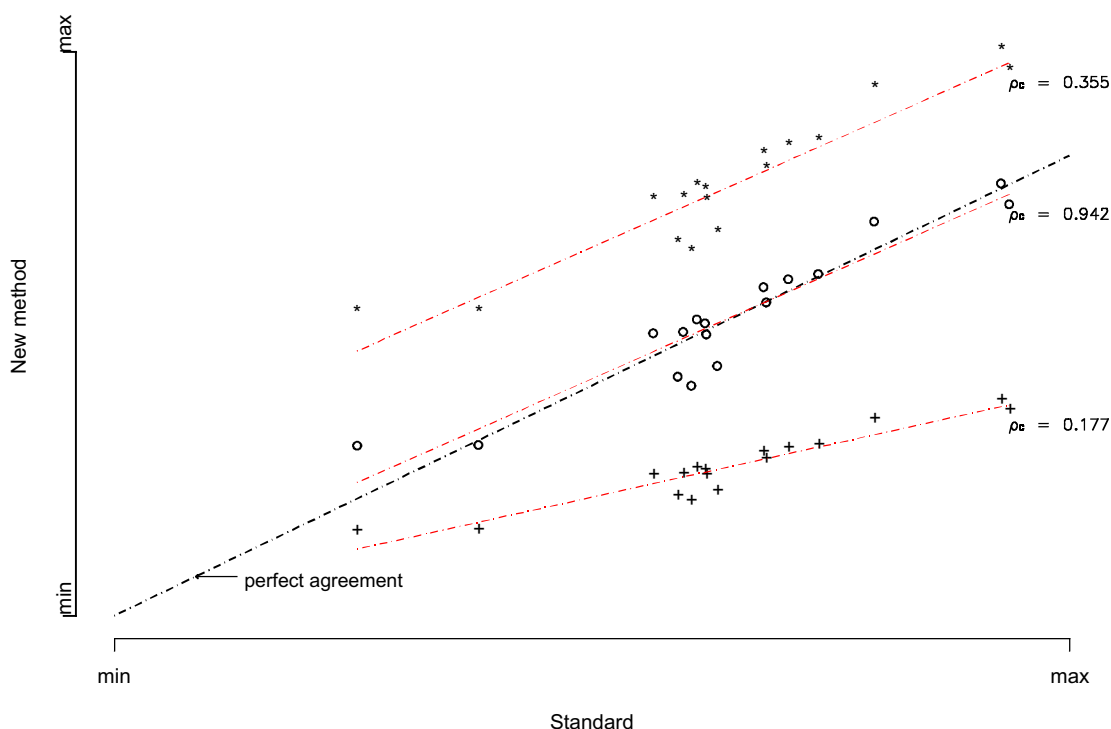
Part 1 - Assessing Agreement

By Dr. Peter Volkers, PEI, (D)

To demonstrate the validity of a new (alternative) method an approach often applied is to plot the results obtained with the new method against those from the ‘old’ method (standard) and to calculate the correlation coefficient between both sets of measurements. But, this provides only part of the information asked for. Substantially different extents of agreement might result in the same correlation coefficient. Thus the correlation coefficient alone is not a sufficient measure to analyse agreement.

To assess not only the linear association measured by the correlation coefficient but the concordance Lin (1) proposed a modified measure for assessing agreement, called ‘concordance correlation coefficient (ρ_c)’. This concordance correlation coefficient takes values between -1 (indicating perfect reversed agreement) and 1 (indicating perfect agreement). A value of 0 for the concordance correlation coefficient indicates that the correlation of both methods to be compared is 0 .

The figure below provides the concordance correlation coefficient for three datasets with identical correlation coefficient (0.943). In dataset (*) both methods differ in their mean value (while the variances are similar) while in dataset (+) the results of both methods differ with respect to mean as well as variance.



How to calculate the concordance correlation coefficient:

Assume that n pairs (y_{1j}, y_{2j}) of measurements with both the new and the standard ('old') method are available and that the data follow a normal distribution. Then the following steps are to be taken to calculate the concordance correlation coefficient ρ_C :

1. Calculate for each of the two methods ($i= 1,2$) the mean : $\bar{y}_i = \frac{1}{n} \sum_{j=1}^n y_{ij}$ and the

$$\text{maximum likelihood estimate for the variance: } S_i^2 = \frac{1}{n} \sum_{j=1}^n (y_{ij} - \bar{y}_i)^2 .$$

- 2 .Calculate the maximum likelihood estimate for the covariance

$$S_{12} = \frac{1}{n} \sum_{j=1}^n (y_{1j} - \bar{y}_1)(y_{2j} - \bar{y}_2)$$

3. Estimate ρ_C by $\hat{\rho}_C = \frac{2 * S_{12}}{S_1^2 + S_2^2 + (\bar{y}_1 - \bar{y}_2)^2}$

Example:

The table below presents the potency of 9 samples measured using two different methods:

Sample	1	2	3	4	5	6	7	8	9
Method 1	1228	1335	1243	1339	978	1034	1121	1194	1324
Method 2	1120	1292	1212	1201	998	989	1220	1210	1286

Thus

$$\bar{y}_1=1199.6, \bar{y}_2=1169.8, S_1^2=15407.8, S_2^2=11112.2, S_{12}=11067.6$$

therefore the concordance correlation coefficient is estimated as

$$\hat{\rho}_C = \frac{2 * 11067.6}{15407.8 + 11112.2 + (1199.6 - 1169.8)^2} = 0.808$$

Using some algebra one can show that the concordance correlation coefficient is the product of the usual correlation coefficient and a factor measuring the difference in location (i.e. means) and scale (i.e. standard deviations) between both methods to be compared.

Hypothesis testing, sample size

Based on the asymptotic normal distribution of the Z-transformed of the concordance correlation coefficient ($Z_{\rho_C} = 0.5 * \ln((1+\rho_C)/(1-\rho_C))$) it is possible to perform statistical inference (e.g. hypothesis testing, sample size estimation) in order to assess agreement of two methods.

To calculate prior to an experiment the sample size necessary to assess the validity of a new method by means of the concordance correlation coefficient with a prespecified statistical confidence one has to make assumptions. Especially one has to prespecify the maximal acceptable deviation with respect to precision (described by ρ^2 , ρ being the usual Pearson

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correlation coefficient), and accuracy (described by differences in means (μ_1 , μ_2) and variances (σ_1^2 , σ_2^2) respectively), i.e. one has to specify

- the maximal acceptable deviation x in precision (resulting in the least acceptable correlation $\rho' = \sqrt{\rho^2 - x}$)

as well as the

- the maximal acceptable (relative) shift in means (defined by $u = (\mu_1 - \mu_2) / \sqrt{\sigma_1 \sigma_2}$)
- the maximal acceptable shift in variances (defined by $1-v$, where $v = \sigma_1 / \sigma_2$)
- the Type I error α (usually 0.05) as well as the Type II error β (e.g. 0.1 or 0.05) for a specific alternative (e.g. $\rho_{c,least} = \rho$).

Based on these specifications one can calculate to the least acceptable concordance

correlation coefficient ($\rho_{c,least} = \frac{2 \cdot \sqrt{\rho^2 - x}}{[v+1/v+u^2]}$) as well as the number of (paired) replications

necessary for testing of a concordance correlation coefficient larger than the least acceptable $\rho_{c,least}$. For details of these calculations, please refer to (2).

Example:

Assume a study to be conducted to assess the reproducibility of a new assay (T, easy to perform), and to compare it to an old standard (R). Assume further that, under ideal conditions the assay T could explain 97% of R ($\rho = .985$). If one determines that a 2% loss in precision ($x = 0.02$), a 12.5% shift in location ($u = 0.125$) and a 10% scale shift ($v = .9$) would be acceptable, the least acceptable concordance correlation coefficient $\rho_{c,least}$ would be 0.962 and 51 paired samples are needed in order to control both Type I and Type II error rates at 5% (see (2)) in a one-sided test for testing ρ_c larger than $\rho_{c,least}$.

References

1. Lin I.-K.L. A concordance correlation coefficient to evaluate reproducibility. *Biometrics*, **45**, 255-268 (1989)
2. Lin I.-K.L. Assay validation using the concordance correlation coefficient. *Biometrics*, **48**, 599-604 (1992)

Part 2 - Reducing the number of animals by moving from multiple dilution assays to single dose assays

Mr Arnold Daas, (EDQM), Council of Europe

The possibilities of reducing the number of animals by moving from routine multiple dilution assays to single dose assays is illustrated with a few numerical examples and some simple guidelines.

An example from practice:

Below is given a typical example of results from a lethal challenge assay in guinea pigs for tetanus vaccines (adsorbed)

Standard		Test	
Assigned potency	250 IU/ml	Potency	? IU/ml
Doses	Survival rates	Dilutions	Survival rates
4.0 IU (= 16 µl)	12/12	1/125	12/12
2.0 IU (= 8 µl)	8/12	1/250	7/12
1.0 IU (= 4 µl)	3/12	1/500	0/12
0.5 IU (= 2 µl)	0/12	1/1000	0/12

Probit analysis of these results yield the following statistics:

Estimated potency	393 IU/ml
Lower 95% confidence limit	283 IU/ml
Upper 95 % confidence limit	546 IU/ml
Slope	2.63 probit(y)/ln(dose)

For batch release purposes this assay gives much more information than necessary because it is sufficient to show that the lower confidence limit is not less than 80 IU/ml (assuming 0.5 ml per human dose)

The number of animals can be reduced dramatically by adopting another approach: An optimised single dose assay. A possible assay of the above test vaccine could for example be as follows:

Standard		Test	
Assigned potency	250 IU/ml	Potency	? IU/ml
Dose	Survival rates	Dilution	Survival rates
1.0 IU (= 4 µl)	3/8	1/80	8/8

The survival rates at these specific dilutions justify the conclusion that the lower confidence interval of the potency is more than 80 IU/ml (one sided p-value = 0.013 using Wilcoxon-Mann-Whitney's exact test).

The above example shows how the number of animals necessary for one assay is reduced from 48 animals per vaccine to only 8 animals per vaccine, a reduction of more than 80 per cent.

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In practice it may be desirable to include some positive and negative control animals to monitor the quality of the assay, but the possibility of an important reduction of the use of animals is evident.

Useful guidelines to determine the optimal doses and number of animals required:

Determine the following key-values:

A: The minimum required potency. In the example this is 80 IU/ml.

B: The target potency. This is the minimum potency that the assay must be able to discriminate from A with a high probability. The closer this value is to A, the more animals are needed. If for example production batches vary between 200 and 400 IU/ml, the target potency might be set at 200 IU/ml.

C: The expected ED50, based on historical data. The laboratory in the example has probably an ED50 of approximately 1.5 IU.

D: The expected slope of the regression based on historical data. The assay in the example had a slope of 2.63 probit(y)/ln(dose). The flatter the slope, the more animals are needed. To be on the safe side, a slight underestimation of the expected slope might be taken. If for example the slope varies between 2.3 and 2.7 probit(y)/ln(dose) a value of 2.4 could be taken.

So let us assume the following values:

A = 80 IU/ml

B = 200 IU/ml

C = 1.5 IU

D = 2.4 probit(y)/ln(dose)

The optimal dose of the standard can then be calculated as

$$C \times \sqrt{\frac{A}{B}} = 1.5 \times \sqrt{\frac{80}{200}} \approx 0.95 \text{ IU}$$

And the optimal dilution of the test vaccine is

$$C / \sqrt{AB} = 1.5 / \sqrt{80 \times 200} \approx 1/84.4$$

Remark: The rules for rounding are slightly different than usual in order to warrant the statement that the minimum requirement of 80 IU/ml is met. The usual rounding rules could push the test-limit below the required value.

The value $D \times \ln\left(\frac{B}{A}\right) = 2.4 \times \ln\left(\frac{200}{80}\right) \approx 2.2$ can be linked to the number of animals necessary to discriminate between A and B with a given probability (e.g. 95 per cent). The following tables can be used as a suggestion for the number of animals to use. (The tables were obtained with Monte Carlo computer-simulations).

Table 1: Minimum number of animals per group to be used when it is accepted that B cannot be discriminated from A in approximately 5 per cent of the assays.

$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals
≥ 1.0	40	≥ 1.4	22	≥ 1.8	12	≥ 2.2	10
≥ 1.1	32	≥ 1.5	18	≥ 1.9	12	≥ 2.3	10
≥ 1.2	28	≥ 1.6	16	≥ 2.0	11	≥ 2.4	9
≥ 1.3	24	≥ 1.7	15	≥ 2.1	10	≥ 2.5	8

Table 2: Minimum number of animals per group to be used when it is accepted that B cannot be discriminated from A in approximately 10 per cent of the assays.

$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals
≥ 1.0	32	≥ 1.4	17	≥ 1.8	11	≥ 2.2	9
≥ 1.1	26	≥ 1.5	16	≥ 1.9	11	≥ 2.3	9
≥ 1.2	23	≥ 1.6	12	≥ 2.0	10	≥ 2.4	8
≥ 1.3	21	≥ 1.7	12	≥ 2.1	9	≥ 2.5	8

Less than 8 animals per group should never be used.

Of course this is only a first suggestion. Other (empirical) considerations may play a role in the choice of dilutions and number of animals.

Extension to quantitative data:

This reasoning can easily be extended to assays depending upon quantitative responses. As an example, let us consider a serological assay yielding quantitative antibody-titres that should replace the former example of lethal challenge.

Again, four key-values have to be determined:

A and B are taken as before. In the example this is 80 and 200 IU/ml respectively.

C: The dose which is expected to give the steepest slope (or the best regression). Usually this is the value located in the middle of a sigmoid dose-response curve (the point of inflexion). This value is not necessarily the same as in the challenge assay (and may even be very different). For this example, however, we will assume the same value of 1.5 IU.

D: The expected slope at dose C, divided by the expected standard deviation of the responses at dose C. The flatter the slope and the higher the standard deviation, the more animals are needed. Let us assume that this value is about $2.2 \ln(\text{dose})^{-1}$.

The optimal doses are calculated in the same way as for quantal responses. The value $D \times \ln\left(\frac{B}{A}\right) = 2.2 \times \ln\left(\frac{200}{80}\right) \approx 2.0$ can be linked to the number of animals needed to discriminate between A and B with a given probability.

Table 3: Minimum number of animals per group to be used when it is accepted that B cannot be discriminated from A in approximately 5 per cent of the assays.

$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals
≥ 1.0	24	≥ 1.4	13	≥ 1.8	8
≥ 1.1	20	≥ 1.5	11	≥ 1.9	7
≥ 1.2	17	≥ 1.6	10	≥ 2.0	7
≥ 1.3	14	≥ 1.7	9	≥ 2.1	6

Table 4: Minimum number of animals per group to be used when it is accepted that B cannot be discriminated from A in approximately 10 per cent of the assays.

$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals	$D \times \ln\left(\frac{B}{A}\right)$	Animals
≥ 1.0	20	≥ 1.4	10	≥ 1.8	7
≥ 1.1	16	≥ 1.5	9	≥ 1.9	6
≥ 1.2	14	≥ 1.6	8	≥ 2.0	6
≥ 1.3	12	≥ 1.7	7	≥ 2.1	6

Less than 6 animals per group should never be used.

An example of assay-results (listed are the antibody-contents in IU/ml per animal):

Standard		Test	
Assigned potency	250 IU/ml	Potency	? IU/ml
Dose	0.95 IU	Dilution	1/84.4
Animal	Antibodies	Animal	Antibodies
1	<0.001	1	0.070
2	<0.001	2	0.056
3	0.001	3	0.038
4	0.002	4	0.042
5	0.001	5	0.087
6	0.006	6	0.023

Wilcoxon-Mann-Whitney's exact test is highly significant (p-value < 0.001) and it can therefore be concluded that the test vaccine contains more than the required 80 IU/ml.

Note: Wilcoxon-Mann-Whitney's exact test is equivalent to Fisher's exact test in the case of binomial data. Wilcoxon-Mann-Whitney is a more universal test because it also applies to quantitative data and scores.

Selected further reading

1. R. Dobbelaer, P. Kight, J. Lyng. (1995). Manual of laboratory methods for testing the potency of final vaccines used in the WHO expanded programme on immunization: Use

and validation of a single vaccine dilution assay for testing the potency of diphtheria, tetanus and combined vaccines, WHO/BLG/95.1, 178-191.

2. Holzhütter H-G, Archer G., Dami N., Lovell D.P., Saltelli A., Sjöström M. (1996). Recommendations for the application of biostatistical methods during the development and validation of alternative toxicological methods. ECVAM biostatistical task force report 1. *ATLA* **24**, 511-530.
3. Bland J.M., Altman D.G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* i: 8 February, 307-310 (1986)
4. Govindarajulu Z. (2001). *Statistical techniques in bioassay*. 2nd ed. Karger, Basel.
Wernimont G.T., Spendly W. (ed) (1985). *Use of statistics to develop and evaluate analytical methods*. AOAC, Arlington, VA.

Session III: Implementation of the 3Rs: Needs and practical experiences

WORKSHOP VI: IN-HOUSE VALIDATION VS.

INTER-LABORATORY VALIDATION

CONCLUSIONS AND RECOMMENDATIONS

Mr K. Redhead, Intervet International (UK) and Mr A. Akkermans, RIVM (NL)

Proper validation of test procedures is the key for a successful application of a method within a laboratory. Guidelines for validation of a method have been drawn up by ICH (document Q2A and Q2B) and are most helpful for an in house validation study. After a successful in house validation a method can be evaluated in an inter-laboratory validation study. For inter-laboratory validation studies the ICH guidelines are less applicable. Furthermore, results obtained between laboratories are susceptible to variation due to:

Different methodologies:

- Same methodology - different interpretation
- Variations in result reporting
- Data processed differently prior to reporting
- Equipment differences
- Reagent differences
- Variations in conditions
- Use of inappropriate statistical methods

This situation results in different approaches to the design of inter laboratory validation studies and gives rise to discussions on the conclusions that can be drawn from the results obtained.

Reasons for an inter laboratory validation study could be:

- Transfer of assay (reference etc) to another geographical location
- Transfer of assay (reference etc) to another group of people
- To establish an assay as a generally acceptable (replacement) method
- To establish reference preparations

Bearing this in mind, the workshop discussion led to the following conclusions:

The first step in inter-laboratory validation should be the prevalidation, which is in line with the recommendations given by ECVAM. Besides the usual issues, topics to take into account during the prevalidation stage are:

Identifying key reagents, which are of good quality and in sufficient amount to guarantee continuity of the method (availability of monoclonal antibodies, process to replace reagents at end of shelf life).

Involvement of interested producers at an early stage. This can reduce animal usage as some reagents, such as sera, may be “by products” from manufacturing and testing.

Due to the increased number of parameters there will almost always be a larger variation in inter-laboratory validation as compared with intra-laboratory. The degree of variation that is acceptable must be assessed and decided upon, on a case by case basis, in consultation with a biostatistician.

How to deal with the correlation of an *in vitro* method with the relevant *in vivo* method?

In general the statement: an *in vitro* method shall not give pass results when the *in vivo* method would reject the product, is valid. However, this should only apply if the *in vivo* method is accepted as a fail-safe test. Otherwise correlation with efficacy studies should prevail.

How to get an alternative test method for a (new) product into a procedure for inter laboratory validation and possible adoption in the monograph?

- A proposal for evaluation of the model can be send to ECVAM, complete with all relevant information about the test and *what test it is replacing*.
- If the main goal of the validation study is the inclusion of the method in the Pharmacopoeia monograph, EDQM has procedures in place to either develop a monograph or revise / replace mandatory methods published in the European Pharmacopoeia. For information on the available procedures, contact the secretariat of the EDQM for information on the applicable procedures.
- Another option is to contact division IV of EDQM.

SESSION IV: THE 3 RS CONCEPT: THE FUTURE DEVELOPMENTS

The EDQM Biological Standardisation Programme

Mr J.-M. Spiesser, EDQM, Council of Europe

New approaches to vaccine quality control: Veterinary industry's experience

Dr K. Redhead, Vaccine Research and Development, Intervet (UK)

Discussions

Conclusions Workshop VII: Antigen qualification and quantification tests

Dr M. Duchene, Glaxosmithkline Biologicals, (B) and Dr D. Dusek, USDA (USA).

Conclusions Workshop VIII: Advanced immunological techniques

Dr M. Leenaars, RIVM (NL) and A. Van de Moer, Vetoquinol S.A. (F)

Session IV: the 3Rs concept: The future developments
THE EDQM BIOLOGICAL STANDARDISATION PROGRAMME

Mr J.-M. Spieser, (EDQM), Council of Europe

My presentation consists of giving you an indication on what the Biological Standardisation Programme BSP is. This is a programme which is unique to our system and for which we have a very specific role, independent from the European Pharmacopoeia itself. But of course, at the end of its projects we have direct links with the European Pharmacopoeia Commission and its expert groups to whom we deliver the final conclusions whenever there is a need for follow-up and integration of the outcome in the monographs, general texts or methods.

In the framework of the current QC for production batch-testing for release by manufacturers and control authorities for biologicals for both human and veterinary use we have indeed a great interest in the alternatives to animal testing and possible solutions, to the problems encountered with the animal tests are part of our regular objective settings.

My presentation will be a general presentation highlighting the origin of the biological standardisation programme, why we created it and its working methods, key issues based on examples. I will give you some indication on the resources and procedures which we applied, the achievements and progress made before ending with some of the perspective for the future.

The origin of the standardisation programme:

This started in 1992 through a contractual collaboration with the EU Commission because this was the time when we almost implemented the Directives on biologicals which were established in 1989, Directive 89/342EC for the vaccines and the 89/381EC for the blood and plasma derivatives. I was detached at the Commission in Brussels during this period and when we looked through the situation which existed within Europe during that time concerning vaccines and blood products, there was a clear need for having a better standardisation and better standards available to foster the mutual recognition of data as built in the Directives.

The description:

It is a specific programme aimed at contributing to the standardisation of biologicals through a harmonised approach with unique tools, being it methods or standards to guarantee high level quality biological products for Europe of course, but also in practice, this extends far beyond Europe as we have a lot of collaboration with our US colleagues, WHO colleagues, Australia, Canada and others.

There are three major activities within our programme:

1) Establishment of common European reference standards.

What are the reasons for having these standards? Our goal is to have these standards in sufficient amount for use as working standards as we realise that making a lot of sub-standards creates more divergence than standardisation. This does not preclude the fact that major manufacturers if they wish to establish their own in-house standards against our standards can do it but then they have to recalibrate regularly these material to reassure that they are still of good quality and fulfil the requirements for in-house standards.

There is no mandatory obligation to use our standards. However, we know that over time more and more manufacturers prefer to use our standards which are 'ready to use' as they do

not need to develop their own qualification. The detailed report of the collaborative studies establishing the EDQM/ European Pharmacopoeia standards is always published (Pharmeuropa-Bio) and carried out state of the art. Furthermore, these materials are regularly monitored.

2) Establishment of validated and standardised assay methods.

We have established a certain number of assay methods for biotech products, and blood products. By doing this we also rapidly began to enter into the development of coordinated validation of alternative methods for QC in application of the 3Rs concept and we have developed a certain number of these alternative methods.

3) The BSP alternative to animal testing

This has been developed within the context of the European legal environments we exist, two Conventions one from the Council of Europe and the one from the European Union Commission. Mrs Lwoff has made an excellent presentation on this subject and I have nothing to add. There is an incentive in the scientific world to use the state of the art *in vitro* methods to characterise biologicals which are different nowadays in a great number of cases, as they are better purified and well characterised products and there is pressure from the public opinion to apply the 3R concept for qualifying and controlling these products. Nevertheless, why we must be scientifically sound in our decisions – it must be practical and easy to implement - as this involves quality of products and patient safety and efficacy (which includes animals when we encompass veterinary products).

Who are our stakeholders?

The European Pharmacopoeia's Commission who have all rights to say that they 'need' or could you 'involve' or that 'they need a standard for the application or a given monograph' etc.

The network of the official medicines control laboratories (OMCLs) who could say that they need a standard method, or that they need given reference materials or specific reagents.

Also, the EU Commission who have already commissioned us in the past and we are currently discussing possible future project on specific product.

The EMEA and its CPMP/CVMP Quality Working Parties and other Working Parties who also request methods or standards and of course lastly, but not least, the manufacturers who can give us access to essential preliminary studies and then we can develop something altogether with all interested parties.

The whole programme is based on team-work between the competent partners. We have at the European Directorate for the Quality of Medicines (EDQM) a dedicated division; my colleagues and I (Division IV) have the task to manage the system, coordinate and execute the programmes which of course within the EDQM will interact with our own laboratory, Division III, and with Division I - the secretariat of the European Pharmacopoeia and its groups of experts where appropriate. The BSP is advised by a Steering Committee which is formed by 11 members and 2 observers. The members are, by definition in our terms of reference and procedure, the Chairs of the biological Experts Groups 6 and 6B, 15 and 15V of the European Pharmacopoeia. The Chairs of the IVMP Group of the CVMP and the Biotech Working Group (BWP) of the CPMP as well as two members of major OMCLs or major independent biological organisations within Europe and 2 observers from WHO as we try to coordinate as much as possible with WHO in order not to duplicate or jeopardise or mutual resources.

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Our work and development is based on important key actors who are the project leaders – the project leader is a scientific expert in the appropriate field, generally originating from an OMCL but not always, which is in my opinion the best person who knows best the project in depth on which we are going to work and he/she advises the EDQM on the study and makes his/her own feasibility preparatory work and is also involved in the preparation and follow-up of the study project as well as in the reporting together with the EDQM building ideally like scientific leadership in that area of interest. Another important key players are also the donors, manufacturer who give donations of suitable products for candidate reference materials or, testing samples if it is something related to developing methods.

All proposals are submitted to the Steering Committee for advice and scrutiny.

Participating is voluntary but the system always implies that invitation is addressed to the different OMCL involved eg, those involved in official batch-release for human biologicals, which does not mean by obligation all 17 EU/EEA OMCLs but only those involved on a given product and which have developed the necessary technical competence(s). The manufacturers present on the European market for that product so there is never a project which we would develop without a very close interaction with the manufacturers involved as well as third parties to foster global approaches and harmonisation like WHO, USA/FDA-CBER, USDA, Japan, Cadreac etc.,.

The final report with clear conclusions on the outcome of the study is approved by the participant firstly, and we will hold a meeting if necessary and then it will go to the Steering Committee for approval. The final document revised after consideration of all comments and remarks is then officially adopted by the European Pharmacopoeia's Commission and then later, it will go to the stakeholders for consideration and implementation. New methods will go to the relevant group of experts to foster necessary revision and update of monographs or general texts or methods, guidelines etc and standards are added to the catalogue of reference materials available from EDQM.

The detailed results and conclusion of the collaborative study are always published in Pharmeuropa-Bio and if appropriate in a peer reviewed additional international scientific journal publicly available. This gives the applicant the possibility to fully document for example in a marketing authorisation file, the use of the new method or standard without having to further validate it. We are now referenced in Medline since the end of 2000 and this should help cut down the need of having a second presentation in a peer reviewed publication unless it is a very interesting project where we would like to communicate the information as much as possible to the interested scientific world (new method) . From now on, by making use of the search in Medline you will find our publications because in the past it was difficult to find if you did not know that Pharmeuropa-Bio existed and where to order it.

Key issues based on examples.

I would like to explain why are we developing alternatives to animal testing and to share with you some of our experience.

One typical example of investing in replacement of animal testing is because animal testing is very costly and it requires a large number of animals, especially if you wish to have a sound study statistically valid.

In addition such tests often require very skilled personnel and it now becomes evident that the number of performing laboratories are diminishing – laboratories who are really able to run appropriate animal testing - and people get into difficulties in maintaining the competence of the personnel.

Either these tests represent a very general systemic test, the ones concerning the assay of human growth however or some safety tests for vaccines where there was just the measurement of a weight gain or weight loss – what does this really mean in terms of checking the quality of a biological? Or, the otherway round is that they are very sophisticated. It is very rare to have skilled personnel able to measure micro metres of growth of tibial bone of rats after having treated human growth hormone and doing this on 64 animals is a big challenge! These are all good reasons for fostering replacement of animal testing.

There is also parallel to these considerations the fact that technical development occurred in production and purification together with the application of GMP - the development of biotech products instead of extractive products has contributed enormously in making other types of products available for the same biological. Analytical tools have changed, molecular biology appeared and a great variety of HPLCs with all their different combinations for detection and different immunological methods were developed which made us have a different approach towards biologicals using these new techniques making them well characterised molecules.

But these recently developed approaches is new and there is a learning phase which needs to be implemented for all these methods. These are also very expensive -analytical testing if you consider the development with very specific reagents such as monoclonals or high sophisticated instrumentation. How will people consider the PCR v. safety target animal testing for poultry vaccines? Availability of suitable reagents – in quantity, consistent quality, and continuity in time is a prerequisite, a typical example is the *in vitro* assay for Hep B/ Hep A vaccines based on commercial kits which will be withdraw from the market very soon. This is not without great concern to manufacturers and authorities and could theoretically have dramatic consequences such as disruptions of availability of products.

There is also limitation as regards correlation studies as both methods, the new *in vitro* and the historical *in vivo* tests do not measure the same properties of the product. We are often falling into the trap that the *in vitro* is a functional test and if you want to measure the function of your well characterised molecule you will probably have, in replacement of the animal test, with a set of different combinations which measures the impurities, some specific functional properties, the receptor binding etc. This can then end up in the need for a series of tests rather than being one single *in vivo* based also on the state of the art assessment/ evaluation of these “old” products. Therefore, some reluctancies!

It then turns rapidly into a full validation rather than a correlation and then of course if you wish to validate that you need to have to have access to “bad samples” and no-one has “bad samples” yet for demonstration you need these to prove that your new method would give satisfaction and is fully validated.

Full validation can mean as much as five years or more of dedicated laboratory work to make sure that we do have a fully validated new method, which of course will involve both manufacturers and OMCLs, which is an expensive exercise. You will often see and demonstrate that you can no longer use the available international standard or the available European standard because either it is given in a presentation and a formulation which is not appropriate, injecting on an HPLC column something which has 20% of the actives and 80% of BSA or HSA is not appropriate and therefore you have to develop a new standard with appropriate new validated / stable formulation. You are also faced, often with a very important issue that can bring you as far as having to give a full new licensing, because you will have new expression of results; and this because you measure something different in your products i.e. the potency will no longer be indicated in IUs but will be expressed in ELISA

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unit or a percentage of a peak of HPLC, or.... This needs to be considered and could take a lot of time...

Also, we need to take into account implications for the patient-dosing – for example we have developed fully validated physico-chemical testing for expressing the content of the active moiety of human insulin but yet the patients are still using international units due to habit. Consequently, the need to accept double labelling and expression of IUs as calculated values based on specific activity - yet another hurdle!

Our working principles are that we always have a feasibility study prior to a large collaborative study, which is scientifically sound generating enough data for statistical evaluation. There is also a political involvement of majority of interested partners as it will help to transpose the new method in mandatory requirements – we have discovered that it is easier to convince assessors if they have been a part of the game. It is easier to convince manufacturers if they have been part of the study then if they just received the results. This facilitates the decision making process and gains much time for the future implementation.

Our work does not always bring a ready-to-use solution. Sometimes this is the case but not in each case. This is only a generic approach and you will have to have in-house individual products specific validation. This reduces the part of your validation because the applicability and the robustness of the method will already have been demonstrated.

Standards:

We always base our standard policy on the primary standard, which is the International Standard, to have our own European standard like US and Japan and to cut as much as possible the secondary levels (national standards, in-house standards) and local or regional trade association standards as the multiple generation of standards create divergence.

What are our resources?

EDQM division IV, consist of a Biometrician, 4 scientist, a secretarial assistant and our own EDQM laboratory (*in vitro* techniques), the valuable contribution of the project leaders and study participants. Our funds come from the EU Commission and our own-self-financing which is generated by the sales of standards for an amount nearing about 50% of our budget.

We support our project leaders through appropriate contributions and try to help them support part of the costs of their special laboratory work related to the project. We never give funds to a person, only to institutions.

Development and monitoring programmes are in place for all the standards which are available on an on-going basis and we do have sometimes, when it is a very costly programme, to bring in extra contributions from sister institutions to make the programme a success. We have had the support of ECVAM in the past, from ZEBET where the feasibility has been done through the funding of ZEBET at PEI (D) etc.

We have procedures in place which ensures efficiency and customer satisfaction. I heard people asking if we could guarantee the continuation of reagents, and that is the reason why they would prefer to use their internal standards; unless there is a very problematic situation in a sudden increase of demand because there is a development of new methods or generics, however, we could be in the situation like those with an in-house standard, that some exceptional situations could happen - but we would always ensure continuity of the products on the quality and on the intended use the whole team with EDQM is dedicated to this important mission. We have also a very developed storage and shipment with Good Practices in places.

Since the beginning of the programme, 10 years ago the achievements have been as follows:

We have developed 63 projects which were initiated and 41 projects which are finalised. Amongst these, 14 projects involving alternative to animal testing have been finalised by the end of 2001. 5 projects are still in development.

4 hormones have been fully developed towards physico chemical characterisation only which are: Somatropin, human insulin calcitonin, oxytocin and standards and methods are available also for these which no longer use animal testing, we replaced definitely the bio-assays for these products.

We have also replaced the pyrogen testing by endotoxin testing (for gram negatives).

We have developed for human vaccines standards and replacements of *in vitro* methods for Hep A, Hep B and Tetanus, which is very recent, with the appropriate standards; also specific reagents such as the guinea-pig anti-sera for tetanus, the mouse anti-sera for the acellular pertussis have been established.

For 5 veterinary vaccines the following was established: guinea pig and rabbit antisera for Tetanus, Clostridia rabbit antisera (5 components), swine Erysipelas challenge strains (2) as well as ELISA antigen coating for that vaccine.

We also have 3 human vaccines projects in progress which are Diphtheria, Diphtheria gp antiserum, Hep B and 2 veterinary vaccines projects: clostridia perfringens and Newcastle Disease for which the replacement of animal testing is our goal.

Our perspectives are double on one hand the replacement of existing standards when they become exhaustive, an on-going procedure and on the other hand, to continue to develop alternatives to animal testing especially for veterinary vaccines, but also it might be that in the future we go also more towards standardisation for advanced therapies such as gene therapy if there is a need. We also give some support to our clinical colleagues through tentative contributions to standardise the biological methods used for example in measuring antibodies for which it is of prime importance to have standard methods.

We will intensify the tools for facilitating implementations of alternatives to animals testing for veterinary immunologicals, whether it is for methods, specific reagents or new standards.

Session IV: the 3Rs concept: The future developments

NEW APPROACHES TO VACCINE QUALITY CONTROL: VETERINARY INDUSTRY'S EXPERIENCE

Dr K. Redhead, Intervet (UK)

Introduction

The quality control (QC) testing of vaccines is one of the major uses of animals in the veterinary biologicals industry. Therefore, it has always been desirable to reduce this animal usage and, if possible, to develop new *in vitro* test alternatives. These new approaches tend to take one or both of two general forms. One is the development of new or the modification of existing assays and methodologies. The other is the novel analysis or interpretation of test data. Most veterinary vaccine manufacturers have pursued these aims with varying degrees of success. However, in 1995 several companies came together to form a collaborative group known by the acronym In-VITRO (International Veterinary Industry Test Replacement Organisation) under the independent chairmanship of Roger Lucken. These collaborators hoped that by pooling their experience and information they would be able to achieve more as a group than as individuals.

The purpose of In-VITRO has been to develop, validate and harmonise alternatives to *in vivo* methods for veterinary vaccine QC testing. In other words, the application of the 3Rs, as described by Russell and Burch (1), to the assays used in the QC of veterinary biological products. The emphasis has tended towards the reduction and refinement of animal usage in potency testing, particularly of clostridial vaccines, and the approach has been multifactorial. The companies are committed to the exchange of relevant information, methodologies and reagents. They have participated in collaborative studies involving the pooling and analysis of existing QC test data, the evaluation of putative reference preparations and the assessment of novel In-VITRO assay methods. Whenever possible the results of such studies have been published and this together with liaisons with the European Directorate for the Quality of Medicines (EDQM), the European Pharmacopoeia (Ph. Eur), the United States Department of Agriculture (USDA) and national regulatory authorities has helped to disseminate the groups findings. In the following text specific examples of the work of In-VITRO are presented to demonstrate it's application of the principles of reduction and refinement to vaccine QC. This is followed by the brief description of an approach applying the principle of replacement to the potency testing of veterinary vaccines.

***Clostridium chauvoei* potency test**

The potency test for *Cl. chauvoei* is a challenge assay in vaccinated and control guinea pigs. The European Pharmacopoeia's requirements for this assay in 1997 (2) were, briefly, that 10 guinea pigs are injected with vaccine on two occasions 28 days apart. Fourteen days after the second injection the 10 vaccinated guinea pigs and five control animals are challenged with virulent *Cl. chauvoei*. The vaccine complied with the test if all of the control animals died and all of the vaccinates survived. If more than one vaccinate died the vaccine failed, if only one vaccinate died the test could be repeated using a further 15 animals.

Each manufacturer within In-VITRO knew that a significant proportion of their own vaccines did not pass the initial test but most of those that were eligible for retesting did pass on the repeat test. When all the manufacturers shared their results and the data were pooled and analysed (Table 1) an obvious pattern emerged. This pattern was consistent between different

manufacturers producing various *Cl. chauvoei* containing vaccines even when the challenge comprised different strains administered in different forms.

Table 1. Pooled results of potency testing of *Cl. chauvoei* vaccines

Test	Number of batches	Number of fails	Number of passes	Percentage passing
Initial	332	37	252	76
Repeat	43	9	34	79

From the combined test data of five manufacturers it can be seen that 252 out of 332 vaccines passed the initial potency assay giving a pass rate of 76%. Of the 80 vaccines which did not initially pass the test 43, where only one vaccinated guinea pig had died, were eligible for repeat testing. When retested, 34 of these vaccines passed the potency assay to give a pass rate of 79%, which is very similar to that obtained in the initial testing. That vaccines rated as borderline should upon retesting have an almost identical pass rate to the initial testing suggests that a single death in the vaccinated group is less an indication of possible vaccine failure and more probably a result of innate variation in the immune responses of the guinea pigs.

On the basis of this data analysis it was proposed that the requirements for the potency test for *Cl. chauvoei* vaccines could be relaxed slightly. This proposal was accepted by the Ph. Eur. and as of 1998 the monograph for *Cl. chauvoei* vaccine (3) states that a vaccine complies with the potency test if not more than one of the vaccinated guinea pigs dies after challenge and a retest is allowed if not more than two die. This modification led to an immediate reduction in the number of repeat potency tests required and, as a result, a significant reduction in the number of guinea pigs used.

Multicomponent clostridial reference serum

The pharmacopoeial monographs for most veterinary clostridial vaccines detail *in vivo* potency assays. In these assays the vaccines are injected into rabbits to induce serum antibody responses which are then quantified on the basis of their ability to neutralise the effects of toxin challenge in mice. The monographs also allow for the use of validated alternative methods. One obvious alternative, which would reduce animal usage, would be the use of an *in vitro* method for measuring the antibody responses of the vaccinated rabbits in place of the mouse toxin neutralisation test.

Some companies already had ELISA systems which they used in the potency testing of various clostridial vaccines. However, these ELISAs differed between companies in various aspects of equipment, reagents, methodologies and reference preparations. Such methods were therefore specific to the products of the individual manufacturers. This has made it difficult to compare and analyse test data from different manufacturers. It has also meant that any company which wishes to develop such assays has had to independently validate their methods and references, so using more animals, and may have encountered additional problems in obtaining regulatory approval. One way to overcome these problems would be to establish a multicomponent rabbit reference serum with independently assigned unitages. This serum could be used by all the manufacturers to set their own in-house reference sera for the different clostridial potency ELISAs of interest, particularly for *Cl. perfringens* types B, C and D, *Cl. septicum*, *Cl. novyi* and *Cl. tetani*.

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After much discussion between *In vitro* members, regulatory authorities and Ph. Eur. representatives it was agreed that no one serum would be representative of the full range of clostridial components used by the different manufacturers. It was therefore decided to produce a serum pool comprising sera generated by the different manufacturers during the routine potency testing of their various clostridial containing vaccines. This was most likely to result in a reference preparation with the desired spectrum of antibody responses and would also remove the need to use additional animals to raise sufficient quantities of a suitable serum.

An international collaborative study was organised by EDQM to produce and establish such a reference serum (4). In the first phase pools of sera, raised against a wide range of clostridial antigens, were provided by the manufacturers. A pilot serum blend, together with samples of the individual serum pools, were redistributed to the donor manufacturers. The manufacturers titrated them in the full range of their own *in vivo* and *in vitro* assay systems to determine whether the blended serum pool would allow the different laboratories, using their own various *in vitro* methods, to obtain results which were similar to each others and to the activities as determined by the *in vivo* assays. Due to the small number of laboratories involved and the limited replication of the assays the amount of data available was limited. However, it did suggest that the serum blend could provide an appropriate reference preparation for use in both *in vivo* and *in vitro* assays for antitoxin responses to the five clostridial components of most interest.

On the basis of the results from phase one it was decided to progress to the second phase of the study. A production scale serum blend, of identical composition to the pilot blend, was prepared, filled and freeze-dried by EDQM. This candidate biological reference preparation (cBRP) was distributed to the five manufacturers and four official medicines control laboratories which had agreed to participate in the study. These laboratories compared the activities of the rabbit reference serum with the existing equine monovalent international standards according to the methods described in the current monographs, i.e. by *in vivo* toxin neutralisation assays, to provide definitive values for the antitoxin activity of the reference preparation in respect of the five components of interest. After detailed analysis of the results it was decided that the cBRP was suitable for use as a definitive preparation with the assigned activities shown in Table 2. This BRP can now be used in *in vitro* potency assays so contributing to the reduction and refinement of animal usage.

Table 2. The assigned activity of the clostridial rabbit antiserum BRP

Antitoxin	Activity per vial
<i>Cl. perfringens</i> beta	10.5 I.U.
<i>Cl. perfringens</i> epsilon	11.0 I.U.
<i>Cl. septicum</i>	7.5 I.U.
<i>Cl. novyi</i> type B	11.0 I.U.
<i>Cl. tetani</i>	8.0 I.U.

Antigenic mass assays

For many people trying to improve vaccine QC testing the ultimate aim is the replacement of animals by *in vitro* methods, particularly for potency assays. An approach which many manufacturers would like to see adopted is the use of *in vitro* antigen quantitation assays for potency testing. This is not that novel an approach. The Center for Veterinary Biologics had

by 1992 already proposed a general *in vitro* relative potency assay method for veterinary vaccines based on antigen quantitation, and have now published guidelines for such a method (5). However, for such a method to be acceptable to regulatory authorities it must be correctly designed and validated and be part of a coherent overall strategy.

The approach should be to move away from the potency test as a surrogate measure of efficacy in the target species and to view it as a demonstration of the consistency of the final vaccine. Before this can be accepted certain criteria have to be met:

Consistency of the production process must be demonstrated and monitored by quality assurance (QA).

The final vaccine must be blended on the basis of a fixed antigen content.

The efficacy of the product has to be demonstrated in the target species with the antigen content at the standard level and at a reduced level.

The antigenic mass assay, usually an ELISA, should be shown to measure the protective antigen.

The assay has to be able to discriminate reliably and consistently between the standard and reduced antigen content vaccines.

The variation between different batches of the vaccine must not be greater than the variation between assays on the same batch of vaccine.

In addition to these main criteria there are several peripheral questions to be addressed such as the need for reference and control preparations, demonstration of specificity, assessment of any adjuvant used etc. However, if these challenges can be surmounted it should be possible to achieve accurate and reliable *in vitro* assays which are capable of consistently identifying batches of vaccines containing sub-standard, but still efficacious, levels of the protective antigens. So providing an extra level of assurance to the quality control testing.

Conclusion

To a large extent almost all new approaches to the QC of veterinary vaccines involve the implementation of some aspects of the 3Rs. Reduction can be achieved by the use of historical data and the application of appropriate statistical methods to ensure that the minimum number of animals are used. The application of *in vitro* assays for the analysis of serological responses has also helped to reduce animal usage where these assays have replaced toxin neutralisation tests and has contributed to refinement in the replacement of certain challenge potency assays. Probably the most radical approach is the possibility of moving away, to an extent, from QC testing of final vaccine towards QA of the consistency of the vaccine production process. This offers the opportunity to use antigenic mass assays as potency tests so almost completely replacing animal usage at this stage in vaccine testing.

Acknowledgements

This presentation was made on behalf of *In vitro*. The *In vitro* group comprises Roger Lucken as Chairman and representatives of Intervet, Merial, Fort Dodge, CSL and Schering-Plough.

References

- Russell W. M. S. and Burch R. L. The Principles of Humane Experimental Technique. London, Methuen, 1959.
- European Pharmacopoeia Monograph (0361) Vaccinum clostridii chauvoei ad usum veterinarium., 657-658, 1997.

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Pharmeuropa: *Vaccinum clostridii chauvoei ad usum veterinarium* 10:2, 317, 1998.

Lucken R, Daas A and Esposito-Farese, M-E. Collaborative study for the establishment of a European Pharmacopoeia biological reference preparation for *clostridia* antiserum for serological potency testing of clostridial vaccines for veterinary use. Pharmeuropa special issue BIO 2000-2, 2001, 65-87.

Veterinary Services Memorandum: Guidelines for veterinary biological relative potency ELISA antigen quantification assays and reference preparations, 1998.

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DISCUSSIONS

Question from the floor to Mr J.-M. Spieser: is the Newcastle standard the new common standard?

Reply: the Newcastle disease assay which is proposed is a quantitative ELISA assay and what we used were strains that are used in European vaccines. The cut-off levels are defined according to European Pharmacopoeial guidelines and they will probably not be comparable with what is common in the United States, but we still have to establish that. We have recently received two vaccines that are on the US market and we will assay them and this will probably give us the answer.

Of course, if you want to look at the global approach, you would have to take into account the issue raised that different vaccines work on different continents.

Comment from the floor to Dr K. Redhead: Dr Redhead mentioned that you could use surrogate correlates of protection instead of antibodies directed against your protective antigens – do you have any examples of that and what exactly do you mean by this?

Dr K. Redhead: I have no examples of this I am afraid –it was another possibility that rather than antibodies that you can prove in challenge assay to be protective, other antibodies to the same antigen that you are using which inter-correlates very tightly but that do not show protection in challenge assays, might be accepted if that correlation is tight enough.

Dr R. Dobbelaer: What is interesting concerning this symposium is the continuous confrontation on humane vets and humane humans! I know that Dr Redhead has experience with the Kendrick test for pertussis vaccine and I am always amazed by Dr Redhead's different approach for veterinary vaccine and human vaccines – for this Kendrick test you have three or four groups of 16 to 20 mice including the reference in the very first part of your presentation I see 8 or 10 guinea pigs and you discuss the fact that one dying guinea pig can make the difference between pass and fail. Is there anything that can justify this different approach? Are the humans too stringent or are the vets too lenient?

Dr K. Redhead: The obvious difference is that this is straight forward. If you make an error, or something goes wrong with the human vaccine then you are potentially playing with a human life. On the veterinary side, it is that bit easier. If something goes wrong, you are only dealing with possibly some animals dying.

Comment from the floor: another difference that may explain this difference is that the veterinary vaccines are tested for efficacy in the target species which might explain why afterwards there is reduced dead animals. You do have to have efficacy data in target species as well.

Dr K. Cussler: to Dr Dobbelaer: I think that it is a pity that human and veterinary groups only meet on rare occasions – would it not be good to have more exchange between group 15 and 15V? There are not so many vaccines in common however, there is rabies, tetanus etc and the monographs are very different!

Comment from the floor: Are you of the opinion that a reference used in an *in vitro* alternative, for example an antigen quantification assay, has to be a vaccine or could it be an antigen?

Dr K. Redhead: That is something within the “In-VITRO” group on which we have had a lot of discussion. I think that it is a case of if the antigen by itself behaves in the same way as the

complete vaccine, then there is no reason why you could not use the antigen. It is a bit like if you have a multi-component vaccine, whether you should be using a monovalent or a polyvalent reference vaccine with it as the reference. You have to try it and see if you get variation or not.

Comment from the floor: the background of the question is also that in veterinary manufacturing, a lot of different adjuvants are used and it is impossible to make a reference vaccine for each combination, however, you could make a reference antigen providing that you validate your extraction methods or that you can validate that the antigens is recognized in the matrix that is in the different vaccines. In my view, a reference antigen would then be preferable to a reference vaccine.

Dr K. Redhead: Particularly if you are thinking of applying this across the products of several different manufacturers. Within the "In-VITRO" group, we have approached this with the idea of in-house manufacturer specific-even product specific tests, used as a replacement for the potency assay for that given product. We have also been looking at this from the point of view of bringing these in on new products rather than trying to replace existing potency assays, in particularly *in vivo* ones as I see a lot of problems there, but if we can do this for a new product where you put the product into the target species and show this dilution efficacy and then assay will pick up this difference with the antigen content, then I think that there is a great chance of acceptability.

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WORKSHOP VII: ANTIGEN QUALIFICATION AND QUANTIFICATION TESTS

CONCLUSIONS AND RECOMMENDATIONS

Dr M. Duchene, Glaxosmithkline Biologicals, (B) and Dr D. Dusek, USDA (USA).

Ag quantification for routine quality control of vaccines

In vivo/ in vitro correlation

- target animal efficacy
- not necessarily with *in vivo* potency test

Where the (AQ) Ag quantification test is a relevant indicator of the quality of the vaccine

Necessary for:

- process validation
- assay validation
- consistency and stability has been demonstrated

AQ test could be applied prior to formulation of a multivalent vaccine

Different references across manufacturers is acceptable

AQ test is more readily accepted for well characterized antigens, case-by-case.

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WORKSHOP VIII: ADVANCED IMMUNOLOGICAL TECHNIQUES

CONCLUSIONS AND RECOMMENDATIONS

Dr M. Leenaars, RIVM (NL) and A. Van de Moer, Vetoquinol S.A. (F)

The advanced immunological techniques meant in the title of this workshop are not the serological models currently used more often as a replacement for challenge type tests. From an animal welfare point of view the serological models have advantages compared to the challenge tests. Still there are many disadvantages of using animal models for quality control testing of human and veterinary vaccines.

It was agreed that the serological tests in mice or guinea pigs used for quality control of vaccines do not always tell much about efficacy of these vaccines in target species but only say something about consistency in production. The serological model shows that the vaccine to be tested is able to induce comparable antibody responses in mice as induced by the reference vaccine which has been tested for efficacy. The fact that it seems almost impossible to produce 'bad' tetanus and diphtheria vaccines makes it, for these antigens, even more disputable to perform an animal test which implies an accuracy that is neither relevant nor necessary. In vaccine production Good Manufacturing Practice is generally applied. Quality assurance becomes therefore more and more important. When these aspects are taken into account a test system to prove consistency in production was considered to be satisfactory to confirm quality of vaccines.

Critical steps in the production process have to be monitored in order to confirm consistency in production. In vaccines that include an adjuvant the final mixing of antigen(s) and adjuvant is seen as a critical step in the production process. Data and information on the interaction between adjuvant and antigen(s) are sometimes not available, especially for vaccines that include an oil emulsion. In this case, the serological assay is the only method currently available to ensure the consistency of the final product. Data and information on the ratio between protective antigens and non-protective antigens are sometimes also difficult to obtain or not available. This ratio could however have an impact on the efficacy of the vaccine. Another critical step in the production process is the quantity and quality of the antigen. This could be tested upstream and not necessarily in the final product. In workshop VII antigen quantification was discussed. Consistency in antigen quality and quantity can be studied by physico-chemical and immunochemical techniques. However, it was agreed that in most cases this may not be enough to give an answer to what the biological activity of the product is.

The use of monoclonal antibodies is the answer to this problem for some vaccines. Especially when protective epitopes of the antigen are known, monoclonal antibodies can effectively be used to confirm consistency in antigen quality. However, when these protective epitopes are not known, how can antigen quality be confirmed? It was suggested that this may be studied by advanced immunological techniques meaning: measuring the immune response to a vaccine (antigen) *in vitro* using cell (line)s. As a read-out in the tests cytokine production, cell proliferation, adhesion molecules and antibody production may be used. It was considered to be possible to measure these parameters after *in vitro* stimulation with for example tetanus toxoid. Validation of the model however was considered a problem since no bad batches of tetanus toxoid are available. Problems may occur when complex antigen mixtures are used in these models because of the complex response that will be induced. At this moment these advanced techniques seem to be too advanced and need to be studied in more detail at an

upstream R&D level. A start in this direction may be a study to proof the principle. Research antigens that were suggested are tetanus and rabies because both vaccines are used in human and veterinary field and one is a bacteria and the other a virus.

Recommendations:

More research is needed to study the interaction between antigen and adjuvant (especially for emulsions) in the final product.

A study is needed for proof of principle of advanced immunological techniques applied for instance to rabies and tetanus toxoid antigens.

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CLOSING REMARKS

Dr Agnès Artiges, (EDQM), Council of Europe

We are now reaching the end of this very interesting symposium and my closing remarks will be very brief.

I would like to thank every participant for his or her participation to the very lively debates we have had, especially during the various workshops where various recommendations were expressed.

Of course we will take into consideration these recommendations for future orientations of our work whether for the European Pharmacopoeia monographs or for the Biological Standardisation Programme (BSP).

I would like to summarise in just a few words the major outcomes:

- 1) A clear legal framework exists and we shall all ensure in our own responsibilities to have the 3Rs applied as far as possible based on good sound scientific evidence.
- 2) A lot of excellent development work has taken place and is already largely validated or in the process of being done in the near future, and we shall of course all continue our efforts.
- 3) Action from regulators and industry should be initiated to help in transferring the scientifically based outcome into appropriate regulatory changes: For that purpose, all actors, industry, assessors, controllers etc have a key role to play.
- 4) It appears that everyone needs to be satisfied and that a system must be built for education, mutual recognition, policy making to avoid discrepant situations between these actors and there is no doubt that licensing authorities will be sensitive to the alternatives of animal testing as it is already foreseen in the Marketing Authorisation Directive and variations for implementing new 3Rs concepts should be used more often by industry especially for veterinary vaccines.
- 5) Harmonisation between major key regulatory territories are also important and we were pleased to have during this meeting, participants from USA and Canada and we hope that the progress made to apply 3Rs in Europe will also be implemented by other authorities in particular by USA, Japan, WHO. Here too, we should all interact to reach a common approach and use all routes possible: e.g., ECBS, ICH, VICH.
- 6) It is in the veterinary field that there is room for more progress, and it appears that in the future, an extra effort should be made in this field by involving all partners: industry, regulators, OMCLs, Ph. Eur. in an on-going constructive effort. EDQM will be dedicated to this effort.
- 7) New techniques are being developed for both production and analytical processes and we should make an effort in order to adapt to these new methods.

We will no doubt be vigilant and with the help of your appropriate tools, we will be able to continue these developments.

Thank you again for your active participation in this symposium and your future contribution. Having said that I would like to close this meeting.

I wish you all a safe return journey.

APPENDICES

European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123)

* Scope (Article 1)

The scope of the Convention is, to a large extent, determined in the definition of the word "procedure", in its Article 1:

".. any experimental or other use of an animal which may cause it pain, suffering, distress or lasting harm, including any course of action intended to, or liable to, result in the birth of an animal in any such conditions..."

* Authorized purposes (Article 2)

Article 2 of the Convention lists the authorized purposes for an experiment to be performed on an animal. They can be summarised as follows: prevention and treatment of human, animal and plant diseases including testing of products; research on physiological conditions in man, animal or plants; protection of environment; applied and fundamental scientific research; education and training and forensic inquiries.

* Care and Accommodation (Articles 5 and 10)

It is one of the main objectives of the text. The Convention states that any animal used or intended to be used in a procedure is to be provided with accommodation, environment and care appropriate to its health and welfare. Appendix A of the Convention contains detailed guidelines on accommodation, environment and care for laboratory animals.

* Reduction - Alternative methods (Articles 6 and 7)

Another main objective of this Convention is the reduction of the number of animals used. Therefore, a procedure shall not be performed if another scientific method, not entailing the use of an animal, is available. Furthermore, species and number of animals must be carefully considered.

* Suffering (Articles 8 and 9)

Animal suffering must of course be reduced to a minimum and possibly eliminated through general or local anaesthesia. Where this is not possible, appropriate legislative and/or administrative measures shall be taken to check the need for such a procedure. The Convention goes further for experiments where the animals are expected to experience severe pain by requesting a special authorization from a competent authority or at least a declaration and a justification for the experiment to be carried out.

* Destiny of the animals (Article 11)

The Convention forbids keeping an animal alive after the procedure is over if it is likely to remain in lasting pain or distress. In any case, the decision to keep an animal alive or to kill it has to be taken by a competent person (e.g. a veterinarian). The animal kept alive shall be placed under the supervision of a veterinarian or another competent person.

* Authorization (Article 13)

In principle, the Convention requires that procedures be carried out by persons authorised by the responsible authority or under the direct responsibility of such a person, except if the project itself is authorised.

* Control of breeding or supplying, and user establishments (Articles 14 to 24)

The Convention also provides for a control of the establishments themselves which must be registered with or approved by the responsible authority. Records of movements of animals entering or leaving a breeding or supplying establishment shall be kept. Similar requirement is made for user establishments (see statistics). Dogs and cats must be marked.

The Convention requires that a person in charge of a breeding or supplying establishment be competent to administer or arrange suitable care for the animals. These requirements go further in user establishments where sufficient trained staff shall be available and arrangement must be made for veterinary advice and treatment. Moreover, persons carrying out procedures shall have had appropriate education and training.

In user establishments only animals supplied from registered breeding or supplying establishments must be used unless an exemption has been obtained from the national authority.

* Education and training (Articles 25 and 26)

The use of animals for educational purposes must be notified and is limited to the education and training of those whose professional activities will involve the performance of procedures or the treatment or care of animals (e.g. veterinarians, researchers, etc.).

* Statistical information (Articles 27 and 28)

The Convention attaches great importance to the collection of statistical information on the use of animals, as one of the major tools for monitoring the use of animals. The Convention requires that, subject to the requirements of national legislation relating to secrecy and confidentiality, certain information listed in Article 27 be collected and sent each year to the Secretariat of the Council of Europe in accordance with the tables presented in Appendix B to the Convention to be published. The harmonised presentation of the data in these tables ensures that the final compilation presents a reliable overall picture of developments.

* Recognition of procedures (Article 29)

In order to avoid unnecessary repetition of procedures, each Party is required to recognise the results of procedures carried out in the territory of another Party where practicable and lawful. To the same end, Parties are encouraged to exchange relevant information in this field.

BIOGRAPHICAL NOTES

Dr. M.M.L. Aerts obtained his degree in pharmacy from the Katholieke Universiteit Nijmegen in The Netherlands. He obtained his Ph.D. in 1981. From the year 1990-1993 he was project manager at Intervet International. Since 1994-1996 he was departmental head of the Antibiotic Department R&D at Intervet International. From the year 1996-2002 he has been the departmental head of the Registration Department of Intervet International. Since August 2002 he has been appointed Director R&D The Netherlands at Intervet International.

Mr Arnoud M. Akkermans graduated in 1987 as medical engineer in the Netherlands. In 1988 he became research worker at the Laboratory for the Control of Biological products (LCB), the vaccine production part of RIVM (Bilthoven, NL). Since then he specialised in biostatistics, test validations and laboratory automation. He took part in the management team of the "Ph.Eur. Collaborative Study on Alternative Methods for Potency Testing of Tetanus Toxoid Vaccines for Human Use". In 2001 he is appointed as a scientist at the Laboratory for Medicines and Medical Devices (LGM), the OMCL part of RIVM. He is mainly involved in the batch release procedures of vaccines and biostatistical advice.

Dr Agnès Artiges graduated in pharmacy and has a PhD in the same subject, as well as a degree and a PhD in law, the latter from the University of Paris, France. In her postgraduate law degree she specialised in European Institutions.

She was Assistant and Assistant Instructor in the Toxicology Laboratory of the Faculty of Pharmacy of Bordeaux before joining the French Ministry of Health in 1971. During her career with the Ministry, she has held the posts of Head of the French Pharmacopoeia, Head of the Registration Authority for Medicinal Products for Human Use and Head of the Sub-directorate of Scientific and Technical Affairs.

In addition, she was Chairman of the European Pharmacopoeia Commission from November 1989 to November 1992 and a member of the former Quality Working Party of the Committee for Proprietary Medicinal Products (CPMP) of the EC and was Chairman of this Working Party from December 1991 to March 1993.

Dr Artiges left the French Ministry of Health in April 1993 to take up the post of Director of the European Directorate for the Quality of Medicines (European Pharmacopoeia and European Network of Official Medicines Control Laboratories/OMCL) - Council of Europe.

Dr M.-E. Behr-Gross earned a pharmacy degree at the Louis Pasteur University of Strasbourg (F). After a spell in pharmaceutical practice, she worked in research on immunopharmacology. After completion of her Ph.D. she became a lecturer at the Department of Pharmaceutical Sciences of the Louis Pasteur University and also gained some experience in product development. She is currently a scientific officer at the European Directorate for the Quality of Medicines where she is in charge of monitoring projects belonging to the Biological Standardisation Programme co-sponsored by the Council of Europe and the European Community.

Dr K.-H. Buchheit obtained his degree in pharmacy from the Johann-Wolfgang-Goethe University in Frankfurt/M. (Germany) and his Ph.D. in pharmacology in 1984 from the same university. He joined Novartis (Sandoz at that time) in Basel, Switzerland in 1984 where he stayed until 1999 as research scientist and group leader in pharmacology in various fields (serotonin receptors, potassium channels, immunosuppression) and in drug development.

Since December 1999, he is Deputy Head of Division IV at the EDQM (Council of Europe, Strasbourg, France).

Dr Lukas Bruckner obtained his degree in veterinary medicine from the University of Berne in Switzerland. He studied for his DVM at the Institute of Veterinary Virology at the University of Berne. He obtained his DVM, submitting a thesis on different aspects of BVD virus, in 1982.

Since 1983 he is working at the Institute of Virology and Immunoprophylaxis (IVI), the Official Medicine Control Laboratory for biologicals for veterinary use in Switzerland. He started at the IVI as a research assistant. Meanwhile he is head of the Biologicals Department. Since 1990 he is member of expert group 15V (vaccines and sera for veterinary use) of the European Pharmacopoeia.

Mr Peter Castle graduated in biochemistry from Cambridge University, England in 1968. He worked on drug metabolism and determination of drugs in body fluids at the Pharmaceutical Society of Great Britain for three years before joining the animal health division of Smith Kline & French in the licensing department, working on veterinary vaccines and anthelmintics. Since 1974 he has worked in the Technical Secretariat of the European Pharmacopoeia, now a division of the European Department for the Quality of Medicines (Council of Europe, Strasbourg). He is Secretary to the European Pharmacopoeia Commission and head of the division dealing with development of monographs and general chapters. Member of the Council of the International Association for Biological Standardization.

Dr Klaus Cussler graduated in 1981 in Veterinary Medicine from the University of Giessen and received his Ph.D in 1988 at the University of Mainz (D); In 1987 he was appointed by the Paul-Ehrlich-Institut where he became Head of the Bacterial Section in the Veterinary Department in 1991. Since 1993, he has been the Animal Welfare Office of the Institute. His research activities are focused on the development of alternative methods to animal testing in vaccine quality control. He is a member of the Advisory Group on Alternative to Animal Testing in Immunologicals (AGAATI) and became Chairman of the Foundation in 1998.

Mr Arnold Daas obtained his degree in mathematics (statistics and operations research) in 1993 from the University of Nijmegen in the Netherlands. He has worked as apprentice at the Medical Statistical Department of the same university, and as assistant at the Biometrical Department of the Erasmus University in Rotterdam. Since 1995 he works as Statistician/Biometrician at the European Directorate for the Quality of Medicines – Council of Europe, where he is responsible for the design and analysis of data from collaborative studies, mainly in the context of the Biological Standardisation Programme.

Dr. Michel Duchêne obtained his degree in Agronomy Sciences from the University of Gembloux in Belgium. He worked in the field of food microbiology and bio-technology at the same university and obtained his Ph.D in 1982.

He joined the Company GlaxoSmithKline Biologicals in 1982 as Bacterial Vaccine Production and Development Manager. From 1984 till 1989, he was Vaccine Production Associate Director.

He was appointed Director, Quality Control in 1989 and has been appointed Technical Affairs Director in 1998.

Dr Roland Dobbelaer graduated in Chemistry/Biochemistry from Ghent University in 1964. He obtained his Ph.D. at the Faculty of Medicine of Louvain University in 1986 while

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working at the Section for Biological Standardisation of the Scientific Institute of Public Health – Louis Pasteur (SIPH) of the Belgian Ministry of Public Health. Roland Dobbelaer is currently head of Section and is responsible for the National Control Authority Batch Release of vaccines operating in the EDQM network of OMCL's. He is also involved in advising the Belgian Medicines Board and the CPMP in the licensing of biologicals. As a member of the CPMP Biotechnology Working Party, he is involved in both the ICH and VICH process. As a member of the European Pharmacopoeia Expert Group N°15 he is involved in drafting European monographs on vaccines. As a member of WHO's Expert Committee on Biological Standardisation he participates in drafting WHO Recommendations on Vaccines. He is also currently Chairman of EDQM's Biological Standardisation Programme Steering Committee.

Dr Marlies Halder: studied Veterinary medicine at the Veterinary Faculty of the University of Munich (D) and obtained a PhD from the University of Munich in 1987 for research on crustaceans and fish diseases. She was employed for several years (1985 - 1991) as a research scientist at the Institute for Zoology and Hydrobiology (University of Munich) and the Akademie fuer Tierschutz (Neubiberg, D). Her working areas included the use of *in vitro* methods in ecotoxicology as well as the diagnosis of and epidemiological studies on viral and fungal diseases of fish and crustaceans. From 1991 - 1994 Dr Halder specialised in Medical Informatics and worked as a freelance programmer for medical instruction courses.

Dr Halder started to work for the European Commission in October 1995 and currently holds a position as a scientific temporary agent at the European Centre for the Validation of Alternative Methods (ECVAM; Institute for Health & Consumer Protection, Joint Research Centre) in Ispra, Italy. Her main working areas are Three Rs methods for the quality control of biologicals.

Dr Coenraad F.M. Hendriksen, DVM, PhD, qualified in veterinary science from Utrecht University and continued his postgraduate training in laboratory animal science. In 1989 he obtained his PhD at Utrecht University on a thesis entitled "Alternatives to animal testing in tetanus and diphtheria reserach".

Since 1989 Hendriksen is animal welfare officer at the National Institute of Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. His research activities are focused on the development and validation of methods to replace, reduce and/or refine the use of laboratory animals, especially in the field of the production and quality control of immunobiologicals. He published the volume "Laboratory Animals in Vaccine Production and Quality Control: Replacement, Reduction, Refinement" (Kluwer Academic Publishers, 1988) and "Replacement, Reduction and Refinement: Present Possibilities and Future Prospects" (Elsevier, 1991). He is co-editor of several books and congress proceedings. He is the Dutch representative of the Scientific Advisory Committee of the European Centre for the Validation of Alternative Methods (ECVAM). Since March 1, 2000 he holds a (part-time) chair on Alternatives to Animal Use at the Veterinary Faculty of Utrecht University.

Dr Gábor Kulcsár graduated in 1993 from the University of Veterinary Science and got his Ph.D. in 2000 from the same university. He has been working for the Institute for Veterinary Medicinal Products since 1994. He works on the field of viral vaccine control and registration. In 2001 he was appointed as Head of Department of EU affairs.

Dr ir. P.P.A.M. Leenaars, graduated in 1992 from Wageningen University, The Netherlands. In 1997 she obtained her Ph.D from Erasmus University Rotterdam (The Netherlands). During 1998-2001 she was a postdoctoral fellow at National Institute for Public Health and the Environment (RIVM) in Bilthoven (The Netherlands). Since 2002 she is Animal Welfare

Officer and Scientist at the RIVM in Bilthoven. Her main research area is development of alternatives to animal testing in vaccine quality control with emphasis on *in vitro* immunological models.

Mrs Laurence Lwoff graduated in 1984 from the University of Paris VI – Jussieu (France). She obtained her degree in Agronomy from the Institut National Agronomique Paris-Grignon (France) in 1986 and received her PhD in molecular biology from the same Institute in 1989. She joined the Council of Europe in 1991, where she was entrusted with the responsibilities of the Secretariat of the Conventions concerning the use of animals in the Public Law Department. In 1999, her responsibilities were extended to biotechnology. In July 2002, she left the Secretariat of the Conventions on the use of animals to join the Bioethics Division where she is currently responsible for the activities on the protection of the human embryo and foetus and on human genetics.

Dr David Mackay trained as a vet at the Royal Veterinary College in London. After a period in clinical practice he obtained an MSc and a PhD in veterinary immunology. David worked for ten years on diagnosis control and research into OIE. List A diseases of livestock at the Institute for Animal Health, Pirbright, before joining the Veterinary Medicines Directorate as Head of the Immunologicals Team in 1999. David now acts as Director of Licensing for the Veterinary Medicines Directorate in the United Kingdom.

Prof. D. Morton is professor of Biomedical Science and Ethics at the University of Birmingham. He is the named veterinary surgeon and director of the animal facility and researches into the recognition and assessment of animal suffering and the development of humane endpoints.

Dr Keith Redhead, graduated in 1975 from the University of Bath, UK and received his PhD in 1978 from the Department of Microbiology also at the University of Bath. From 1978 to 1980 he was a postdoctoral research fellow at the Sir William Dunn School of Pathology, University of Oxford. From 1980 he was a Scientist in the Division of Bacteriology at the National Institute for Biological Standards and Control. Since 1995, he has been a Project Manager at Intervet UK Ltd conducting research on bacterial veterinary vaccines. In 1996 he became a member of the International Veterinary Industry Test Replacement Organisation (*In vitro*).

Dr Alain Sabouraud obtained his degree in pharmacy from University R. Descartes (Paris V) in Paris (France). He studied for his Ph. D. at the INSERM, France. He obtained his Ph. D. in 1992. During the period 1992-1993, he was research assistant at INSERM and lecturer at the School of Pharmacy (Paris V). He joined Pasteur-Mérieux in 1993 as the head of potency testing laboratory in the Quality Control department. In 1997, he worked as a researcher at the Center for Disease Control and Prevention, Atlanta (Rabies and Hepatitis laboratories). Since 1999, he has been the Head of the Quality Control department for products under development and test development in Aventis Pasteur France.

Mr Jean-Marc Spieser studied Pharmacy at the University of Strasbourg and obtained his Masters Degree (postgraduate) in Applied Industrial Pharmaceutics at the University of Montpellier in 1973.

After different positions in Research and Pharmaceutical Industry he joined the Technical Secretariat of the European Pharmacopoeia Commission at the Council of Europe in

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Strasbourg. Jean-Marc Spieser is currently Head of Division IV at the EDQM, an independent division which manages the activities of the Biological Standardisation Programme aimed at:

- developing and validating new methodologies and particularly those IN-VITRO methods which are alternative methodologies to *in vivo* animal bio-assays and
- establishing the European working standards and reference materials for biologicals (hormones, vaccines and blood derivatives).

and the OMCL Network including for the time being about 85 participating Official Control Laboratories all over Europe involved in both the human and the veterinary field In 1994, he initiated the inter communication between Official Medicines Control Laboratories (OMCL) within Europe by developing a real European Network governed by general common policies and operational guidelines especially in the areas of :

- Quality Assurance
- market surveillance, for pharmaceuticals commercialised in Europe through both systems (centralised and decentralised) and,
- batch release activities by official control Authorities for biologicals .

Dr Ariane Van de Moer graduated in 1986 from the University of Sciences and Technologies of Languedoc, Montpellier (France) and received her Ph.D. in 1990 from the Faculty of Pharmacy, Unité de Recherche en Immunologie, Montpellier (France). She joined, from 1990 to 1992 as post-doctoral fellow, the Department of Veterinary Pathology and Public Health, Massey University, Palmerston North (New Zealand). In 1992 she was appointed by Pitman Moore (Mallinckrodt Veterinary Ltd, New Zealand) as research scientist in *in vitro* test development, vaccines. She developed her career as team leader test development, vaccines, at Mallinckrodt Veterinary Ltd (New Zealand). In 1997 she joined Rhône-Mérieux (Merial), Lyon, as manager biological analysis and hormonology department and further developed her career as senior manager of the department of biochemistry and methods (Merial, Lyon). She is currently head of biotechnologies at Vétoquinol S.A. Dr Ariane Van de Moer is a member of FEDESA's Biological Working Party, member of the International Association for Biologicals and co-founder and member of *In vitro* (International Veterinary Industry Test Replacement Organisation).

Dr Eva Vítková, M.D., Ph.D, graduated in 1977 from the Medical Faculty, Charles University in Prague and was candidate of Medical Sciences when she did her thesis entitled: Cellular immunity in the experimental and natural parotitis infection, elaborated in the Institute of Sera and Vaccines in Prague, defended at the Committee on Immunology of Czechoslovak Academy of Sciences in 1984. She started her career in 1981 in medical microbiology, Institute for Postgraduate Medical Education in (Prague). From 1982 to 1986 she worked for the Department of Clinical Immunology, University J.E. Purkyn Hospital, Brno. She then worked for the State Institute for Drug Control from 1986. From 1989 to present she is Head of the Department of Immunology, Virology and Pharmacology, she is now head of Department of Biological Methods.

Dr. Peter Volkens obtained his diploma in mathematics (mathematical statistics) from University Göttingen in Germany in 1980. He studied for his Ph.D at the University Göttingen, Germany. He obtained his Ph.D. in econometrics in 1984. During the period 1980-1984 he was research assistant at the Institute for Statistics and Econometrics, University Göttingen. From 1984 to 2000 he worked as statistician in agricultural science and (since 1986) in the pharmaceutical industry, dealing with all phases of preclinical and clinical research & development. In 2001 he started as statistician at the Paul-Ehrlich-Institute, Germany.

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