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Potency Testing of Veterinary Vaccines for Animals: The Way From in Vivo to in Vitro

Editor
C. Jungbäck

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Potency Testing of Veterinary Vaccines for Animals: The Way From in Vivo to in Vitro

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Paul-Ehrlich-Institut (PEI)

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Organization of the Proceedings

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Paul-Ehrlich-Institut (PEI)
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International Alliance for Biological Standardization (IABS)

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Preface

Before being placed on the market inactivated vaccines are predominantly tested in vivo, mainly in laboratory animals.

In recent years substantial efforts have been made either to modify these animal tests in order to reduce the number of required animals and the stress imposed on them (Refinement) or to completely replace these experiments by in vitro tests. The acceptance of these tests differs considerably between vaccine manufacturers and licensing authorities. It is thus comprehensible that vaccine manufacturers hesitate to adopt the new test methods.

To promote further progress the international scientific workshop "Potency Testing of Veterinary Vaccines: The Way From in Vivo to in Vitro" was held at the Paul-Ehrlich-Institut in Langen, Germany on 01-03 December 2010. More than 130 participants from industry, academia and regulatory authorities discussed the current state of the 3R concept, examples of its successful implementation as well as still existing hurdles. This volume presents the proceedings of this international workshop where the current state of knowledge of replacement of in vivo tests was recapitulated. Already existing replacement approaches were presented and their advantages and disadvantages discussed. Furthermore proposals for new ways to ensure the quality of veterinary immunological medicinal products (IVMPs) were made.

Thanks to the excellent presentations and the substantial and constructive contributions to the discussions an intense scientific discussion took place which made the meeting a fruitful one.

The hurdles to be overcome whenever the 3Rs should be implemented in the potency testing of inactivated veterinary vaccines were identified and discussed and a proposal for a road map for future steps towards in vitro testing was made.

In my opinion the workshop can be regarded as a good step forward to replace or reduce in vivo tests for the quality control of IVMPs.

I would like to thank the European Directorate of Quality of Medicines & HealthCare (EDQM) which acted as a cosponsor of this meeting. This sponsorship gave the opportunity to a number of representatives from national authorities to attend this meeting, which cannot be undervalued and was of great benefit for the success of the workshop.

Carmen Jungbäck

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SESSION I

Use of the 3R Approach for Potency (1)

Chairpersons: *Carmen Jungbäck*
Jacques Léchenet

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Introductory Speech

History, Approaches, Legal Situation and Political Pressure, Outlook, Expectations

J.-M. Spieser

European Directorate of Quality of Medicines & HealthCare (EDQM), Strasbourg, France

Introduction

Good morning ladies and gentlemen. It's a great honour to be here as the co-sponsor of such an important event. As professionals you are concerned with the quality of biological medicinal products. That is why we have come together on the occasion of this conference. Here we will examine where we are in terms of the development of alternatives to animal testing in particular in the domain of vaccines for veterinary use, an issue which is close to our hearts. Let me just give you briefly the historical background.

Background

In 1986 the scene was set by the Council of Europe with its Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes, International European Treaty No 123 and a few months later, the European Union Commission with its Directive 86/609, which was more or less the same as the Convention. This framework created by politicians and lawyers elaborated the legal tools which would act as the incentive to boost the implementation of the principles of the 3Rs, which is Refining, Reducing, and finally Replacing animal testing – a real challenge for the years which followed.

Achievements

Almost 25 years later, can we be satisfied? I would say yes and I would say no and that is probably one of the reasons why we are all here together. Yes, because we have already achieved a lot in the application of the 3R concepts. We achieved it thanks to the willingness of all stakeholders and partners who involved themselves enthusiastically and voluntarily. We saw the authorities who often generated the preliminary impulse and incentives. This was done through initiation of feasibility assessments, including initial testing, to demonstrate the interest and value in developing the new methods. We also saw the industry who were always responsive and gave access to representative and often customised samples for hands-on evaluation through collaborative studies.

We did not make any a priori choices when we began but the cases came naturally setting obvious priorities amongst themselves. We started, of course, very rapidly with the general safety tests; the abnormal toxicity test, specific impurity general tests, pyrogen testing. These were really the key issues which drew our attention first. I'm very pleased to thank here publicly all the collaborators from the Paul-Ehrlich-Institut who have devoted a lot of their time to these very important issues.

We also devoted time to so-called well-characterised molecules such as small peptides and small proteins, moving from in vivo potency assays to new modern animal-free testing. A new era began in which the pharmacological activity was no longer seen as the key issue for potency assays. Emphasis was instead placed on checking the quality criteria of specific molecular characteristics of these products. This was a big change. Key methods were of course the molecular separation methods such as HPLC and capillary zone electrophoresis.

The endotoxin LAL test was one of the first to come as an alternative to pyrogen testing but later on, and again through the initiative of the Paul-Ehrlich-Institut, we had the development of monocyte activation tests. This is a great step forward and gives the real possibility to completely eliminate the pyrogen test in the future.

When looking more closely at individual products we see that the choice available for crude biologicals, of which there are still many, especially in the veterinary field, is more limited. Of course for these products we could not apply this idea of molecular characterisation but we rapidly turned to replacing the challenge potency tests by serological assays. That was again a step further. This approach was particularly interesting for bacterial vaccines in the human and veterinary fields and for specific immunoglobulins such as tetanus.

Other key developments were the cell-based assays for complex molecules such as erythropoietin, filgrastim and again a certain number of veterinary vaccines.

Let me give credit publicly here to the Biological Standardisation Programme of the European Directorate for the Quality of Medicines & HealthCare and the Expert Groups of the European Pharmacopoeia for their excellent and efficient work. Their contribution has largely helped bring about the success of the undertakings in all of these areas.

Challenges for Veterinary Vaccines

In the control of veterinary vaccines we have moved away from routine batch testing of abnormal toxicity and we are also now moving away from routine testing for target animal species toxicity. So now the most important thing will be replacement of animal challenge tests by alternatives such as serological tests, cell-based testing or in vitro tests. When it comes to serological tests we have a very crucial issue before us; what is the meaning of these animal tests? When you propose the use of small animal models to test vaccines for cows or horses or other animals we are faced with the same problem that we faced in the human field. What is the meaning of the results obtained and how does the mouse or rat assay transpose to the target species? Is it a meaningful check on the actual potency of your material or is it a consistency test?

Still a lot of work, and not the easiest work, remains to be done. We have developed skills in the field of human vaccines. In the veterinary field we have completed some important projects such as for Newcastle disease and rabies. However we still have a large challenging domain before us in the veterinary vaccines field. This is of course what we will have the chance to scrutinise during the next two and a half days.

Importance of Collaboration

Let me underline the importance of collaboration. EDQM plays an important role in the development in the 3Rs, but there are many other scientific and political partners involved.

Dr. Vieths has already mentioned the important role he and his colleagues at the Paul-Ehrlich-Institut have played at the European Pharmacopoeia level and in the Biological Standardisation Programme. Like the Paul-Ehrlich-Institut, each of the national institutes and control authorities has made important contributions. The European Medicines Agency is also key player. They have an influence on the marketing authorisation files and they too must apply 86/609 and its revisions to make sure that the issue of alternative animal testing has been taken into account. Other scientific and political partners have also made critical contributions and I would specifically wish to mention the ECVAM and EPAA initiatives because they play a very important role, at a political level and at a sponsorship level, and we thank them for their comprehensive co-operation.

There are a lot of others who of course should be mentioned but it is not the aim here to make an exhaustive list. Nevertheless they have not been forgotten and I include a very thankful recognition of their contribution.

Lessons Learned

What lessons have we learned? A major revelation which is perhaps not astonishing in the light of scientific honesty was that often the traditional animal tests were far from being validated in current terms. They were used historically. They were used because it was the first easy thing to do. They were developed by our grandfathers and they served their purpose at the time. They were used by our fathers, by our aunts and so on and we continued to use them. However we never, either at the early stage or later on, devoted time to see: Are they really validated? Do they really do what we ask them to do? Honestly, we have to say that we often discovered that this was not entirely the case.

That is where the difficulties began for making correlation studies for the validation of alternative methods. In fact, it turned out that a certain number of new methods had to be validated from scratch using the appropriate accepted rules and international guidelines for validation. You could imagine that this could turn into a major issue for established products where you would have to reorganise the whole validation process and make direct links to clinical studies. This is of course not what we are looking for. We do not wish to reregister the products. So there is a very challenging issue before us. The correlation between the clinical value in the target animal and the value obtained with the new alternatives is not an easy issue to resolve without a direct link via the 'old' tests.

Overcoming Conservatism

I would also wish to be provocative here as I have been known to be. I would wish to remind you all, whether you are sitting on the side of industry or on the side of the regulators that, from my personal viewpoint, especially in the field of veterinary medicine, we are suffering from over-conservatism. This is really something which we all have to overcome.

Authorities and industry create a lot of harm to future development and the implementation of promising alternatives through this over-conservatism. Nothing is more frustrating than the hurdles which complicate the final application of newly developed methods. Imagine the effort of many people working hard to develop and validate an alternative and then they discover several years after the studies are complete that the method is still not in place. We have to be very honest and address that issue. Why is it?

First of all, let's begin with the authorities: licensing and control authorities, surveillance authorities, i.e. the assessors, the OMCLs and the inspectors. They should in future probably be more open to pragmatic and realistic solutions and avoid blindly applying administrative rules. We have to accept change and bring in a dose of pragmatism. Industry also suffers from a good level of conservatism and reluctance to implementing changes. We can understand that revalidation, training, or retraining of new personnel and procurement of new equipment, including reagents, is not an easy issue and not an easy task. But it should not be the excuse, which is often the case for the time being, to promote stagnation. We should be willing to go ahead and we should be willing to take that last step.

Scope for Improvement and International Collaboration

During the next two and a half days we will have the opportunity to examine among others the Newcastle disease case and the rabies case. These are two excellent examples from which we can learn more so we can in future offer better solutions on how to promote an optimised implementation of the 3Rs. There is no reason that we cannot succeed in the veterinary field as we have done in the human field. We have to be honest. The veterinary field is behind. You are behind your colleagues from the human field. Why is that? There is no reason. They are fundamentally the same biological products. We should be able to find the same solutions.

One major component of future success will lie in the capability of involving our international partners because we know that today the market is a global market. We know that when we define something for Europe, if our colleagues overseas do not have the same approach, we will fail. So we need to have this international approach. Europe cannot stand alone and our colleagues from the USA, Australia, Japan, and all the others will have to be closely involved and contribute to finding commonly agreed solutions. In this context I am pleased to note the interest shown by our overseas colleagues, and their presence at this conference is a good sign of their willingness to be loyal and efficient partners.

Conclusion

To conclude, I wish to thank our partners the Paul-Ehrlich-Institut, and in particular you, Carmen Jungbäck, and the IABS for your enthusiastic willingness to organise this very important conference. I am convinced that it will be a milestone for future developments and contribute successfully to creating the missing international dimension in our task to implement the 3R concept planet-wide. I wish you all a successful and very fruitful discussion and creative solutions. Thank you.

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Recent Progress and Future Directions for Reduction, Refinement, and Replacement of Animal Use in Veterinary Vaccine Potency and Safety Testing:

A report from the 2010 NICEATM-ICCVAM

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Key words: Veterinary vaccines, potency, safety, ICCVAM, recommendations, workshop

Abstract: Veterinary vaccines contribute to improved animal and human health and welfare by preventing infectious diseases. However, testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant pain and distress. NICEATM and ICCVAM recently convened an international workshop to review the state of the science of human and veterinary vaccine potency and safety testing, and to identify priority activities to advance new and improved methods that can further reduce, refine and replace animal use. Rabies, *Clostridium sp.*, and *Leptospira sp.* vaccines were identified as the highest priorities, while tests requiring live viruses and bacteria hazardous to laboratory workers, livestock, pets, and wildlife were also considered high priorities. Priority research, development and validation activities to address critical knowledge and data gaps were identified, including opportunities to apply new science and technology. Enhanced international harmonization and cooperation and closer collaborations between human and veterinary researchers were recommended to expedite progress. Implementation of the workshop recommendations is expected to advance new methods for vaccine testing that will benefit animal welfare and ensure continued and improved protection of human and animal health.

Introduction

Veterinary vaccines contribute significantly to improved animal and human health and welfare by preventing and controlling infectious diseases. However, testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant pain and distress. Accordingly, the USA Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the United States National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) recently identified vaccine potency and safety testing as one its four highest priorities [1].

ICCVAM is an interagency committee of Federal agencies charged by law with evaluating new, revised, and alternative methods with regulatory applicability, and with promoting the scientific validation and regulatory acceptance of test methods that accurately assess the safety of chemicals and products while reducing, refining (less pain and distress), and replacing animal use. ICCVAM members include 15 USA Federal regulatory and research agencies that require, use, generate, or disseminate safety testing data, including theUnited States Department of Agriculture (USDA), which regulates veterinary vaccines, and the United States Food and Drug Administration (USFDA), which regulates human vaccines. ICCVAM is a permanent interagency committee of the National Institute of Environmental Health Sciences (NIEHS) under NICEATM. NICEATM administers ICCVAM, provides scientific and operational support for ICCVAM-related activities, and conducts validation studies on promising new safety testing methods. NICEATM and ICCVAM serve a critical translational role in moving research advances from the bench into safety tools that can be used in regulatory practice to prevent disease and injury.

In order to promote and advance the development and use of scientifically valid alternative methods for human and veterinary vaccine testing, NICEATM and ICCVAM subsequently organized an international workshop on September 14-16, 2010 at the National Institutes of Health in Bethesda, Maryland, USA. The *International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions*, was organized in partnership with the European Centre for the Validation of Alternative Methods (ECVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM) and Health Canada, and was co-sponsored by the Society of Toxicology. The full proceedings of the Workshop will be published in *Procedia in Vaccinology*, which is accessible online at: http://www.elsevier.com/wps/find/journaldescription.cws_home/720522/description#description

Goals and Organization of the Workshop

The goals of the International Workshop were to: 1) identify and promote the implementation of currently available and accepted alternative methods that can reduce, refine and replace the use of animals in vaccine potency and safety testing; 2) review the state of the science of alternative methods and identify knowledge and data gaps that need to be addressed; and 3) identify and prioritize research, development and validation efforts needed to address these gaps and to advance alternative methods that can ensure continued protection of human and animal health.

The workshop opened with a plenary session where expert scientists and regulatory authorities from the USA, Europe, Japan, and Canada outlined the importance of vaccines to human and animal health, and described national and international regulatory testing requirements for both human and veterinary vaccines. Daily plenary sessions were followed by breakout group discussions for veterinary and human vaccines. Each breakout group co-moderator presented the consensus recommendations arising from the discussions in the following plenary session. The breakout groups focused on in vitro replacement methods for potency testing, in vivo refinement and reduction methods for potency testing, and 3Rs alternatives for safety testing.

Nearly 200 scientists from 13 countries attended the workshop, which provided a unique opportunity for interested stakeholders from the human and veterinary vaccine fields to interact and gain important insights on the current similarities and differences in vaccine potency and safety testing procedures across different countries and regions.

Importance of Veterinary Vaccines to Animal and Human Health

Veterinary vaccines are used to prevent and control a wide range of infectious diseases in companion animals, livestock, wildlife, fish, zoo animals, and many other animal populations. They serve a vital role in controlling and preventing foreign animal diseases such as Foot and Mouth Disease (FMD), and emerging diseases such as new influenza strains. The recently announced worldwide eradication of Rinderpest Disease, a devastating livestock disease that has persisted for thousands of years, was only possible because of the availability and implementation of effective veterinary vaccines.

Veterinary vaccines also contribute to human health by controlling zoonotic diseases that can be transmitted from animals to humans, such as rabies. Vaccines are used to reduce foodborne diseases, such as preventing the transmission of Salmonella via infected eggs by vaccinating layer hens. Veterinary vaccines also contribute to human health by ensuring a reliable and cost-effective food supply. An additional important benefit of vaccines is that they support enhanced animal welfare by preventing and reducing debilitating and deadly diseases.

Veterinary Vaccines: Priorities for Future Research, Development, and Validation Activities

The workshop identified criteria that should be used to prioritize veterinary vaccines for future research, development and validation efforts to reduce, refine, or replace animal use for potency and safety testing. The recommended prioritization criteria for vaccine potency testing included:

- Testing that uses large numbers of animals per test, or large numbers of animals due to the number of serials produced annually
- Testing that involves significant animal pain and distress, such as challenge testing and toxin neutralization tests
- Vaccine testing that requires live viruses and bacteria that are highly contagious and/or hazardous to laboratory workers, livestock, pets, and wildlife, including foreign animal diseases
- Vaccines for which the functional protective antigen has been identified and characterized
- Vaccines for which there are ongoing alternative development and validation activities
- New vaccines that are currently undergoing pre-licensing development and evaluation.

Based on these prioritization criteria, the highest priority vaccines recommended for further development of alternative test methods included:

- Rabies vaccines (both human and animal)
- Leptospiral vaccines
- Clostridial vaccines
- Erysipelas vaccines
- Foreign animal diseases, esp. Foot and Mouth disease and Blue Tongue disease
- Poultry vaccines
- Fish vaccines
- New vaccines.

Rabies, *Clostridium sp.* and *Leptospira sp.*, vaccines were identified as the highest priorities because they require large numbers of animals and most involve either challenge or toxin neutralization testing that can involve significant animal pain and distress. Vaccines for which the protective antigen is known would greatly facilitate the development of antigen and antibody quantification methods. The workshop participants also recommended that manufacturers should seek to develop replacement alternatives during the development phase for new vaccines.

Priorities for safety testing included: extraneous agent testing where animals are still used, especially avian vaccines; inactivation testing for killed vaccines, such as rabies; residual toxicity testing; subunit protein and DNA vaccines, and new vaccines.

Veterinary Vaccine Potency Testing: In Vitro Replacement Methods

State of the science

A significant number of veterinary vaccine potency tests for serial release are conducted using in vitro methods. For live viral vaccines, these include culture techniques to quantify microbial content as an indicator of antigenic content of the vaccine. Examples include the Feline Calicivirus vaccine, Marek's disease vaccine, and Infectious Bursal disease vaccine.

Some inactivated vaccines also now use in vitro antigen quantification assays for potency testing, such as avian Newcastle Disease, non-adjuvanted canine Leptospiral (*L. canicola, L. pomona, L. grippotyphosa, L. icterohaemorrhagiae*) bacterins, and feline leukemia vaccines. For feline leukemia virus, the gp70 protein protective antigen is quantified.

Research needs

The workshop identified and recommended priority development and validation activities necessary to advance in vitro potency tests. These included: increasing efforts to identify protective antigens where these have not yet been identified or confirmed; investigating gene cloning to produce purified protective antigens for vaccines that can be more readily quantified; and developing standardized methods and reagents necessary to quantify protective antigens.

Adjuvants are widely used in veterinary vaccines; however, many adjuvants can interfere with antigen quantification assays. The workshop participants therefore recommended that the relative impact of various adjuvants on antigen quantification assays be further investigated, as well as the extraction methods used for vaccines containing adjuvants that can interfere with antigen assays.

Workshop participants also agreed that while there are numerous in vitro methods that have been developed, the primary impediment to broader use is the cost and time required for each company to conduct product-specific validation of the in vitro potency assay as well as requalification and monitoring of the specific references for each of their proprietary vaccines. There was broad agreement that vaccine manufacturers should interact early and frequently with regulators during the development and validation of new potency and safety assays.

Vaccine Potency Testing: Using Serological Methods as Refinement Alternatives

State of the science

Potency testing for inactivated veterinary vaccines has traditionally used challenge testing of vaccinated animals with live microbes to determine the quantity of vaccine necessary to provide adequate protection. Inadequately protected and control animals that become infected usually develop significant

clinical signs of the disease and/or die. However, in recent years, antibody quantification procedures have been developed and validated and subsequently replaced the challenge test for several vaccines. While such testing still requires the use of animals, it provides for significant refinement by eliminating the pain and distress involved in challenge testing procedures because animals are no longer exposed to live pathogens, which avoids the infection and progression of clinical disease in inadequately protected and control animals.

Referred to as serological methods, these methods involve vaccinating animals, taking blood samples and then quantifying the amount of antibody produced by a given amount of vaccine. Vaccines for which antibody quantification assays have been approved for potency testing include swine Erysipelas, non-adjuvanted canine Leptospira (*L. canicola*) and bovine Leptospira (*L. hardjo*).

In addition, the potency testing of several Clostridial vaccines are now assessed by serological methods, where the antitoxin present in serum from vaccinated animals is quantified by a serum toxin neutralization test (TNT). The neutralization tests have traditionally utilized mice, but some Clostridial vaccines (e.g., *Clostridium tetani*) are now tested by the vaccination of guinea pigs and serological evaluation of anti-tetanus toxoid antibodies is conducted by using an indirect enzyme linked immunosorbent assay (ELISA) [2].

Priority research needs

Workshop participants recommended priority research and development activities needed to advance the use of serological methods and in vitro TNT assays. Recommendations included:

- Identify and understand the antibodies involved in protective immunity for specific vaccines
- Develop and validate assays and reagents necessary to quantify protective antibodies
- Conduct research into new methods to assess functionality of antibodies or other immune responses
- Conduct research necessary to convert in vivo Toxin Neutralization Tests (TNT) to ELISA or cell based TNT methods
- Develop specific cell lines to detect enzymatic activity of toxins
- For rabies vaccines, establish a focused working group of both human and veterinary researchers to develop and validate meaningful serological assays
- Develop reference standards and stability assays necessary to support antibody quantification and in vitro toxin neutralization tests
- Develop serological methods for Leptospira vaccines to replace challenge testing for serovars for which protective antigen assays are not yet feasible
- Identify protective antibodies for fish vaccines
- Support activities necessary for broader implementation of available in vitro toxin neutralization tests.

The further development, validation, and implementation of a serological test for both human and veterinary rabies vaccines was the highest priority identified by workshop participants, due to the high variability of the current in vivo potency

test and the large number of mice used. Continued research and development into the potency testing of fish vaccines were also considered a high priority due to the large number of vaccination-challenge procedures currently conducted, which typically use large numbers of unvaccinated controls. Research and development efforts are expected to increase as additional fish vaccines enter the market and more animal health companies develop vaccines for aquaculture use.

Veterinary Vaccine Potency Testing: Using Humane Endpoints as Refinement Alternatives

State of the science

Despite the increasing use of in vitro and serological potency testing procedures for many vaccines, some vaccines still require the use of challenge tests using live microbes. In the past, the death of control animals was required to ensure that the challenge microbe was sufficiently pathogenic to cause death in unprotected animals and to demonstrate the protection of vaccinated animals. However, death is no longer a required endpoint by any regulatory authority, and testing guidelines now allow for humane euthanasia of moribund animals.

Humane endpoints are criteria that can be used as the basis for ending a test procedure early in order to avoid further pain and distress, or ideally, criteria that can be used to end a procedure *before* the onset of animal pain and distress [3, 4]. However, the use of earlier, more humane endpoints must be consistent with the attainment of specific testing objectives.

Earlier humane endpoints have been approved, and are currently in use, for challenge testing procedures for veterinary rabies vaccines and swine erysipelas vaccines. For veterinary rabies vaccines, paresis, paralysis and/or convulsions were determined to be sufficiently predictive of rabies infection and indicative that an animal would not recover [5]. Humane endpoints for rabies vaccine potency testing procedures are now incorporated into several national and international regulatory guidance documents and procedures [6, 7].

For swine erysipelas vaccine potency testing, a satisfactory challenge is evidenced in controls by body temperature increases above a specific threshold (i.e., 105.6° F or higher on at least two consecutive days), or clinical signs including, but not limited to, acute illness with hyperemia of the abdomen and ears, moribundity, with or without characteristic skin lesions (i.e., rhomboid-shaped appearance), stiffness, and/or joint involvement; or any combination of these symptoms and lesions [8, 9]. Humane endpoints for erysipelas vaccine challenge testing have previously been approved for use by several regulatory authorities [9, 10].

Priority research and development needs

Workshop participants uniformly agreed that earlier humane endpoints should be identified, validated, and implemented for all existing challenge testing procedures. At a minimum, all clinical signs should be systematically collected and an evaluation conducted to identify one or more clinical signs that might be useful as an earlier human endpoint. In addition, objective parameters should also be considered and collected, especially if clinical signs are not sufficiently accurate predictors, or if the clinical signs selected as humane endpoints still allow for significant severity and duration of pain and distress. Examples of objective quantitative measures that could be collected include body temperature and body weight changes.

Another recommendation arising from the workshop was the collection of data to identify earlier humane endpoints for unvaccinated control groups that could be used as the basis for terminating the remainder of the animals in a control group. For example, once a specified percentage of controls exhibit a specific clinical sign (or signs) which indicates that a challenge microbe was sufficiently pathogenic to cause clinical disease in unprotected vaccinates, the remaining controls might be humanely euthanized.

Additional recommendations to advance the use of humane endpoints included:

- Providing guidance on the recognition of acceptable clinical signs for use as humane endpoints, and increasing availability of comprehensive training to animal care and technical staff responsible for observation of test animals
- Identifying earlier humane endpoints for Leptospira potency testing still conducted using the challenge test, as well as other vaccines with a relative long latency period before overt disease is evident
- Collecting and evaluating potential humane endpoint data during pre-marketing efficacy tests
- Development of more innovative methods to observe animals.

A thorough understanding of disease progression during a challenge test was identified as an important priority to advance earlier more humane endpoints. To better understand disease progression during challenge testing and to expedite future data collection, workshop participants recommended that existing clinical data for control groups should be analyzed to identify potential earlier humane endpoints. The opportunity for possible application of earlier humane endpoints for control animals for poultry and fish vaccines was identified as a priority.

Further research is needed to develop enhanced innovative methods for observing animals with increased frequency to detect clinical signs that might be used to end studies before animals die or become moribund. This may be achieved with, for example, videotaping or real-time computer monitoring techniques. Furthermore, the frequency of animal observations should be increased to minimize the occurrence of unanticipated deaths. At a minimum, all animals should be observed at least twice daily.

Improved dissemination of information about the clinical signs associated with each disease was recommended, particularly among manufacturers, as well as increased sharing of information between manufacturers and regulators regarding

effective earlier humane endpoints. Manufacturers should be encouraged to implement humane endpoints, but there should be an awareness of the potential costs for increased frequency of clinical observations and advanced equipment to improve clinical surveillance.

Veterinary Vaccine Potency Testing: Reduction Alternatives

State of the science

Historically, a gradual reduction in the number of animals required per test has occurred over time. This can be attributed in part to improvements in the health status of animals, increased control of experimental variables, and other factors. In addition, the number of animals required for challenge studies has been reduced in some cases by replacing multi-dilution assays with single dilution assays, such as for rabies vaccine [11].

Priority research needs

To reduce the number of animals used in vaccination-challenge and serological tests, workshop participants recommended a systematic investigation into the causes of excessive variability in test results, and investigation of the causes for repeat testing due to incomplete or inconclusive results. This should be followed by concerted efforts to avoid, reduce, or control the sources of variation and the causes of incomplete test results. As factors affecting experimental variability are reduced or eliminated, a reassessment of the minimum number of animals required to maintain the necessary statistical power should be conducted. A retrospective review of existing data was also recommended to determine if the size of control groups could be further reduced, while still maintaining statistical power.

Workshop participants also noted that greater flexibility should be incorporated into the regulatory process so that the reduction of animals can be more readily applied on a case-by-case basis, especially for minor use applications of vaccines. In addition, participants also recommended increased efforts to investigate if single dilution assays can be used to replace multi-dilution methods for both vaccination-challenge and serology assays.

Veterinary Vaccine Safety Testing: Application of 3Rs Methods

State of the science

Regulatory authorities require demonstration of the safety of each lot of veterinary vaccine before it is released. This traditionally has involved testing in a small number of target species or a few laboratory animals. In certain situations, animal numbers for the safety evaluation of a veterinary vaccine can be reduced by

using information previously obtained from animals utilized for the potency test. For example, if the potency test requires the use of host animals, the same animals are also observed for any clinical sign of adverse reactions, thereby negating the need for additional animals for a general safety test. Examples include Newcastle Disease Vaccine, Bovine Virus Diarrhea Vaccine, Canine Distemper Vaccine, and Mink Enteritis Vaccine.

Vaccines that use additional animals for safety testing are typically those that require a separate laboratory animal safety test because safety assessment is not assessed in the target or host species during potency testing. More recently, test results for a variety of measurable vaccine parameters associated with consistently negative general safety tests for multiple vaccine lots have been approved on a case-by-case basis for product-specific situations as surrogates for the general safety test.

Priority research needs

The workshop identified several priority activities to further reduce and replace animal use for vaccine safety testing. Participants emphasized the importance of understanding that current safety testing procedures often serve multiple purposes, and that any strategy to reduce, refine, or replace animals must consider and address all of these requirements. Understanding the basis for failed safety tests is essential to identifying alternate methods to detect unsafe vaccines. It is also important to understand the full range of potential changes in a vaccine product that could result in a failed safety test, and to ensure that any alternative safety assessment approach is capable of detecting all of these changes.

The workshop recommended the continued investigation and application of cell culture and PCR techniques for extraneous agent testing. These techniques represent promising approaches for replacement of the in vivo chicken extraneous agent test [12]. Other specific activities included determining if the in vivo veterinary rabies inactivation test could be replaced with cell culture techniques used to confirm rabies virus inactivation for human rabies vaccines. Workshop participants also supported further investigations necessary to identify and develop the various tests that might be incorporated into a consistency approach for specific vaccines that could be validated as a substitute for current general safety tests.

Improved International Cooperation and Harmonization

The workshop participants strongly agreed that greater international cooperation and harmonization efforts would expedite global progress in reducing, refining, and replacing animal use for veterinary vaccine potency and safety testing. The availability of internationally harmonized potency test methods could significantly reduce animal use by eliminating the need for manufacturers to perform multiple potency release tests for the same vaccine lot in order to meet varying regulatory guidelines.

To facilitate international harmonization of potency and safety testing methods, workshop participants recommended that regulatory agencies should develop and seek international harmonization of the general principles for the validation of alternative potency and safety tests. Such efforts should build on existing guidelines for the validation and use of in vitro potency assays [13, 14]. This would be followed by early and frequent communication among national and regional authorities during the design, conduct, and evaluation of validation studies.

Internationally harmonized testing procedures and reagents for in vitro potency assays, and increased availability of internationally recognized reagents were also recommended to facilitate the reduction of animal use. For example, reagents for in vitro potency assays such as specific antibodies and viral and bacterial antigens are currently provided by USDA Center for Veterinary Biologics (CVB) to vaccine manufacturers upon request. Broad international availability of Standard References, supported by national and regional regulatory authorities, would aid in the conversion of animal-based tests to non-animal assays, and significantly reduce animal use for the re-qualification of master references by each manufacturer [15]. International organizations such as the World Organization for Animal Health (OIE) could oversee international Standard References, while centers of excellence such as USDA CVB and the European Directorate for the Quality of Medicines and HealthCare (EDQM) could maintain and distribute such standards.

Workshop participants also recommended improved dissemination and accessibility of information about new initiatives, documents, and guidance on available alternative methods. They also recommended that such information should be readily available in the public domain to increase access and consideration by vaccine manufacturers. International organizations that were suggested to aid in this effort included the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) and the OIE.

Conclusions

This was the first international workshop in the United States to bring together stakeholders from both the human and veterinary vaccine communities to discuss opportunities to further reduce, refine, and replace animal use for potency and safety testing. The collective interactions of experts from various vaccine manufacturers, academia, government, and the international community contributed to recommendations broadly supported by all participants. Enhanced post-workshop interagency and international collaborations, cooperation, and communications are expected to help accelerate progress in this area.

The workshop reviewed the state of the science for existing alternative methods and approaches that could be implemented now to provide for animal reduction, refinement, and replacement for vaccine potency and safety testing. Consideration of these methods and approaches by vaccine manufactures and product specific validation and implementation where appropriate can be expected to reduce animal use and improve animal welfare in the near term.

The workshop identified knowledge and data gaps, and future research, development, and validation activities needed to address these gaps in order to advance alternative methods for vaccine potency and safety testing. Advances in science and technology that can and should be applied to these efforts were highlighted and identified as a priority for future initiatives.

The workshop emphasized the value and role of international cooperation, collaboration, and harmonization in advancing alternative methods for vaccine potency and safety testing. The workshop concluded that increased international cooperation is essential in order to maximize the impact of new methods and to accelerate their implementation globally.

The workshop recommendations are expected to serve as guidance for future initiatives and actions necessary to achieve progress in the refinement, reduction, and replacement of animal use for veterinary vaccine potency and safety testing. Implementation of the workshop recommendations is expected to advance alternative methods for vaccine potency testing that will benefit animal welfare while ensuring continued and improved protection of humans and animals.

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Potency Testing of Inactivated Rabies Vaccines Using a Serological Method

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Abstract: Batch potency testing of rabies vaccines could be done by challenge, measurement of serum response or antigen quantification. Here, we show the development of a serological test that was successfully validated for use in batch release. The serological test is based on serum neutralization (SNT). The correlation to the NIH challenge was demonstrated by batches passing respectively failing equivalently in the NIH and SNT.
The SNT provides information on immunogenicity and exhibits several advantages to the NIH:
1) SNT uses many fewer animals for batch release.
2) SNT allows quantitative information on the individual serum response, in contrast to the "dead" / "alive" interpretation of the NIH.
3) SNT is quicker than the NIH and needs fewer working hours.
4) SNT avoids the highly disturbing intra-cerebral injection and suffering from rabies for mice and spares the staff the emotional stress of massively harming animals.

Introduction

Batch potency testing of inactivated rabies vaccines is traditionally performed by a challenge method. The method was originally developed by the National Institutes of Health (NIH) in the 1950s for batch potency testing of inactivated rabies vaccines for human use. It is now used worldwide. For veterinary vaccines, the NIH method was slightly adapted. The veterinary challenge method according to the European Pharmacopeia (EP) is done by a single immunisation followed by intracerebral infection of the mice with rabies virus two weeks later. From test day 19 onwards, the number of rabid mice is counted. Mice that present typical symptoms of rabies such as shaky movements, trembling, convulsions or paralysis are euthanized to avoid further suffering until reaching lethal endpoints. The reporting of rabid mice is performed until day 28. Finally, all surviving mice are sacrificed.

The NIH challenge method presents a number of problems:

1 A huge number of mice are required due to the multi dose assay format
2 It means severe distress for the mice, and is further emotionally challenging for the lab staff
3 It is highly variable
4 It causes high costs
5 It is time consuming since the mere test takes four weeks.

For these many reasons, an alternative method is really needed. Our laboratory developed a serological alternative method to overcome the problems of the NIH challenge method [1]. The serological method consists of a single immunisation of ten mice per batch and standard. Two weeks later, blood is taken from the mice. Then, the rabies virus neutralising antibodies are detected in the serum samples by serum neutralisation test (SNT). The results are available the latest at test day 21.

The characteristics of the serological alternative method are:

1 The immunisation with a single dose of vaccine and of standard (biological reference preparation [BRP4])
2 The detection of neutralising antibodies in vitro
3 The alternative method uses less mice and causes less suffering
4 It needs less time and is less labour-intense, i.e. means less cost
5 In Germany, the serological alternative method is used for batch release of veterinary inactivated rabies vaccines for years
6 The results are accepted EU-wide.

Discussion

The beginning of the serological alternative method goes back to the 1990s. Over the years, the assay underwent several modifications with respect to the injection dose, the animal number, the read-out and the plate type. In 2009, a collaborative study (BSP105) was prepared by the European Directorate for the Quality of Medicines (EDQM). The collaborative study provided valuable input for optimisation of the assay. The injection dose of the BRP4 was adapted to the minimum potency requirement for veterinary rabies vaccines in the EU, i.e. 1 IU/dose compared to BRP4. The adaptation enables us to obtain a more precise value of the minimum standard performance.

Further, we changed the read-out from manual interpretation to statistical calculation. Originally, we performed manual read-out of how many individual mouse titres were above the mean BRP4 titre. Following the contributions of EDQM, now we apply a statistical read-out using the EDQM programme CombiStats. Certainly, we calculated all results with both methods and obtained the same interpretations on batch performance, showing read-out equivalence.

We also increased the animal number from six to ten mice per group to get a wider result data base and to reduce the number of invalid runs.

Moreover, we modified the plate type. The monograph for veterinary inactivated rabies vaccines contained a brief description of a general alternative serological test. This description referred to the rapid fluorescent focus inhibition

test (RFFIT) that is explained in detail in the monograph 0723 of human rabies immunoglobulin. The corresponding plate type is the so-called chamber slide. Chamber slides consist of only eight wells. They are expensive themselves, are complicated to handle and require a high volume not only of serum, but also of detecting reagents. Therefore, the use of chamber slides results in high costs. Consequently, we carried out a suitability study on microtitre plates. We demonstrated concordance between both plate types so that we switched to routine use of microtitre plates. Since this meant a variation to the original monograph method, we referred to our method as "modified RFFIT" However, this term seems rather cumbersome. Considering that the biological principle of the serological alternative test is virus neutralisation by antibodies, we now prefer to use the easy assay nomenclature "serum neutralisation test", abbreviated SNT.

Regarding the immunisation route, the monograph suggested a subcutaneous (s.c.) or intramuscular (i.m.) immunisation. However, we obtained very low serum responses after s.c. or i.m. injection. So, we performed a comparison study using the intraperitoneal (i.p.) route versus s.c. or i.m. The study showed that i.p. vaccination was clearly much more efficient. It is therefore used in the serological assay.

In contrast to the several doses of batch and standard that are applied in the NIH test, the serological assay is based on a single dose injection only. The standard is tested to set the level of minimum performance. Therefore, the standard BRP4 has to be adjusted to the minimum potency requirement of 1 IU/dose. Since the current BRP4 contains 11 IU/dose, it is diluted 1:11 in PBS. The test vaccine batch only needs to be adjusted in case that a manufacturer claims a higher potency value than 1 IU/dose. According to the monograph, one fifth of the dose volume is injected which is equivalent to 0.2 ml per mouse.

Two weeks following vaccination, blood samples are drawn and the SNT is run on BHK cells. Serum samples are diluted serially and tested in four replicates. Virus is added and after two days, non-neutralised virus is detected by fluorescent antibody. The ED50 of each serum sample is calculated statistically. As for all SNTs, the virus titre influences the ED50 of the sample. In order to quantify the serum response independent of the virus titre of the individual experiment, a quantification standard is further included in the plate layout. This quantification standard is the WHO International Standard for Rabies Anti-Immunoglobulin (RAI). RAI is added in a concentration of 2 IU/ml and titrated identically as the serum samples. The comparison of the ED50 of the serum samples to the ED50 of the RAI enables the quantification of the serum activity in IU/ml independent of the virus titre. Therefore, we can plot the serum activities of the ten mice per group in a trend chart. This allows the semi-quantitative performance interpretation of the BRP4.

To know whether a batch has passed the release test or not, the serum activities of the batch group are compared to the serum activities of the BRP4 group. This is done by the CombiStats programme using a one-sided test. It tells whether the batch is significantly better than the standard. The statistical level of significance is set at 0.05, meaning that maximally 5% false positive test results may be obtained. Hence, the acceptance criterion of the alternative serological assay is that the p-value be equal or below 0.05.

Importantly, the serological assay was validated against the NIH challenge assay. Parallel testing in the serological and the NIH challenge assay showed a similar titration response. Further studies showed that the serological assay is suitable for the major European rabies vaccine strains including Virus fixe Pasteur, Flury LEP, Pasteur RIV, Pasteur VP12 and SAD Vnukovo.

The critical issue of any test is the detection of non-conforming batches. There is different nomenclature for non-conforming batches. In contrast to the commonly used term "subpotent", the term "sub-standard" is preferable. It reflects the fact that a "fail" result in a mouse test does not necessarily tell about the true potency in the target species. To validate the serological assay on sub-standard batches, we performed parallel testing of acceptable and of sub-standard batches that were either expired, accidentally subformulated or of unknown failure reason. The studies demonstrated the equivalence of pass, fail or borderline decisions. The results were obtained at PEI and in the collaborative study involving 13 different laboratories in Canada, Europe and the USA. The correlation between serological assay and NIH challenge assay proved to be good.

In daily use of the serological assay for batch release, we monitor the ED50 of the plate challenge virus and the ED50 of the quantification standard RAI. The data confirm the stable performance of the cell-based assay. The monitoring of the serum activity pattern of the BRP4 group shows reproducible performance of the standard. Once sufficient data are available, assay validity criteria can be set to the mean serum activity of the BRP4. If we have a closer look on the BRP4 group, we see that not all mice respond to vaccination with 0.2 ml of BRP4 adjusted to 1 IU/ml. If the serum activity is 0.0 IU/ml, the mouse is called a non-responder. The number of non-responder mice should be limited. Otherwise, the assay is declared invalid. The frequency of non-responders was monitored in the collaborative study BSP105 and found to correlate to the potency of the vaccine.

The data base was analysed statistically to determine the acceptable number of non-responders depending on the group size. Two non-responders out of a group of ten mice can be expected to occur randomly and thus are allowed in a valid run. Considering the reproducible performance of the BRP4, in future there might be the potential to reduce the use of the BRP4 standard vaccine.

Apart from the assay validity parameters and the performance of the BRP4, we further monitor the serum activities of the vaccine batch group. This provides us the semi-quantitative interpretation of the batch-to-batch consistency of each vaccine. Individual batches that fail a test clearly show a reduced serum activity pattern compared to passing batches. Thus, our first experience suggests that the trending of the batch serum activity pattern provides information on the batch performance independent of the BRP4 standard.

Conclusions

The NIH challenge assay is unsatisfying in various regards. A serological method was internationally validated and is now available as alternative potency test for inactivated veterinary rabies vaccines. The serological method analyses

the immunogenicity, but shifts the virus infection from the mouse to in vitro. Therefore, the serological assay means much less distress for the mice and much less ethical concern for the staff involved. Importantly, the serological method has further financial benefits: the results are generated a week faster than using the NIH challenge test. The serological method needs fewer working hours and many fewer mice, i.e less cost. The current format is a single dose assay. Thus, it provides the relevant information for batch release: whether the batch is better than the standard or not.

Overall, the serological method can be regarded as semi-quantitative: The individual serum activities of the mice vaccinated with the test batch allow batch-to-batch consistency trending already at a single dose in vivo. This is a clear advantage to the NIH challenge test in which the read-out of the mice is only "dead" or "alive". In consequence, the serological method provides more information with fewer mice.

Taken together, the serological method provides an important improvement compared to the NIH challenge assay. The serological method was successfully validated on passing, failing and borderline batches, respectively. The serological method shows stable performance in routine use and is especially attractive for new labs since no biosafety level S3 stable is required.

We are currently adapting the serological method to human rabies vaccines. Furthermore, we are investigating a fully quantitative read-out of the serological method for quantitative purposes such as vaccine stability monitoring.

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Potency Testing of Veterinary Rabies Vaccines: Replacement of Challenge by in Vitro Testing

Considerations for Development of Alternative Assays

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Key words: rabies vaccine, potency testing, vaccine challenge

Abstract: Vaccination of domestic animals against rabies creates a critical barrier between wildlife reservoirs and the human population. Ensuring these vaccines are potent and effective is paramount in preventing human exposure to this deadly and costly disease. The National Institutes of Health (NIH) test is, at present, the most widely used and internationally recommended potency assay for batch testing inactivated rabies vaccines. This test has numerous inherent limitations and disadvantages, including a lack of precision. The NIH test requires a large number of animals and involves unrelieved pain and suffering. A relevant in vitro assay should provide a more accurate, reproducible, rapid, safe, and humane rabies vaccine potency test.

Introduction

Rabies vaccines for veterinary use are essential for safeguarding the public from exposure to the rabies virus, as vaccination of domestic animals provides a barrier between humans and the wildlife reservoirs of the virus. Ensuring rabies vaccines are potent and effective is paramount in preventing human exposure to this deadly and costly disease. In the United States, the US Department of Agriculture's Center for Veterinary Biologics (CVB) is responsible for ensuring that veterinary biologicals, including rabies vaccines, are pure, safe, potent and efficacious [1, 2]. Potency testing of conventional inactivated rabies vaccines is conducted in accordance with Title 9 of the Code of Federal Regulations (9 CFR), §113.209 [1, 3].

Potency evaluation of inactivated rabies vaccines has long been the subject of continued research, and identification of a single method by which all of the necessary parameters of the numerous types of inactivated rabies vaccines can be satisfactorily measured has proven to be a daunting task [6]. In vivo methods play a critical role in the assessment of potency and safety of vaccines [4]. Evaluation of the protective activity of the test vaccine is the goal of any vaccine potency test, and demonstration of the test vaccine's ability to protect the species for which the vaccine is intended is the most direct and reliable way [5]. Testing each manufacturing batch of Rabies Vaccine in the intended target species, however, is cost-prohibitive and impractical.

The NIH Potency Test

The National Institutes of Health (NIH) test, fully described in chapter 37 of "Laboratory Techniques in Rabies," Fourth Edition (1996) published by the World Health Organization (WHO), is the most widely used and internationally recommended potency assay for batch testing inactivated rabies vaccines [7-11]. Developed in 1953 by the NIH, the assay describes the minimum standards for achieving compliance with US potency test regulations and defines minimum recommended batch potency requirements. Even in the face of the scientific and technological advances, this assay has been carried out without any major modifications or improvements for over five decades [6, 7, 12].

Functionally, the NIH test is an antigen extinction method that compares a lot of the test vaccine to a reference vaccine in mice [6, 10, 12]. Dilution sequences of each vaccine are prepared, and the 50% endpoints (ED50) for protection against challenge are estimated by the method of Spearman-Karber. The relative potency (RP) of the test lot is the ratio of the estimated ED50s[10]. A test is considered valid by the CVB only if it fulfills the validity criteria listed in 9 CFR 113.209 [3].

The NIH test calls for at least three dilutions in each sequence, with each dilution administered to a group of at least 16 mice. Often four or more dilutions are used. The mice are vaccinated by the intra-peritoneal route and challenged with live Challenge Virus Standard (CVS) by the intra-cerebral (IC) route. A set of unvaccinated mice, comprised of at least three groups of ten mice, are used to estimate the median lethal dose of the challenge inoculum.

For a test to be considered valid by the CVB, the dilutions of both vaccines must widely encompass the ED50. That is, mortality must be at least 70% in the mice vaccinated with the highest dilution and no more than 30% in the mice vaccinated with the lowest dilution [13]. The WHO manual [12] states these criteria only for the reference vaccine, presumably to allow for the possibility of an obviously super potent vaccine lot.

Limitations of the NIH Potency Test

The NIH Potency Test has numerous limitations and disadvantages. The routes of vaccination and challenge differ from the accepted immunization and natural infection routes in domestic animals [7, 10, 14]. Consequently, differences between the clinical response in the NIH test and the response in naturally exposed target species vaccinates is possible. It has been established that, following IC injection, inoculum enters the bloodstream, allowing for the neutralization of challenge virus to occur in peripheral circulation [7, 14, 15]. Five minutes after IC administration only 2-8% of the original inoculum was recoverable in the brain and associated meninges. Additionally, the trauma associated with IC injection results in the production of immunoglobulin M (IgM), which is not the case in naturally exposed domestic animals [14].

Most inactivated rabies vaccines are aimed at producing an immunologic response to the rabies virus glycoprotein (G protein) [5], a protective antigen found in all rabies virus isolates. Although protection demonstrated by the assay correlates to G protein serum virus neutralizing antibody (VNA) titers, differences in infectivity and pathogenic characteristics of the CVS challenge virus and wild-type viruses have been shown to markedly affect measured vaccine potency [7, 10, 14]. The NIH test discriminates in favor of vaccines derived from the same parent strain as that of the challenge material [7].

This test requires a large number of animals, and those animals used may experience unrelieved pain and distress [6]. At least 200 mice are required to test a single vaccine lot, although some conservation of mice can be achieved by testing multiple vaccine lots simultaneously [3, 6, 7, 12, 13].

The NIH assay has been widely criticized for its variability, and individual tests frequently fail to meet validity criteria. This results in the need to conduct retests [7, 11].

In spite of its limitations, there are no documented cases wherein a batch of product that passed the NIH test was subsequently associated with widespread failure to protect host animals. In this regard, the test appears to have served its purpose well.

Perceived Roadblocks and Proposed Solutions

The NIH potency test is perceived as a "gold standard" for potency testing of rabies vaccines, in large part because vaccine failure in the field is rarely reported [2]. Transition to new methods of potency testing of veterinary rabies vaccines will require overcoming a number of challenges.

Current validation practices would require any new assay method(s) to be compared to vaccination/challenge studies or the NIH assay. Each manufacturer would need to validate the assay for its own product, as each vaccine may be unique in its composition and method of manufacture. Requiring each manufacturer to conduct its own vaccination/challenge studies would result in the use of hundreds of host animals.

This would conflict with the goal of reducing animal use. In addition, these studies are of one- and three-year duration, and the animals must be kept in containment. The cost and other factors associated with requiring vaccination/challenge studies for validating new assay methods would likely discourage manufacturers from conducting the work.

Validation of new methods against the NIH potency test is also problematic. It may be difficult to validate a new assay by direct correlation due to the NIH test's lack of precision. Direct correlation may also be undesirable if a candidate assay is superior to the NIH assay in meaningful ways.

These limitations should not be allowed to slow progress toward replacing the NIH assay. Consideration should be given to accepting an indirect link between the potency measurement of the candidate assay and efficacy in the target species. For example, validation studies for replacement assays may be designed so that some type of vaccination-challenge in mice, patterned after the NIH test, serves the necessary role in the conceptualization stage of the validation process. Depending on the analytical principle and architecture of the new assay, other laboratory studies may support target species efficacy.

To be considered as a replacement for the NIH test, the alternative approach must be able to provide a more precise and humane method to evaluate batch potency of inactivated rabies vaccines effectively. Attention should be paid to the inclusion of new and emerging technologies. Even in the absence of an in vivo assay, potency evaluation should continue to include qualitative information about the structure and conformation (immunogenic activity) of protective epitope(s) together with a quantitative measure of the protective antigen(s).

Traditionally, potency testing of rabies vaccines is based on a single assay conducted on final product. Regulators should consider the possibility of utilizing multiple assays to evaluate batch potency, including "in process" assays, as part of the potency assessment. In addition, consideration should be given to the impact of stringent manufacturing process controls to ensure consistency of production.

The transition to alternative methodology will require open dialogue and more importantly the establishment of a partnership between regulatory authorities and the product manufacturers. A major shift such as this can only occur through cooperation, sharing of information, and the establishment of a clearly defined common goal.

Conclusion

The National Institutes of Health test lacks precision, is time consuming, and causes severe pain and distress to the test animals. This archaic test has remained unchanged since the 1950s and has been viewed as a *necessary evil* required for the potency evaluation of inactivated rabies vaccines. The change from in vivo testing of rabies biologics is overdue. Development of alternate methodology to evaluate the potency of inactivated rabies vaccines is supported by scientific and technological advances and the need to reduce unrelieved animal pain and suffering. To accomplish this goal, regulatory authorities and vaccine manufacturers need be willing to work together and take a fresh approach to product potency evaluation.

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SESSION II

Use of the 3R Approach for Potency (2)

Chairpersons: *Rodolfo Bellinzoni*
Wim Hesselink

Jungbäck C (ed): Potency Testing of Veterinary Vaccines for Animals: The Way From in Vivo to in Vitro. Dev Biol (Basel). Basel, Karger, 2011, vol 134, pp 37-44.

Towards in Vitro Potency Testing of Inactivated Erysipelas Vaccines

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Key words: swine erysipelas; vaccine; batch potency; alternative method; 3R; consistency approach

Abstract: Ph. Eur. Monograph 0064 "Swine erysipelas vaccine (inactivated)" currently advises mouse serology for batch potency testing. However, technological advances in vaccine production, improved quality control systems and comprehensive post marketing surveillance increasingly promote the acceptance of non-animal approaches for batch release testing.
Protein and immune profiles of inactivated swine erysipelas vaccines obtained by SDS-PAGE and Western Blot might offer a convenient global and functional in vitro alternative. Characteristic and consistent protein and immune profiles could be obtained for aluminium-adjuvanted vaccines. Immunoreactivity of polyclonal sera raised in mice differs markedly from reactivity of swine sera.

Swine Erysipelas

Swine erysipelas is caused by the bacterium *Erysipelothrix rhusiopathiae* which is found in most if not all pig farms. Most surveys indicate that 20-40% of pigs are carriers. Most prominent clinical signs are sudden death, fever, characteristic skin lesions, lameness and reproductive failure [1, 2]. The bacilli are excreted via saliva, faeces or urine. They are also found in many other species, including birds and sheep. The organism has the ability to persist for short periods in most soils. Thus, eradication is difficult and vaccination is still essential. [2]

European Pharmacopoeia (Ph. Eur.) Testing Requirements

Swine erysipelas is tightly linked with the history of vaccine development and vaccine control. The first live vaccines were in use as early as 1882, immune sera since 1893. Inactivated vaccines became available in 1947 [3]. Since a long time, batch potency testing has been performed in mice, which are highly susceptible to erysipelas septicemia. Multiple dilution challenge assays had been in use since 1952. Ph. Eur. Monograph 1997:0064 "Swine erysipelas vaccine (inactivated)" advised a three dilution challenge test in 106 mice per batch, some 50% of which died after severe suffering. In 2002, the test procedure was modified to a one point challenge assay in ten mice. This resulted in a considerable reduction with regard to the number of animals needed. However, potency was difficult to assess due to the high variability of results when dealing with such a small number of animals. Moreover, since confined to a single dose, this approach did no longer allow for a quantitative estimate of potency. In 2004, the monograph underwent a renewed revision and now advises mouse serology. Along with further reduction this means substantial refinement since testing is based on a vaccination bleeding experiment with subsequent immunochemical determination of antibody titers instead of a lethal challenge procedure. Although – similar as with the one point challenge test – mice receive just one dilution of a single vaccine dose, subsequent serum dilution and testing of replicates increase certainty of measurement and yield quantitative data.

Quality Assurance Aspects

Prior to amendment of the Ph. Eur. Monograph, basic suitability and transferability of the serologic approach had been confirmed by two international studies [4, 5]. Studies revealed that mouse serology is a suitable alternative to the challenge procedure. However, vaccine adjuvant and the mouse strain used may influence the antibody response to a large extent. Subsequently, a thorough in-house validation was performed according to VICH Guidelines 1 and 2 – Validation of analytical procedures to set suitable test validity criteria and to implement adequate test controls [6]. In-house validation was finalized in 2000 and the erysipelas ELISA was accredited in 2002. In 2005 erysipelas vaccines were included in the restricted list according to Article 82 of Directive 2001/82/EC as amended. Since then, we tested more than 180 batches by mouse serology for the German and EU market. This equals about 50 million vaccine doses.

Consistency Approach

In the Ph. Eur. chapter "General principles", reference is made to the *European Convention on the protection of animals used for experimental and other scientific purposes* and it is stated that the Commission is committed to the reduction of animal usage wherever possible in pharmacopoeial testing.

It is known from the field that killed erysipelas vaccines are efficacious and adverse effects on vaccinates are relatively rare. Consistent test results have been obtained over time. So, it is perceived that in vivo testing could be replaced by a panel of in vitro test according to the consistency approach. The consistency approach implies that the extent of batch release testing should reflect the level of consistency in production obtained with the vaccine. A set of parameters is used to constitute a product profile to ensure similarity to a batch of proven clinical efficacy and safety. Consistency testing relies very much on the implementation of quality systems and on the use of in vitro analytical tools [7, 8]. In case of veterinary vaccines, the consistency approach currently is mainly put into practice by using antigen/adjuvant quantification in conjunction with additional physico-chemical analysis of vaccines.

Antigen quantification has been approved for batch potency testing of numerous vaccines and a huge variety of antigens. For the time being, Ph. Eur. monographs for Newcastle disease, rabies, and canine leptospirosis [9-11] expressly allow for antigen quantification. However, for various reasons, in case of rabies and Leptospira vaccines for use in dogs current testing is still based on animal experiments. In the USA, the possibility of antigen quantification is generally addressed in paragraph § 113.8 9 C.F.R. on in vitro tests for serial release.

Main concerns are that antigen quantification is used under the critical assumption that by the evaluation of one or a few key antigen(s) it is possible to estimate the potency of the complete product and that antigen quantification is only a measure of quantity and not necessarily of biological activity [12, 13]. Moreover, test systems often rely on the use of monoclonal antibodies specific to individual vaccines and manufacturers. This further hampers the extensive, bilateral use of antigen quantification by industry and OMCLs and diminishes its contribution to the 3Rs.

Recent Investigations

Since recently, we use SDS-PAGE in conjunction with immunoblotting to evaluate batches of inactivated erysipelas vaccines. Vaccine profiling is a global approach, which verifies the immunologic functionality of product batches and relies on commonly available test components.

Currently, four inactivated erysipelas vaccines are available on the German market (Table 1). All vaccines contain *Erysipelas rhusiopathiae* serovar 2. They are adjuvanted either by aluminium phosphate, aluminium hydroxide or by a vitamin E derivative. Three vaccines are manufactured by alkaline lysis of bacteria; one vaccine is based on formaldehyde inactivation. Preservatives are either formaldehyde or thiomersal. Serum is added to the cultivation media of two vaccines.

Table 1: Inactivated erysipelas vaccines currently available on the German market.

Vaccine	*E. rhusiopathiae*	Parvovirus	Inactivation	Adjuvant	Other excipients
1	1 strain	1 strain	NaOH	$AlPO_4$	Horse serum, Bovine serum; Lactalbumine
2	1 strain	-	NaOH	Vit. E derived	CH_2O
3	1 strain	1 strain	NaOH	Vit. E derived	CH_2O
4	3 strains	-	CH_2O	$Al(OH)_3$	Horse serum, Oleic acid, Thiomersal

SDS-PAGE was performed using 4–12% Bis/Tris gels under denaturing and reducing conditions. Gels were Coomassie stained. Blots were probed with polyclonal mouse pool sera, pig pool sera or individual pig sera and revealed with 3-Amino-9-ethylcarbazole (AEC) peroxidase substrate solution on nitrocellulose membranes.

Whereas, when examining the vaccines without any pre-treatment, one vaccine readily yielded characteristic profiles, initially neither protein nor immunoprofiles were detected in case of three vaccines (Fig. 1 A, B). Sodium-citrate pre-treatment according to Ph. Eur. monograph 01/2008:0444 on Diphtheria and Tetanus vaccine [14] resulted in protein and antigen bands in fractions of one vaccine (Fig. 2 A, B). Interestingly, most antigens have been identified in the sediment instead of the supernatant. SDS-PAGE and western blotting yield complementary profiles (Fig. 1 A, B; Fig. 2 A, B). Protein profiles of untreated inactivated vaccines are far less complex than profiles obtained for bacterial lysates or live vaccines (Fig. 3 A, B). Apparently, this is not exclusively caused by protein denaturation or fragmentation during manufacture but is also linked to antigen adsorption to the adjuvant. As expected, antigen desorption was successful in case of aluminium phosphate (Figs1 and 2). A quantitative improvement of profiles could be obtained by subjecting the aluminium hydroxide adjuvanted vaccine to Trypton desorption followed by centrifugation. Due to their unique formulation, it is especially difficult to obtain meaningful profiles of alphatocopherol based vaccines. For the time being, blurred bands could be obtained by sodium deoxycholate treatment [15] and subsequent protein precipitation with trichloroacetic acid.

Batches of aluminium based products yielded consistent profiles. Older batches of vaccine four appear to lack the double bands around 40 kDa (Fig. 4). Proteins in the range of 64–66 kDa and 39–40 kDa have been identified to be involved in immunity [16]. Whereas specific and relevant immunogenic fractions are detected by both, mice and pig sera, there are also fractions exclusively detected with pig sera (arrows in Fig. 5).

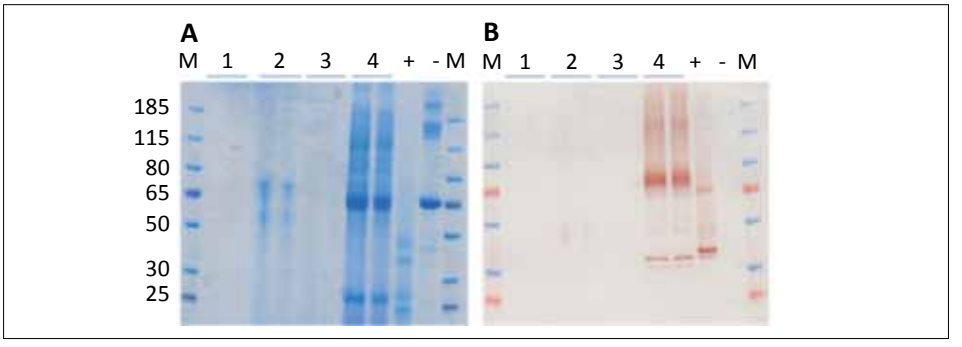

Fig. 1: Coomassie stained 4-12% gel (A) and AEC revealed immunoblot (B) probed with pool serum raised in mice. 1-4 different inactivated erysipelas vaccines, + positive control (erysipelas lysate), - negative control (inactivated, non-adjuvanted Leptospira vaccine), M marker.

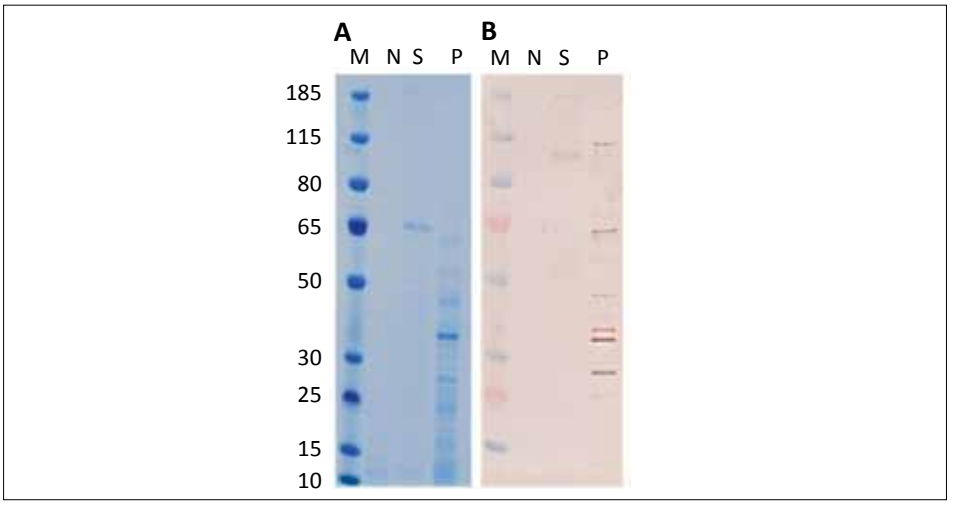

Fig. 2: Coomassie stained 4-12% gel **(A)** and AEC revealed immunoblot **(B)** probed with pool serum raised in mice: Vaccine 1, protein and immunoprofiles after sodium citrate pretreatment. **M** marker, **N** native vaccine, **S** supernatant, **P** pellet.

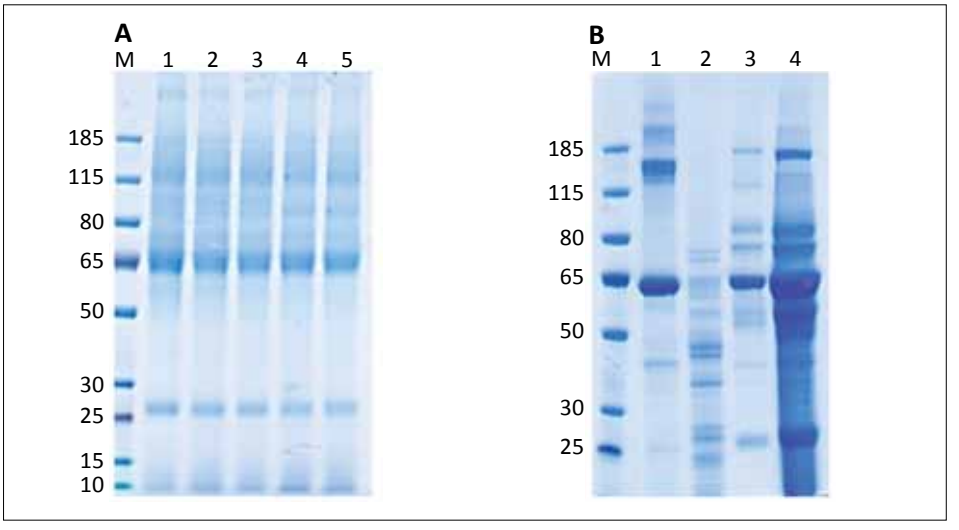

Fig. 3: Coomassie stained 4-12% gels
A: 1–5 Vaccine 4, different batches in order of production, diluted 1:10 with purified water, **M** marker
B: 1 inactivated, non-adjuvanted Leptospira vaccine, 2 erysipelas lysate (ELISA coating antigen, Biological Reference Preparation), 3 equine erysipelas hyperimmune serum, 4 erysipelas live vaccine, M marker.

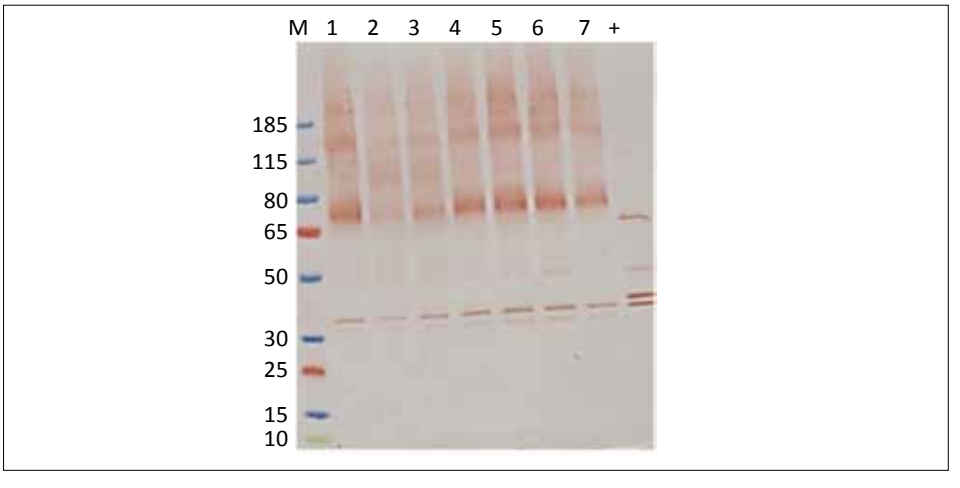

Fig. 4: AEC revealed immunoblot probed with pool serum raised in mice. 1–7 Vaccine 4, different batches in order of production, no pretreatment, M marker, + positive control (erysipelas lysate, ELISA coating antigen, Biological Reference Preparation).

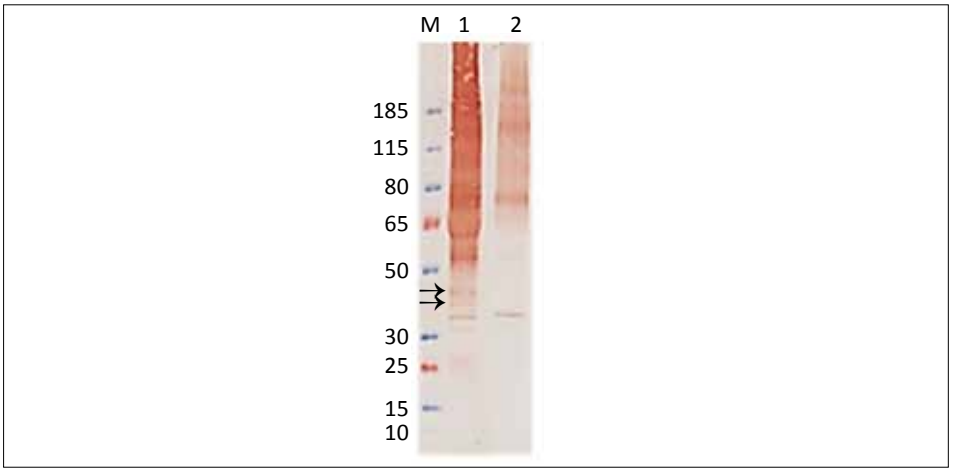

Fig. 5: AEC revealed immunoblot: Vaccine 4, comparison of immunoprofiles, blots probed with sera raised in pigs (1) and mice (2), M marker. The two arrows indicate fractions exclusively detected with pig sera.

Conclusion

There is proof of principle that consistent and characteristic vaccine profiles can be obtained by SDS-PAGE and Western blotting. Detection of immunogenic fractions verifies the immunological functionality of product batches. There is potential to obtain data relevant for the target species by utilizing corresponding sera.

Differences in physiology make the extrapolation of test data between species, gender and breed of animals difficult. Although laboratory animal testing is of value in some cases, most experimental designs do not reflect route of administration and vaccination schemes in the target animals. It is thus a questionable approach to rely on adapting in vivo methods in surrogate species when developing new vaccines. Instead, efforts should be undertaken to develop and validate animal-free test strategies. Vaccine profiling by one – or two – dimensional gel electrophoresis, possibly combined with techniques like mass spectrometry or chromatography, presents a global, convenient, in vitro approach. Rapid technological advance in life sciences will further diversify our understanding and our tools how to assess vaccine quality.

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Testing of Veterinary Clostridial Vaccines: From Mouse to Microtitre Plate

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Abstract: Vaccines to protect against clostridial diseases are among the most common veterinary biologicals. Each batch of these materials is subjected to a variety of toxicity and antigenicity tests. The potency of the final vaccine is then assessed by Toxin Neutralisation Test (TNT). All of these tests use mice and have lethal endpoints. Development of alternatives for potency testing was based on ELISAs able to measure antibody levels to the specific toxins relative to a standard serum with a defined unitage. These alternative assays were shown to correlate with the relevant TNTs and have been accepted by European Regulatory Authorities as batch release potency tests. Recently we have developed in vitro cell line alternatives for the toxicity and antigenicity tests for *Cl. septicum* using the VERO cell line. With this cell line it has been possible to develop in vitro assays which, when compared with the in vivo tests, gave correlations of 87% to 100%. Having shown proof of principle, similar cell line assays have been developed for *Cl. novyi* and *Cl. perfringens* types C and D.

Introduction

Pathogenic clostridial species such as *Clostridium perfringens* types A, B, C and D, *Cl. septicum, Cl. novyi, Cl. tetani* and others are ubiquitous throughout nature and responsible for a wide range of animal diseases including necrotic enteritis, lamb dysentery, struck, pulpy kidney, braxy, blackwater and tetanus. As a result, vaccines to protect against these diseases are among the most common veterinary biologicals. In the vast majority of clostridial mediated diseases the causative factors are soluble toxins and the appropriate vaccines comprise chemically toxoided forms of these toxins. The quality control testing of these materials begins the moment the fermentation process stops and continues through to the blended final product. Much of this testing involves the use of animals. For each batch of these materials the initially produced toxin is subjected to toxicity testing in Minimum Lethal Dose (MLD) tests and antigenicity assessment in L+ tests. After inactivation the resulting toxoids are checked for freedom from toxicity using MLD tests and assessed for antigenicity in Total Combining Power (TCP) tests. All of these in-process tests use mice and have lethal endpoints.

The potency of the final blended vaccine batch is usually assessed in a version of the relevant original Ph. Eur. monograph potency test. These tests are based on the ability of the vaccine to stimulate an appropriate antibody response in rabbits. The specific antitoxin concentrations in the rabbit sera are subsequently measured in vivo by Toxin Neutralisation Tests (TNT) in mice [1-3], which again usually have lethal endpoints. It is estimated that in Europe alone tens of thousands of mice are used annually for the testing of veterinary clostridial antigens and vaccines.

It should be noted that in all of the above tests the mice serve only as indicators for the presence of active toxin. At various times over the past 20 years we have investigated ways to replace the mice in these tests with in vitro methods mainly centered around the use of cell line or immunosorbent assays performed in microtitre plates. Here, as examples of the progress achieved, we present details of the in vitro replacement assays that have been developed for *Cl. septicum* antigens and vaccines and their performance in comparison with the in vivo tests.

Materials and Methods

In vivo in-process tests

The minimum lethal dose (MLD) test simply measures the minimum concentration of the fermenter culture supernatant that will kill mice. This gives an approximate measure of the amount of biologically active toxin present. The bacteria were removed from a sample of *Cl. septicum* final fermenter culture supernatant by centrifugation and sterile filtration. Ten-fold serial dilutions were made of the supernatant in physiological saline solution to give dilutions down to 10^{-5}. For each dilution, 0.5 ml was inoculated intravenously into each of two mice. The mice were monitored for three days and any which died or had to be euthanased were recorded. The MLD is the dilution containing the smallest amount of toxin which caused the death of both mice inoculated with that dilution.

The L+ test gives a measure of the antigenicity of the toxin by determining the lowest concentration of fermenter culture supernatant, which when allowed to react with a set amount of a standard neutralizing antitoxin, will still kill mice. For *Cl. septicum*, a sterile sample of final culture supernatant was diluted over a suitable range, such as 1/5 to 1/30, in physiological saline solution. An equal volume of a standard *Cl. septicum* neutralizing antitoxin solution at 1.0 International Units (IU)/ml was mixed with each dilution and incubated at 37° C for one hour. For each toxin-antitoxin mixture, 0.5 ml was inoculated intravenously into each of two mice. The mice were monitored for three days and any which died or had to be euthanased were recorded. The ideal end-point of the test is when one of the mice injected with a specific toxin-antitoxin mixture survives and the other one dies resulting in an exact L+ value for the toxin. However, in many cases both mice which have received one toxin-antitoxin mixture die and the two mice which received the mixture containing the next lowest concentration of toxin survive. In these cases the L+ value for the toxin is a range.

The total combining power (TCP) test is similar to the L+ test but is used to measure the antigenicity of the toxoided antigen based on the ability of the toxoid to bind the appropriate neutralizing antitoxin. For *Cl. septicum*, a sterile sample of toxoided antigen was diluted over a suitable range in physiological saline solution. An equal volume of a standard *Cl. septicum* neutralizing antitoxin solution at 2.0 International Units (IU)/ml was mixed with each dilution and incubated at 37° C for one hour. Then an equal volume of *Cl. septicum toxin*, at 1.0 L+ units/ml was mixed with each dilution and incubated at 37° C for one hour. For each toxoid-antitoxin-toxin mixture, 0.5 ml was inoculated intravenously into each of two mice. The mice were monitored for three days and any which died or had to be euthanased were recorded. Again the ideal end-point of the test is when one of the mice injected with a specific toxoid-antitoxin-toxin mixture survives and

the other one dies resulting in an exact TCP value for the toxoid. However, in many cases both mice which have received one mixture die and the two mice which received the mixture containing the next lowest concentration of toxoid survive. In these cases the TCP value for the toxoid is a range.

In vitro (cell line) in-process assays

For the in vitro equivalents of the *Cl. septicum* MLD, L+ and TCP tests the relevant toxins and toxoids were prepared for testing in essentially the same way as for the in vivo tests. It has previously been demonstrated that VERO cells are very sensitive to the cytopathic effects of *Cl. septicum* toxin [4, 5]. Therefore, the in vitro assays are basically the same as the in vivo tests except for the use of VERO cells rather than mice, as indicators for the presence of active toxin, as described below.

The day before the preparation of the relevant toxin or toxoid, VERO cells were seeded into a microtitre plate at a concentration of 2 x10^5 cells/ml and incubated overnight at 37° C in 5% CO_2. The next day, two 100µl portions of each dilution of prepared toxin, toxin-antitoxin mixture or toxoid-antitoxin-toxin mixture were transferred to two individually marked wells in the plate containing the VERO cells. One column of wells on the plate was used as a cell control, where the cells were not exposed to any toxoid, antitoxin or toxin and were thus at optimal conditions. The plate was then incubated overnight at 37° C in 5% CO_2, after which each well was stained using crystal violet. Once dry, the ODs of the wells were read at 630nm and the endpoints of the test samples were determined by comparing the ODs with those obtained from the column of cell control wells. As with the in vivo tests the ideal end-point of these assays is when one of the duplicate wells inoculated with a specific dilution shows greater than 50% cell survival and the other well, which received the identical dilution, shows less than 50% cell survival resulting in an exact value for the toxin or toxoid. However, in several cases both wells which have received the same dilution show greater than 50% cell death and the two wells which received the mixture containing the next lowest concentration show less than 50% cell death. In these cases the relevant value for the toxin or toxoid is a range.

Correlation of in vitro and in vivo in-process tests

The testing of three samples of *Cl. septicum* culture supernatants, with substantially different levels of toxicity, in the mouse and the cell line MLD assays was initiated on the same day. Five samples of *Cl. septicum* culture supernatants, with differing levels of antigenicity, were tested in the mouse and the cell line L+ assays on the same day. Five *Cl. septicum* toxoid samples, with various levels of antigenicity, were tested in the mouse and the cell line assays on the same day. The results of all the above assays were recorded for analysis.

The correlation between the cell line and mouse assay results was evaluated by calculating the Pearson correlation coefficient. Linear regression analysis was applied to quantify the relationship between the cell line and mouse assay results. Linearity was judged by visual inspection of the regression plot, the residual plot and the coefficient of determination (R^2). In cases were non-linearity was observed data were log transformed. Where the results gave a range of values, for the MLD and L+ assays the lowest values of the range were used for statistical analysis, and for the TCP assay the median (midpoint) value of the range was used.

In vivo potency assay

The sera from a group of 10 rabbits, which had been vaccinated with a production batch of clostridial vaccine containing *Cl. septicum* antigen, were pooled. The serum pool was then assessed for potency in the toxin neutralization test (TNT) in mice according to the appropriate Pharm. Eur. monograph [3]. Briefly, dilutions of the serum pool were mixed and incubated with a mouse lethal dose of the relevant clostridial toxin and the resultant mixture injected into mice to ascertain whether the serum dilution contained enough specific antitoxin antibodies to neutralize the lethal effect. The third British Standard Antitoxin for *Cl. septicum* was assayed in parallel so allowing potency, in units, to be assigned to the original vaccine in relation to that particular clostridial antigen.

In vitro (ELISA) potency assay

The *Cl. septicum* antitoxin ELISA was performed as previously described [6]. Briefly, a microtitre plate was coated overnight at 5° C with purified *Cl. septicum* toxin (Wellcome Research Laboratories). The plate was then blocked by incubation at 37° C for one hour with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (Sigma). The test and reference rabbit sera were appropriately diluted in PBS containing 0.05% Tween 20 (PBST) and added to the plate. The plates were again incubated at 37° C for one hour before the addition of diluted goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma) in PBST and further incubation at 37° C for one hour. Tetramethyl benzidine solution (ICN Biomedicals) was added as substrate and the colour allowed to develop for 15 minutes at room temperature then stopped by the addirion of 1 M sulphuric acid. The plate was washed three times with PBST prior to the addition of each new reagent. The absorbance was measured at 450 nm using a Titertek Multiscan spectrophotometer (Flow Laboratories) relative to a row of control wells. The antitoxin concentration of the test samples was determined by comparison of absorbance with those of the calibration curve generated using the reference serum.

Correlation of in vivo and in vitro potency assays

Pooled rabbit sera were available for 80 production batches of *Cl. septicum* containing vaccines which had been assessed for *Cl. septicum* potency by the TNT in mice. These sera were also assessed in the ELISA potency test. The correlation between the ELISA and mouse TNT results was evaluated by calculating the Pearson correlation coefficient. Linear regression analysis was applied to quantify the relationship between the ELISA and mouse TNT results. Linearity was judged by visual inspection of the regression plot, the residual plot and the coefficient of determination (R^2).

Results

Correlation of in vitro and in vivo in-process tests and potency assays

The results of the statistical analysis of the correlation and of the quantifying of the relationship between the in vitro and in vivo assays using linear regression analysis are summarized in Table 1.

The Pearson correlation coefficient, which was higher than 0.99 for the MLD and TCP assays, indicates a very high correlation between these cell line and mouse assays. For the potency ELISA / mouse TNT assay and the L+ assay the Pearson correlation coefficients of 0.95 and 0.87 respectively, while lower than for the MLD and TCP, are still high.

The R^2 values (Table 1) and the regression plots (not shown) for the MLD and TCP assays demonstrate that there is a linear relationship between the in vitro and in vivo results. The R^2 values for these linear regression fits are very high at 0.99. The R^2 value (Table 1) and the regression plot (not shown) between the in vitro (ELISA) and in vivo (mouse TNT) potency assays show a linear relationship with a high regression fit of 0.88.

For the L+ assay the results of both the cell line and mouse assays had to be $\log_2$ transformed to obtain a satisfactory fit. This fit with a R^2 value of 0.80 (Table 1) and the regression plot (not shown) indicate that the log linear relationship, while not as good as for the MLD, TCP and potency assays, can still be considered to be satisfactory, given the purpose of the assay.

Table 1: Correlation and linear regression analysis between in vitro and in vivo assays

Assays compared	Pearson correlation coefficient (p-value)	Linear regression equation	R^2 (regression)
cMLD / mMLD	0.9996 (0.0187)	cMLD = 650.4 x mMLD + 597.2	0.9991
cL+ / mL+ (log_2 transformed)	0.8747 (0.0411)	$log_2(cL+) = 0.990 \times log_2(mL+) + 0.434$	0.7981
cTCP / mTCP	0.9989 (<0.0001)	cTCP = 1.07 x mTCP + 6.91	0.9979
ELISA / mTNT	0.9450 (0.0225)	ELISA = 1.05 x mTNT – 0.38	0.8849

c = cell line m = mouse

Discussion

There is a strong desire to apply the principles of the 3Rs, particularly replacement, in the field of biological products development and control [7]. Replacing mouse tests with in vitro cell culture or ELISA technology is an important step in reducing the use of animal models for measuring vaccine characteristics. The mMLD is an important in-process analysis test used to quantify toxin levels in supernatant of production fermenter batches of *Cl. septicum* and also to monitor reducing toxicity during antigen toxoiding. The sensitivity of the mMLD test is such that it can differentiate only between batches of toxin with a ten-fold or greater difference in concentration levels, whereas, the cell line assay is much more sensitive and can differentiate between batches of toxin with only two-fold differences in concentration. Owing to this limitation of the mMLD test, batches of toxin must have concentration differences of at least ten-fold for the test to differentiate between them, therefore, it was only possible to use three batches of differentiable toxin which had concentrations within the normal range of production batches. Since the correlation and regression fit between the cMLD and mMLD assays are so good it is clear that the cell line assay would provide a more than adequate replacement for the mouse MLD. In fact the cell line assay would be a far more accurate method than the mouse assay to discriminate between *Cl. septicum* batch toxicity levels.

The mTCP is the most important of the mouse tests used to assess clostridial antigens. The mTCP is used to quantify the antigenic content of the toxoided material and vaccines are blended on the basis of this TCP unitage. The correlation data generated for these assays is the most important in maintaining confidence that consistency of vaccine blending can be achieved using the replacement in vitro assay. The correlation and the linear regression fit for the cTCP and mTCP assays are very good and the toxoids used to generate this data adequately cover the range of TCP values that are normally produced. Therefore, once again the cell line assay would be an excellent replacement for the mTCP.

The mL+ test is a useful assay for quantifying the antigenicity of pre-toxoided toxin production batches as part of in-house monitoring of consistency of production. However, it is far less critical for the production of effective vaccines than either the mMLD or mTCP tests. The data generated for the correlation between the cL+ and mL+ assays are satisfactory and regression analysis showed that there is linear relationship for the $\log_2$ transformed values. The toxins used to generate this data cover the expected range for normal production toxins. The correlation and the linear regression fit are sufficient to suggest that the cL+ assay is a suitable replacement for the mL+ assay.

The potency test is the most important assay employed in the testing of clostridial vaccines as release of the final vaccine batch is dependent upon it passing this test. Eighty batches of final vaccine, containing *Cl. septicum* antigen, were assessed in both the mouse TNT and ELISA potency assays and the correlation and the linear regression fit for the in vivo and in vitro assays are very good demonstrating that the ELISA is a good replacement for the mouse TNT.

Conclusion

The results show that all four in vitro assays are suitably sensitive and accurate replacements for the respective mouse assays. It is estimated that if these four types of in vitro assay were used in place of the relevant in vivo tests for the appropriate clostridial toxins, toxoids and final vaccines the numbers of mice used in the control testing of these materials could be reduced by tens of thousands per annum in Europe alone.

Acknowledgements

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The Quantitative ELISA for Inactivated Newcastle Disease Virus Antigen:

Development of the Test System and the Way to a Ph. Eur in Vitro Potency Test

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Abstract: The development of an alternative in vitro potency test required experimental studies, which were performed in-house and in collaboration with other laboratories (Official Medicines Control Laboratories, Manufacturers), coordinated by EDQM (European Directorate for the Quality of Medicines & HealthCare).
This paper provides background information concerning the development of the quantitative ELISA for inactivated Newcastle disease virus (NDV) antigen, which was added in the European Pharmacopoeia monograph as an in vitro batch potency test.

Introduction

Despite the general opinion, that measurement of only antigen in an adjuvanted vaccine for potency testing is not acceptable, the Central Institute for Animal Disease Control (CIDC) - Lelystad (since 2008: Central Veterinary Institute / CVI) started the project to develop an in vitro Newcastle disease virus (NDV) antigen estimation, with the aim: an alternative to the batch potency testing of inactivated Newcastle disease vaccines as described in the European Pharmacopoeia monograph which at that period which required the use of chickens.

Steps

Methods have been developed to quantify the haemagglutinin-neuraminidase (HN) protein of NDV [7: Standard Operation Procedure]. Vaccination experiments with inactivated ND vaccines indicated that the in vitro quantified HN protein of NDV is a reliable indicator of the protective serological response after immunisation [1-5]. In studies performed for inactivated ND vaccines of different manufacturers, a high correlation was demonstrated between the results of this candidate in vitro potency assay and the results of the (HI) serological potency assay [1]. Correlation between in vitro (NDV-HN antigen content) and in vivo (previous Ph.Eur. 0870 test A and B, respectively serology and vaccination challenge) potency assays was confirmed in a collaborative pre-validation study (EDQM BSP055-1), participated by three Official Medicines Control Laboratories (OMCLs: PEI-Germany, AFFSA-France, CIDC-Netherlands) [6].

Promising outcome of the pre-validation study resulted in organization of a large-scale collaborative study (EDQM BSP-055-2) to validate the candidate in vitro method and the suitability of the candidate Biological Reference Preparation (BRP). Fourteen laboratories participated in the collaborative study: AFFSSA (France), CEVA (Hungary), CIDC (Netherlands), CODA VAR (Belgium), EDQM (Europe), Fort Dodge (Netherlands), Intervet (Netherlands), HIPRA (Spain), ISCVBM (Czech Republic), IVI (Switzerland), LNIV (Portugal), Lohmann (Germany), Merial (France), and PEI (Germany). The results of this study indicated that the antigen content could be determined with high precision and good repeatability as well as reproducibility were found. All laboratories found a similar ranking of the vaccines, based on the antigen content. A threshold relative antigen level of 7.0 antigens units per dose would discriminate between batches with sufficient and insufficient potency [7].

Conclusion

The in vitro measurement of the antigen content of inactivated ND-vaccines with the proposed method is a reliable alternative potency assay.

In 2007 the quantitative ELISA has been added as an alternative in vitro potency test in the European Pharmacopoeia monograph 0870 on inactivated ND vaccines.

In such a project, collaboration with other laboratories (OMCLs and manufacturers) and the central coordination by EDQM are crucial.

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The Quantitative ELISA for Inactivated Newcastle Antigen:

Experience Report From an OMCL

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Key words: potency test, ELISA, inactivated, Newcastle disease, vaccines

Abstract: The relative haemagglutinin-neuraminidase (HN) antigen content of inactivated Newcastle disease virus (NDV) vaccines from different manufacturers was determined by means of an Enzyme-Linked Immunosorbent Assay (ELISA) according to Monograph 870 of the European Pharmacopoeia (Ph. Eur.). Wide ranges of reactivity of the different products were observed. When comparing the antibody responses from chickens vaccinated with vaccines showing either high or low reactivity in the antigen ELISA it was found that approximately the same titres of antibodies were induced in the chickens. One hypothesis is that the inactivation procedures used to inactivate the Newcastle disease antigen may alter the antigenic determinant recognised by the monoclonal antibody used. An alteration of the antigen would influence the binding by the monoclonal antibodies used as catching and detection antibodies in the ELISA which may result in a lower ELISA reactivity.
It was also found that HN antigen of two inactivated Paramyxovirus 1 (PMV-1) vaccines for pigeons could not be measured in the ELISA. For these vaccines the antigen-ELISA based on monoclonal antibody IDNDV134.1 cannot be used.
Our experience shows that a thorough knowledge of the products tested with the ELISA and their influence on the test method is essential to avoid misinterpretations of the test results. The level of ELISA reactivity should not be used for the comparison of vaccines. Furthermore, prediction of the ability of an unknown vaccine to induce antibodies based on the level of ELISA reactivity is not possible. The results (level of reactivity) of the antigen ELISA for the in vitro potency testing of inactivated Newcastle disease vaccines should therefore be carefully interpreted. However, by knowing the performance characteristics of the NDV antigen ELISA and the characteristics of the vaccines to be tested it becomes a valuable tool for the control of inactivated Newcastle disease vaccines in our laboratory. The implementation of this ELISA method for the batch release testing markedly reduces the number of chickens and the time required for batch release testing.

Introduction

According to Ph. Eur. monograph 870, for batch release testing of avian inactivated Newcastle disease vaccines an in vitro ELISA potency assay can be used alternatively to the serological test or the challenge assay in chickens. Suitable reagents to perform the test are available as a biological reference preparation by the European Directorate for the Quality of Medicines & HealthCare (EDQM). These reagents include a lyophilized monoclonal coating antibody, a monoclonal antibody conjugated with horseradish peroxidase (HRPO), a reference antigen and a control antigen.

Since the implementation of the testing of the HN antigen content by ELISA in the European Ph. Eur. in 2007 [1], numerous inactivated Newcastle disease vaccines from different manufacturers have been tested at the Paul-Ehrlich-Institute's Veterinary Official Medicines Control Laboratory. In the following, the experiences made with the NDV antigen quantification assay by this laboratory are reported and the results are discussed.

Materials and methods

Vaccines

In total, 235 batches of commercially available inactivated oil-emulsion NDV vaccines from six different manufacturers (coded A-F) were tested. The vaccines differed in the NDV strains used, their combination with other viruses (coded A1, A2 …), the route and dose of application, the adjuvants used or the applied inactivation procedure. Tested vaccines are summarized in Table 1.

Table 1: Characteristics of the tested vaccines.

Vaccine	Strain	Target species	Inactivation	Adjuvant (per dose)	Dose (ml/dose)
A1 to A10	NDV Clone30	Chicken	Formaldehyde	215 mg Paraffin	0.5
A11	NDV Clone30	Turkey	Formaldehyde	215 mg Paraffin	0.5
A12	PPMV-1 P201	Pigeon	Formaldehyde	138 mg Paraffin	0.25
B1, 2, 3	NDV Ulster 2C	Chicken	BPL	170-186 mg Paraffin	0.3
B4	NDV Ulster 2C; PMV-3	Turkey	BPL	170-186 mg Paraffin	0.3
C + D	NDV LaSota	Pigeon	Ethylenimine	Montanide ISA 206VG (mineral-oil, W/O/W)	0.5
E	NDV LaSota	Pigeon	Formaldehyde	Carbomer 934P	0.2
F	PPMV-1 988M-ca	Pigeon	BPL	Montanide ISA763A VG (non-mineral oil, W/O)	0.3

Method of antigen extraction

Two ml of the vaccine samples were mixed with 8 ml isopropyl myristate-98% (cat.-no.: 172472, Sigma-Aldrich, Steinheim, Germany) and mixed for one minute on a MS1 Minishaker (IKA-Works INC, Wilmington, USA) at maximum speed (2500 rpm). The mixture was then centrifuged for ten minutes at 1000 x g using a cooled centrifuge at +8° C. The lower aqueous phase containing the antigen was collected and used immediately for examination.

NDV antigen ELISA

For the determination of the NDV antigen content an ELISA kit (Newcastle Disease Vaccine (inactivated) BRP, cat. no.: Y0000388, EDQM, France) was used. The test was performed according to the published standard operation procedure (SOP) [2] as follows:

Coating of microtitre plates

The monoclonal anti NDV HN coating antibody was reconstituted in 1.5 ml aqua dest and diluted 1:20 in carbonate buffer pH 9.6. The cavities of 96-well flat bottom high binding microtitre plates (Corning Costar 3590, Fisher Scientific GmbH, Germany) were filled with 100 µl coating antibody and sealed with a foil. The plates were then incubated for two hours at +37° C. After this the plates were washed three times with 350 µl PBS/Tween 80 (0.05%) using an ELISA washer (Tecan HydroFlex™, Tecan Deutschland GmbH, Crailsheim, Germany).

Incubation of samples, reference and control antigen

100 µl of extracted and 1:4 in ELISA-buffer (phosphate-buffered saline containing 0.05% Tween 80, 5% NaCl and 1% bovine serum albumin) prediluted vaccine samples, undiluted reference and control antigen were filled into the cavity of the microtitre plates according the scheme described in the SOP. Serial two-fold dilutions of samples, reference antigen and control antigen were prepared on the plates according to the SOP. Each sample, reference and control antigen was tested in duplicate.

Incubation of antigen

The microtitre plates were sealed with a foil and incubated for four hours at +37° C. After this the plates were washed three times with 350 µl PBS/Tween 80 (0.05%). To dry the plates these were tapped on absorbing paper.

Incubation of detection antibody HRPO conjugate

The monoclonal HRPO conjugated detection antibody was reconstituted in 1.5 ml aqua dest and then diluted 1:20 in ELISA buffer. The cavities of the plates were filled with 100 µl of the prediluted detection antibody.

After incubation for one hour at +37° C the plates were washed three times with 350 µl PBS/Tween80 (0.05%). Then 100 µl tetramethylbenzidine (TMB Chromogen Substrate, Prionics Deutschland GmbH, Germany) was added and incubated for 15 minutes at room temperature in the dark. The reaction was stopped by adding 100 µl 0.5 M H_2SO_4. Thereafter, the optical density was measured at 450 nm with an ELISA reader (Tecan Sunrise™, Tecan Deutschland GmbH, Crailsheim, Germany).

Statistical analysis

For the quantification of the NDV antigen ELISA results CombiStats software (EDQM, Strasbourg, France) was used. With this software the relative potency of the vaccines using a parallel line model was calculated by comparison to the reference antigen preparation. The relative potency of a vaccine was expressed in antigen units (AU) per dose.

As the vaccines differed with respect to their water phase and their dose volumes a correction factor (f) had to be applied according to the following formula:

$$f = \frac{(V_T)}{0.5} \times \frac{(W_T)}{25}$$

where
f = correction factor
V_T = volume per dose
W_T = water phase (%)

In-house validation of NDV antigen ELISA

In order to validate the NDV antigen ELISA and to establish an appropriate value in antigen units (AU) for the control antigen, an in-house validation of the assay was performed including the following parameters: accuracy, specificity, measurement range, precision/uncertainty of measurement (intra- and inter-assay repeatability), linearity and parallelism.

Vaccination of SPF chickens

Per batch of vaccine ten chickens hatched from SPF eggs (Lohmann Tierzucht, Cuxhaven, Germany) were immunized with a full vaccine dose at an age of four weeks. Application of the vaccine was performed subcutaneously or intramuscularly according the manufacturer's product information. Blood samples were taken three weeks (vaccine A12: four weeks) after vaccination.

Measurement of NDV antibodies by ELISA

A commercially available antibody test kit from Idexx was used to detect antibodies to NDV in the chicken sera. The ELISA was performed as prescribed by the manufacturer: Chicken sera were diluted 1:500 in sample diluent buffer. 100 µl of the prediluted samples were dispensed into the wells of the antigen coated ELISA plates in duplicate. The plates were then incubated for 30 minutes at room temperature. After incubation the plates were washed three times with 300 µl of aqua bidest. 100 µl goat anti-chicken horseradish peroxidise conjugate were dispensed into each well and incubated for 30 minutes at room temperature. Following a washing step as described above, 100 µl of TMB substrate were added to the plates. The enzymatic reaction was stopped after 15 minutes with 100 µl of stop solution. OD values were measured at 650 nm against air as blank. The sample to positive control (S/P) ratios and titres were calculated in line with the manufacturer's instructions. Calculation of titres was performed according to the following equation: Log10 titre = 1.09 (Log10 S/P) + 3.36. S/P ratios greater than 0.2 or titres greater than 396 are considered to be positive for NDV. Results were expressed as mean titres of the immunised chicken group.

Measurement of NDV antibodies by HI test

The haemagglutination inhibition (HI) test was performed according Monograph 870 of the Ph. Eur. All test sera were inactivated by heating at +56° C for 30 minutes. 25 µl of inactivated serum were plated into the first row of wells in a microtitre plate with U-shaped wells. Then 25 µl of phosphate buffered saline at pH 7.2-7.4 were added to the rest of the wells. Two-fold dilutions of the sera were prepared across the plate. To each well 25 µl of a suspension containing four haemagglutinating units of inactivated NDV strain LaSota (VLDIA039 HAG-NDL, Gezondheidsdienst voor Dieren, Deventer, The Netherlands) were added. For the titration of two vaccines for use in pigeons (vaccines A12 and F) PPMV-1 strain P201 (Intervet, The Netherlands) was used as antigen. The plates were incubated at +4° C for one hour. Then 25 µl of a 1% (v/v) suspension of red blood cells collected from SPF chickens were added. The plates were incubated at +4° C for one hour. The HI titre was read as the highest dilution that produces complete inhibition. On each assay, respectively, one negative control serum and two positive control sera with high and low antibody titres were included.

Recovery of antigen from adjuvants

In order to investigate the influence of the adjuvant (matrix) with respect to recovery or alteration of the NDV antigen a spiking experiment was conducted. Two matrixes of vaccines showing either high or low reactivity in the NDV antigen ELISA were tested. As an adjuvant Montanide ISA 206VG had been used in the production of vaccines C and D (both showing high reactivity in the antigen ELISA). The adjuvant was spiked with different amounts of NDV strain LaSota provided as raw material by the manufacturer of vaccine C. Before spiking the titre of this NDV antigen was determined by HA test and adjusted to 2048 HA units (HAU) in PBS. Then two-fold serial dilutions were prepared (2048 to 8 HAU). These antigen dilutions were finally mixed with the adjuvant in a ratio 50/50 according to the manufacturer's instructions for the adjuvant.

A vaccine from manufacturer A (containing Egg Drop Syndrome Virus instead of NDV as antigen) sharing the same matrix composition was spiked with the prepared NDV antigen quantities in the same way. After the mixing had been finished the antigen was extracted as described and the antigen levels were measured using the NDV antigen ELISA.

Results

In-house validation of ND Antigen ELISA

The validated value of the control antigen at the PEI laboratory was estimated as 2.30 AU/vial (n = 30). Alert limits used to monitor the consistency of the method were calculated to be 1.61 to 3.22 AU/vial corresponding to 70% to140% of the validated value.

Accuracy was determined by showing that the mean value and 95% confidence interval of repeated measurements of the control antigen were completely within the range of the validated alert limits.

Only a limited specificity test was performed by testing two inactivated oil emulsion vaccines containing infectious bronchitis virus (IBV) or turkey rhinotracheitis virus (TRTV) as antigen. The ELISA did not react with the two antigens.

The measurement range corresponding to the given dilution scheme for the reference antigen was 0.0125 to 0.4 AU per 100 µl sample.

The precision/uncertainty of measurement was estimated by repeated measurements of the control antigen and also by repeated testing of three different inactivated vaccine samples from two manufacturers. For intra-assay precision a coefficient of variation of 8.0% was calculated when using the control antigen as a sample. Using vaccine samples the coefficient of variation of intra-assay repeatability ranged between 1.6% and 10.5%.

For inter-assay precision a coefficient of variation of 1.9% was calculated when using the control antigen as a sample. Using vaccines as samples the coefficient of variation ranged between 1.1% and 18.4%.

The uncertainty of measurement based on the data of the repeated measurement of the control antigen was calculated to be 8.2%.

Resulting dilution curves from reference, control antigen and samples were shown to be parallel and linear as analysed by CombiStat software (Fig. 1).

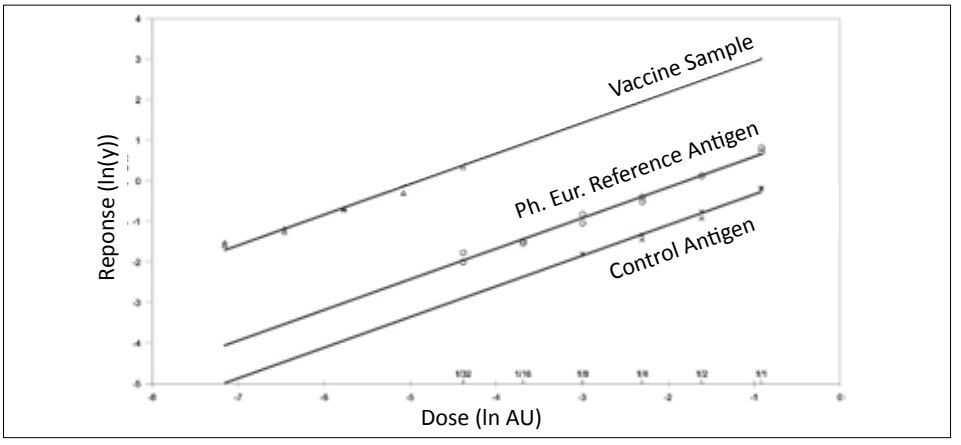

Fig. 1: Example of a NDV-antigen-ELISA assay. Parallelism and linearity of dose/response curves of the reference, control antigen and a vaccine sample.

Relative potency of vaccines

Figure 2 shows the results of the ELISA reactivity of 235 measured batches of inactivated NDV oil-emulsion vaccines. The vaccines A1 to A10 produced by the same manufacturer A showed an average relative potency of 9 AU/dose.

A vaccine for use in turkeys (vaccine A12) produced by the same manufacturer showed 22 AU/dose on average whereas batches of a pigeon vaccine (A11) from the same manufacturer containing the PPMV-1 strain P201 did not react in the NDV antigen ELISA.

Vaccines from manufacturer B (B1- B4) showed an average of 95 AU/dose. Compared to the vaccines from manufacturer A this level is approximately ten times higher.

The highest relative potency was measured in vaccines C + D (408 AU/dose). Both vaccines are produced and marketed by manufacturer C; vaccine D is also distributed under a different name by company D.

An average value of 31 AU/dose was determined in vaccine batches from manufacturer E. The relative potency of this vaccine ranged between the vaccine batches from manufacturers A and B.

For two batches of a PPMV-1 vaccine from manufacturer F containing PPMV-1 strain 988M-ca no reactivity in the ELISA could be measured.

Antibody response after vaccination

Because of the substantial differences found in the NDV antigen ELISA reactivity between the vaccines of different manufacturers the capability of two selected vaccines to induce antibodies was investigated. SPF chickens were vaccinated with a full dose of inactivated NDV vaccines of manufacturer A (showing low relative potency in NDV antigen ELISA) or manufacturer B (showing on average a ten times higher reactivity than vaccine batches from

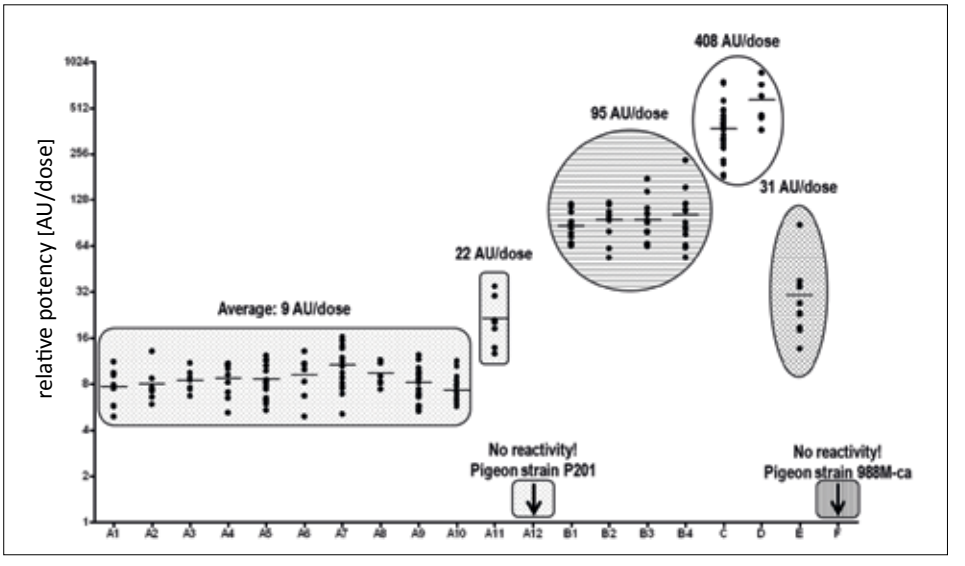

Fig. 2: Relative potency of inactivated NDV oil-emulsion vaccines from different manufacturers measured with the NDV antigen ELISA.

manufacturer A), respectively. The results are summarized in Fig 3. The antibody levels induced in the SPF chickens were found to be at the same level independent of the method used to measure the antibody titres (ELISA or HI).

Testing of the two vaccines (A11 and F) which did not react in the NDV antigen ELISA showed that they are able to induce antibodies in chickens when tested in HI test only (Fig. 4). The level of antibodies induced by PPMV-1 vaccine batches of manufacturer A was slightly higher compared to antibody levels induced by vaccine batches from manufacturer F.

However, the antigen used in the HI test was homologous to the antigen included in vaccine A11 but heterologous (other strain) to the antigen included in vaccine F.

Antigen extraction from adjuvants

In order to investigate the influence of the adjuvant (matrix) with respect to recovery or alteration of the NDV antigen the matrixes of two vaccines were spiked with defined amounts of NDV LaSota antigen. Thereafter the antigen was extracted and the relative potency was measured with the NDV antigen ELISA. A clear correlation between the amount of spiked NDV antigen and the relative potency was detected. The relative potency of the extracted NDV antigen showed the same level of ELISA reactivity irrespective of the matrix from which the antigen was extracted (Fig. 5).

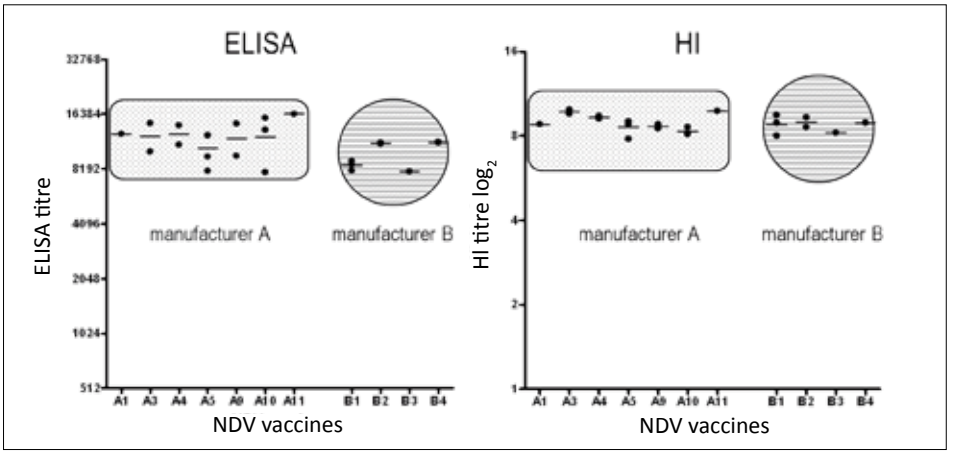

Fig. 3: Antibody response of four week old SPF chickens after vaccination with a full dose of an inactivated NDV vaccine from manufacturer A or B, respectively. Blood samples were taken three weeks after vaccination and measured by ELISA and HI test. Each dot in the graphs represents the mean NDV antibody titre of ten vaccinated chickens.

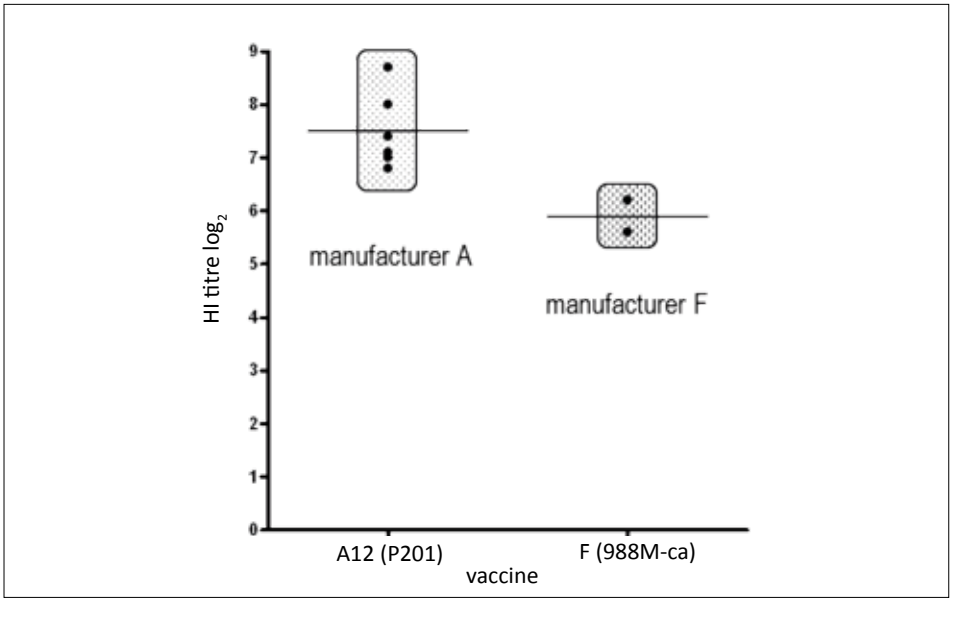

Fig. 4: Antibody response of SPF chickens after vaccination with a full dose of an inactivated PPMV-1 vaccine from manufacturer A or F, respectively. The vaccines contain either strain P201 (manufacturer A) or 988M-ca (manufacturer B). Chicken sera of both groups were tested by HI test using PPMV-1 strain P201 as antigen.

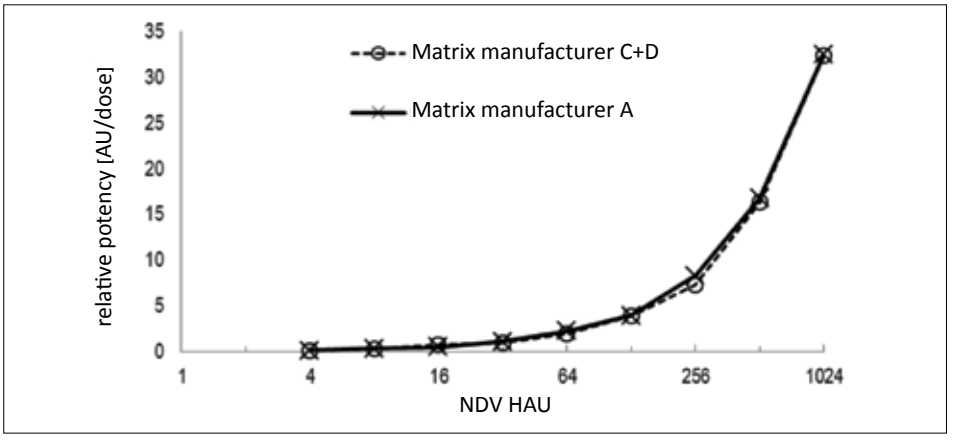

Fig. 5: Recovery of NDV from two different vaccine matrixes (adjuvants) spiked with defined amounts of NDV strain LaSota.

Discussion

According to Monograph 870 of the Ph. Eur. official batch release testing of avian inactivated Newcastle disease vaccines should be carried out by an in vitro ELISA potency assay wherever possible. If the in vitro potency assay gives valid results with a given product, measurement of the antigen content should be performed instead of the serological or challenge assay. In our laboratory the in vitro potency assay (NDV antigen ELISA) was implemented after an in-house validation of the assay.

According to the leaflet coming with the test kit (Newcastle Disease Vaccine inactivated BRPs) each individual laboratory should establish an appropriate value in AU for the control antigen during validation of the method in their laboratory. Neither a relative content of the control antigen nor a release specification for vaccines is stated on the leaflet. But information is included that examples of values for the control antigen of different laboratories can be found in the report of a collaborative study [3]. By comparing our results with these results it was noticed that our results for the control antigen (2.30 units/dose) were in the lower range of all values (combined values: 2.23-6.86 units/dose). Results from other laboratories may be different, so that specifications for individual vaccines may vary between laboratories.

Only a limited specificity validation was performed on two antigen vaccines containing IBV and TRTV as antigen. Based on literature all NDV strains of 20 inactivated vaccines can be detected by the NDV antigen ELISA [4]. Because of these studies and because of the intrinsic antigen specificity by using monoclonal antibodies in the ELISA an extensive validation of the specificity was not performed.

Validation studies by R. Mass et al [5] found variation coefficient (CV) values of 2% for intra-assay variability and 5.1% to 13.5% for inter-assay repeatability of three participating laboratories. The between laboratory variation of three laboratories testing six different vaccines was in the range between 0% to 30.14%.

Results of our laboratory (intra-assay CV: 1.6% to 10.5%, inter-assay CV: 1.1% to 18.4%) fit well with these results. The repeatability of the ELISA is considered to be good taken into account that the variation of the method is affected by the combination of sample extraction and the subsequent ELISA procedure.

In the past, 235 batches of inactivated Newcastle disease vaccines from different manufacturers have been tested with this ELISA at the Paul-Ehrlich-Institut.

By plotting the results it was noticed that the relative potencies of vaccines produced by the same manufacturer ranged always within a certain level of reactivity. Therefore vaccines produced by the same manufacturer could be grouped (boxes and cycles in Fig. 2). The tested vaccines showed a wide range of reactivity in the NDV antigen ELISA. After comparison of vaccine groups from different manufacturers based on average AU/dose a calculated factor of about 45 was found between low and high reacting vaccines. This finding is in line with the results of R. Mass et al where a 100-fold difference in antigen content was found between the vaccines with the highest and the lowest antigen content [4].

This wide range seems unexpectedly high as the use of unnecessarily high amounts of antigen is normally avoided by manufacturers for economic reasons.

The average relative potency of vaccine A11 (22 AU/dose) was approximately twice as high as that of other vaccines from the same manufacturer containing the same NDV Clone 30 strain. According to the manufacturer's information this is likely because vaccine A11 is formulated with double the amount of NDV antigen. Although the antigen measurement within the same group of vaccines (sharing the same production process) seems to be accurate, it is questionable whether the observed differences between vaccines from different manufacturers really exist. The established amount of antigen for the production of an inactivated ND vaccine is determined by the manufacturer which depends in part also on the properties of the used adjuvant. But it seems very unlikely that the huge differences in antigen amount between different products can be explained by that fact alone.

Comparison of the antibody responses of chickens immunized with vaccines from manufacturer A or manufacturer B, respectively, showed that there is no relevant difference in the in vivo antibody response between these vaccines. In theory, vaccines from manufacturer B (measured 10-fold antigen amount) should be able to induce higher antibody levels than vaccines from manufacturer A. As this was not the case it was assumed that the results of the ELISA did not reflect the actual antigen amount of the vaccines. ELISA results are therefore probably influenced by other factors. One of these factors could be the adjuvant used in the production of the vaccines. Adjuvants may influence the effectiveness of the antigen extraction or may alter the antigen in such a way that the binding by antibodies is negatively affected. The spiking experiment performed showed that there was no negative effect of the adjuvant that could explain the differences in relative potency of the different vaccines measured with the antigen ELISA.

Another factor that could have an impact on the ELISA outcome is the specificity of the antibodies used. Although absence of reaction was observed in the NDV antigen ELISA with the two PPMV-1 vaccines for use in pigeons (strains P201 and 988M-ca) both vaccines were able to induce antibodies in chickens. The non-reaction of the vaccines in the NDV antigen ELISA is probably due to the inability of the antibodies to detect these strains. The binding antibody and the horseradish-peroxidase conjugated detection antibody bind the same epitope. They specifically recognise a linear epitope of the NDV HN-complex (amino acids 335-355) [2]. Ujvári [6] describes that the length of the HN proteins varies between different NDV genotypes. It is therefore most likely that the epitopes of the HN proteins from PPMV-1 strains may differ from that of LaSota NDV strains which would explain the absence of reaction.

A further possible explanation for a reduced binding of the NDV antigen is that the different inactivating reagents (formalin, ß propiolactone, ethylenimine) and the conditions of inactivation may lead to an alteration of the antigenic structure. Recently, Jagt et al. [7] showed that treatment of NDV antigen with formalin or PBL influenced the outcome of the ELISA measurement which would explain the differences in relative potency between NDV vaccines from different manufacturers.

Conclusions

The NDV antigen ELISA is a useful tool for the batch release testing for most of the inactivated Newcastle disease vaccines. Using the NDV antigen ELISA will significantly reduce the number of animals and the time needed for batch release. As the ELISA is not able to detect pigeon paramyxovirus using the monoclonal antibodies provided with the test kit the in vivo test is still necessary for these vaccines. For unknown inactivated ND vaccines the suitability of the ELISA has to be tested first.

Care should be taken when comparing ELISA results of inactivated ND vaccines from different manufacturers. Adequate interpretation of the ELISA results of vaccines with different characteristics has to be taken into account when specifications for a minimum antigen amount are specified. The specifications have to be defined for each group of vaccine. As the level of ELISA reactivity does not allow a prediction of the antibody response in the animal, misinterpretation of low or negative antigen-ELISA test results of unknown inactivated ND vaccine samples has to be avoided.

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SESSION III

In Vivo / in Vitro, a Critical Analysis

Chairpersons: *Tim Miller*
Marlies Halder

Jungbäck C (ed): Potency Testing of Veterinary Vaccines for Animals: The Way From in Vivo to in Vitro. Dev Biol (Basel). Basel, Karger, 2011, vol 134, pp 69-73.

The Validation of Potency Tests: Hurdles Identified by EMA/CVMP/IWP

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Key words: veterinary vaccines, potency tests, validation

Abstract: The biological nature of IVMPs leads to some unavoidable batch to batch variation in production. The potency test is part of the quality control of the finished product intended to confirm consistency of production and that each batch is formulated equivalent to batches that have been demonstrated to be efficacious. Adequate validation of potency tests is essential to ensure that the results of the assays accurately reflect the amount, titre, or potency of the active substance measured and to indicate the limitations on the accuracy of the measurements to be expected from the test used.
The CVMP/IWP published their conclusions concerning validation of potency tests in a Reflection Paper in March 2010. The test validation must demonstrate a dose response and the precision of the result should enable reliable detection of a sub-standard batch. However, the inherent variability in experimental animals often leads to unacceptably wide confidence intervals for in vivo tests which limits their ability to detect slight changes of the antigen amount. The development of in vitro methods as alternatives to in vivo potency tests is encouraged.

Introduction

Technical requirements for veterinary vaccines in the European Union are defined in Annex 1 to Directive 2009/9/EC [1]. This requires that a quantification of the active substance shall be carried out on each batch to show that each batch will contain the appropriate potency or titre to ensure safety and efficacy. It should be noted that, while the general aim is to ensure that each batch of vaccine will be equally efficacious, the required test is part of the quality control of the finished product intended to confirm consistency of production and that each batch is formulated equivalent to batches that have been demonstrated to be efficacious.

The biological nature of veterinary vaccines leads to some unavoidable batch to batch variation in production. Manufacturers, therefore, set limits rather than absolute parameters for most processes. The test methods available to quantify the active substance during and at the completion of production are also subject to biological variability.

They often only provide indicators of the quality, quantity and the reproducibility of the product batches rather than precise measures. Adequate validation is essential to ensure that the results of the assays accurately reflect the amount, titre, or potency of the active substance measured and to indicate the limitations on the accuracy of the measurements to be expected from the test used. This applies as much to in-process control tests used to measure antigen prior to blending or inactivation as it does to the control of the active substance in the finished product. All these factors influence the confidence that can be put into the tests for their capacity to determine accurately the titre or potency of the product and the extent to which the value given for the potency reflects the actual or likely potency of the batch.

The Immunologicals Working Party (IWP) of the European Medicines Agency Committee for Veterinary Medicinal Products (CVMP) discussed the difficulties associated with validation of batch potency tests and published their conclusions in a Reflection Paper in March 2010 [2].

Current Methods Used for Control of the Active Substance in the Finished Product and Problems Identified

The following methods are commonly used to control the active substance in the finished product:

1 Direct measurement of the active substance: Titration of living vaccine organisms is commonly used to control live vaccines but direct measurement of vaccine antigens in inactivated vaccines is often difficult because of interference by other components of the vaccine, e.g. adjuvants and preservatives.
2 Challenge of vaccinated animals with virulent organisms: Typically the numbers of diseased or dead animals are compared to a non-vaccinated challenged group. These tests do confirm the batch being tested is efficacious but often require large numbers of animals for significant results and may be relatively insensitive to small changes in quality or quantity of the active substance. The consequences for animals that succumb to the challenge infection, particularly the non-vaccinated controls, are often severe and humane end points may need to be set to minimise the welfare implications.
3 Measurement of a serological response in vaccinated animals: An initial in vivo stage may be followed by an in vitro test. Both stages need to be validated and while the in vitro test may be adequately reproducible the variable responses of vaccinated animals often leads to a wide confidence interval for the test as a whole. The method may therefore be relatively insensitive to small changes in formulation.

For the routine batch release potency test, European Pharmacopoeia [3] monographs usually propose an alternative test to that required for immunogenicity testing. For inactivated vaccines, this is usually a test in laboratory animals and is described in some detail. In some cases, it contains suggestions for alternative approaches e.g. different types of animals, number and size of doses administered and a range of days from vaccination to time of collecting blood samples or use of an in vitro method. In all cases, the tests described are given as examples

of the type of test that may be carried out and are not per se validated [4]. It is the applicant's responsibility to develop and validate a suitable test to use for batch release and a method that is totally different from the test suggested by the European Pharmacopoeia can be used.

An additional problem that has been encountered, especially in respect to older vaccines or those that have been developed over a period of time, is where potency tests may be changed during vaccine development and several different types of test may have been used for developmental studies and for batch release. While it is understandable that vaccine manufacturers will wish to take account of scientific advances and improvements in technology this can often lead to difficulties in comparing the results of different studies and setting appropriate validated limits for batch release. A particular problem exists when validating alternatives to older "standard" tests, that may not have been fully validated, because it may not be possible to establish a satisfactory correlation.

Validation of the Control of the Active Substance in the Finished Product

The control of the active substance in the finished product is an analytical procedure and needs to be validated against the criteria described in VICH guidelines GL1 (Validation of analytical procedures: definition and terminology) [5] and GL2 (Validation of analytical procedures: methodology) [6]. The following points should normally be addressed to correctly validate the control of the active substance:

- As a minimum, the test validation needs to be able to demonstrate a dose response (i.e. that it is able to respond to changes in the vaccine that affect its efficacy, such as active substance concentration) and the precision of the test (i.e. repeatability, intermediate precision and repeatability between laboratories if relevant). The control method should be sufficiently sensitive to be able to distinguish between a batch containing the correct quantity of active substance and a batch with less active substance.
- If the potency test for an inactivated vaccine involves an initial in vivo stage followed by an in vitro test, validation requires the inherent variation in the in vitro test and also the in vivo stage to be taken into account. Due to the inherent variability in experimental animals this often leads to an unacceptably wide confidence interval, which in turn draws into question the ability of the test as a whole to discriminate a sub-standard batch. For inactivated vaccines, the methods used for the control of the finished product are in general not sufficiently sensitive to detect slight changes of the antigen amount. Thus, reasonable dilution factors should be applied to the vaccine batch when the dose-response relationship is investigated. Moreover, the biological significance of the statistical analysis of the results should always be examined. For instance, if there is a good dose-response relationship, and a significant difference can be shown between a standard batch and a batch with, for example, half the amount of antigen (thus making possible the detection of a defective batch containing only 50% of antigen), the efficacy of a batch containing half the amount of antigen should be considered.

Implementation of in Vitro Methods for Inactivated Vaccines

In the spirit of reduction, refinement and replacement of animal tests (3Rs) and in accordance with Directives 86/609/EEC [7] and 2010/63/EU [8], the development of in vitro methods as alternatives to in vivo potency tests to control the quality of vaccines is encouraged. The revised Annex 1 of Directive 2009/9/EC [1] refers to the quantification of the active substance in the finished product instead of the previously required assay of biological activity of the active substance(s) in the finished product. Therefore, control of the active substance by an in vitro method may be considered acceptable to demonstrate the quality of the vaccine batch under test and to show the consistency of production and could be considered to be more in line with the requirements of the revised Annex 1 than currently used in vivo methods.

The in vitro approach is not commonly implemented by applicants at present so there is currently insufficient information to establish a clear position on the future of these methods. It is acknowledged that implementation of an in vivo method may be quicker and less expensive than developing suitable in vitro methods. However, in vitro methods have a number of potential advantages including a reduction in the numbers of animals used for batch control testing, more reproducible results because they are not dependant on the responses of experimental animals, improved reliability, with less chance of failing an acceptable batch or needing to repeat the test because of problems with the test, and speedier results enabling batches to be released sooner.

Applicants are therefore encouraged to develop in vitro tests for the control of active substances in the finished product. The following points should be taken into account:

- Consistency of production: Support for the use of an in vitro test can be provided by taking a consistent approach to the manufacturing process. The aim of this concept, which includes GMP, process validation and in-process and finished product tests, is to demonstrate that a manufacturing process produces batches of finished product which reliably fulfil all the specifications laid down in the quality file and which can hence be considered to be as safe and efficacious as the batches used to demonstrate safety and efficacy in the dossier for the marketing authorisation.
- Control of the active substance: The assay of the active substance alone may not be sufficient to demonstrate the consistency of the production process. The Applicant will need to select and justify the antigen(s) to be measured and investigate if a correlation can be established between the quantity of the antigen(s) and the ability of the vaccine to protect. The methods of control (in-process and on finished product) should be able to detect sub-standard batches containing less active substance than standard batches.

- Control of the adjuvant: The properties of adjuvants are important factors in the efficacy of vaccines that contain them. The quality and quantity of the adjuvant should therefore be controlled by validated tests during the production process and, if possible, in the finished product.
- The in vitro methods used for in-process and finished product tests should be validated against the criteria described in VICH guidelines GL1 [5] and GL2 [6].

Conclusions

In vivo potency tests tend to have a number of inherent problems that make them difficult to validate and present problems in interpretation. While it is not possible at present to recommend general solutions to the issues identified above, the development of in vitro methods as alternatives to in vivo potency tests is encouraged.

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In Vitro Vaccine Potency Testing:

A Proposal for Reducing Animal Use for Requalification Testing

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Key words: potency testing, in vitro potency assay, veterinary vaccine testing, vaccine testing

Abstract: This paper proposes a program under which the use of animals for requalification of in vitro potency tests could be eliminated. Standard References (USDA/CVB nomenclature) would be developed, characterized, stored and monitored by selected reference laboratories worldwide. These laboratories would employ scientists skilled in protein and glycoprotein chemistry and equipped with state-of-the-art instruments for required analyses. After Standard References are established, the reference laboratories would provide them to the animal health industry as "gold standards". Companies would then establish and validate a correlation between the Standard Reference and the company Master Reference (USDA/CVB nomenclature) using an internal in vitro assay. After this correlation is established, the company could use the Standard References for qualifying, monitoring and requalifying company Master References without the use of animals. Such a program would eliminate the need for animals for requalification of Master References and the need for each company to develop and validate a battery of Master Reference Monitoring assays. It would also provide advantages in terms of reduced costs and reduced time for requalification testing. As such it would provide a strong incentive for companies to develop and use in vitro assays for potency testing.

Introduction

Post-licensing production of vaccines requires potency testing of each lot to ensure that the vaccine will provide a protective immune response in vaccinated individuals. While the potency of most live vaccines is now accomplished using in vitro assays, inactivated vaccines remain a challenge for conversion from animal potency testing to faster and more efficient in vitro potency testing. Although significant improvements have been made in this area and worldwide regulatory agencies have accepted some in vitro assays as potency tests, the strict regulatory requirements necessary to ensure vaccine potency have discouraged their

continued development and use in the animal health industry. In the USA, such regulatory requirements involve repeat animal requalification requirements for each in vitro potency assay, and development and validation of batteries of Master Reference monitoring assays by each vaccine manufacturer. Laboratory animal use may have been reduced somewhat but the use of host animals that must undergo pain and suffering from challenge testing as a result of the requalification requirements has increased dramatically.

This paper proposes a program wherein the use of animals for post-licensure requalification of in vitro potency testing could be eliminated in any country that requires repeat animal requalification of vaccine references. It is a concept proposal but, as we move forward with development and validation of new in vitro potency tests, such concepts must be seriously considered in order to reduce animal use and increase efficiency. It is understood that the initial demonstration of vaccine efficacy would still require use of animals.

In Vitro Vaccine Potency Assays

Most animal health companies are developing or already using in vitro assays for potency testing of inactivated vaccines. The most common format is the ELISA wherein the tested vaccine is compared against a Master Reference and a Relative Potency (RP) or quantification of antigen is determined. An RP of 1.0 or greater must be obtained for a satisfactory RP potency test or for a quantification test, an acceptable level of antigen is required. The Master Reference may be a final adjuvanted product, a functional antigen, an extract, a purified protein or even a live organism that has been accepted by regulatory agencies. Prior to VSM439 Draft, the dating for the Master References was based on the storage conditions (from three to five years) and continued stability monitoring. The proposed VSM439 extends the dating period for "legacy products" to as much as 15 years if monitoring indicates stability. Requirements for Vaccine Master Reference qualification, requalification, and stability monitoring

Master References must be established as correlating to protection in an immunogenicity test (host animal efficacy vaccination/challenge test or field study) and monitored for stability on a routine basis using a battery of validated stability assays. Master References must also be requalified in animals on a routine basis to demonstrate that they still provide a protective immunogenic response (every two to five years, depending on their storage) [1]. As such, the use of in vitro assays requiring Master References still requires the use of considerable numbers of animals and has significant added costs involved with the development and validation of the battery of stability monitoring assays. These requirements have made the continued development and use of in vitro assays difficult for industry to justify, especially for older products. Figure 1 shows a diagram of the major steps required by each company to qualify, requalify, and monitor the stability for each Master Reference used for in vitro potency testing of each antigen in a vaccine.

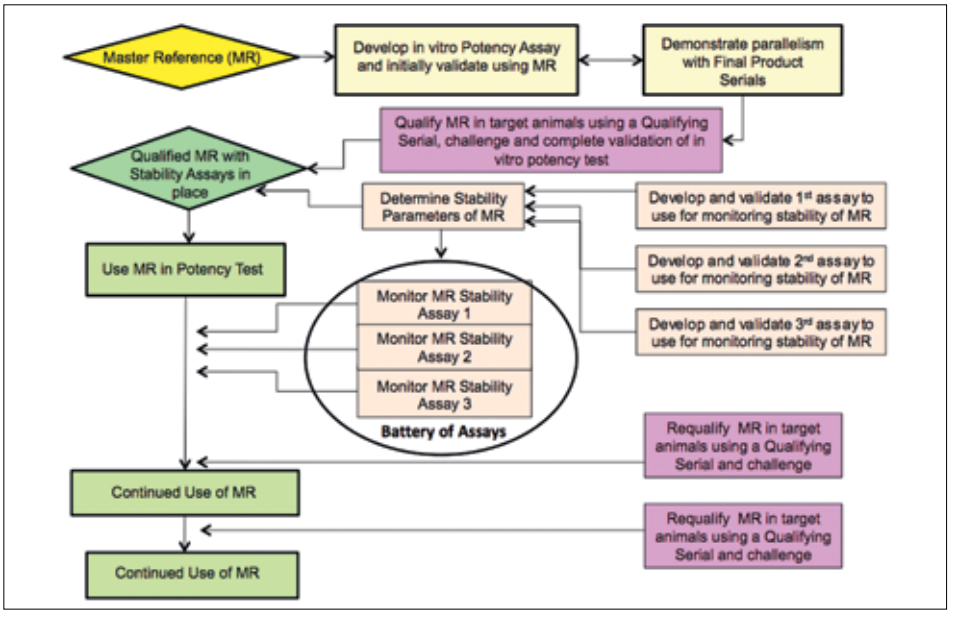

Fig. 1: Current Process for Vaccine Master Reference qualification, requalification, and stability monitoring.

In order to provide an idea of the impact of animal use for requalification of Master References on the animal health industry, an example of a 5-way multivalent feline vaccine will be used (Fig. 1). Each antigen in the vaccine would have to be tested by a different in vitro potency test. Therefore, every 2-5 years, the Master References must be requalified in kittens that have to undergo vaccination and then challenge with the respective live pathogenic agent. Such tests generally require 20 vaccinates and 20 controls for each antigen being tested. That means that 200 kittens would have to undergo a challenge every two to five years to requalify a Master Reference for one five-way feline vaccine (see Fig. 2).

The animals must be pre-bled for determination of serological status and held in quarantine prior to any vaccination (Fig. 2). Therefore, such testing alone can take 9-12 months (of the two to five years) to complete. In some cases the animals seroconvert before they can be vaccinated. Those kittens must be euthanized and another group of animals located, held and retested. One can quickly see how overwhelming such requirements can be for even the largest of companies. In 2010, there are approximately 40 feline antigens in killed vaccines in the USA If all of these use in vitro potency tests for release, under the current USA animal requalification requirements, a minimum of 2 000 kittens would be used in challenge testing involving pain and distress every two to five years. The number of animals required, the size of laboratory and the number of scientists needed for requalification is considerably larger for a typical company routinely using 50 Master References.

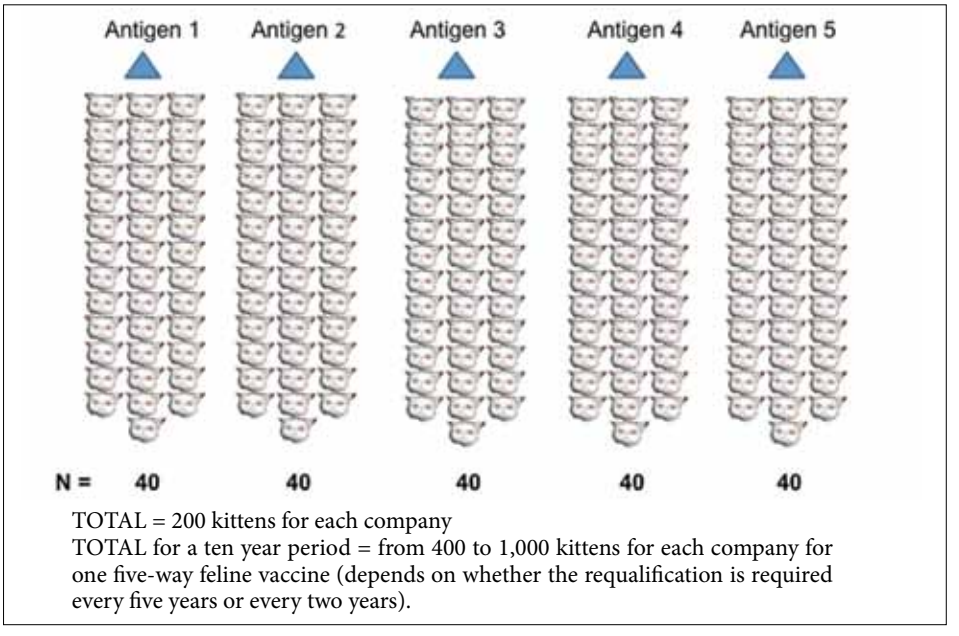

Fig. 2: Example of number of kittens required for requalification of one five-way multivalent feline vaccine by one company.

Worldwide Standard References

Eliminating the requirement for requalification of Master References in animals could be accomplished by establishing reference laboratories that maintain Standard References for use by all animal health companies. These laboratories could be located within worldwide government agencies or institutions that could carefully control, monitor and in collaboration with other interested countries, dispense them as "gold standard" reagents for use during qualification, requalification and monitoring of in-house Master References. The Standard References would be stored frozen, characterized using optimal protein chemistry methods that may not be available or cost effective for individual companies, and controlled in a manner suitable for use as worldwide references. The institution controlling the Standard Reference would need to identify the functional antigen(s), produce and characterize the Standard Reference, develop and validate stability monitoring assays, and monitor the stability of the Standard Reference using its validated methods.

Worldwide Reference Laboratories

A worldwide collaboration among the Reference Laboratories would be established to coordinate the Standard Reference development. Each laboratory

could focus on a specific group of antigens. For instance, the USDA Center for Veterinary Biologics has identified protective antigens for five Leptospira species [2]. Therefore, they could focus on development of Standard References for Leptospira-containing products. Rabies protective antigens have also been identified and could be an excellent candidate for development and distribution of a Standard Reference by a Reference Laboratory [3]. Tetanus and other similar toxoid antigens that are already identified could also be candidates for Standard References that could be distributed by a Reference Laboratory.

Use of Standard References

The Standard References would be used by each company during the qualification of its internal Master Reference, for monitoring of the Master Reference and for requalification of the Master Reference. See Figure 3 for the diagram of this process. The qualification of each company's Master Reference would involve demonstration of a specific relationship between its own Master Reference and the Standard Reference using one specific in vitro method that is acceptable to regulatory agencies. By validating this relationship and establishing a CV for the assay, the company could then routinely monitor and requalifiy its Master Reference against the Standard Reference using this in vitro method (Fig. 3).

Using Standard References to qualify a replacement Master Reference (Fig. 3)

The company could also qualify a new Master Reference, if necessary, using the Standard Reference. The new Master Reference would need to be prepared according to the procedure used to prepare the original Master Reference and be qualified against the Standard Reference according to its original qualification/validation method. Note that each company would develop its own method for correlating the antigen content of the Master Reference to the Standard Reference. This correlation would be conducted prior to or during the conduct of the host animal immunogenicity test. The vaccine used in the host animal immunogenicity test would either be the Master Reference itself or a qualifying serial prepared to be equal to the Master Reference within the CV of the assay used for quantification. Therefore, the amount of antigen established as protective would be correlated to the amount of antigen in the Master Reference. The correlation of Master Reference to host animal protection would be conducted at the same time as the in vitro correlation of the Master Reference to the Standard Reference. Figure 3 shows a simplified diagram of Master Reference qualification and requalification if a certified Standard Reference was provided. Note that no animal testing is required post qualification of the Master Reference.

Use of Standard References by individual laboratories

It is not necessary for each company to use the same types of assays for correlating the Master Reference to the Standard Reference nor is it necessary for each company using an ELISA to have the same relative correlation back to the Standard Reference. The only critical factor is that a relationship between the

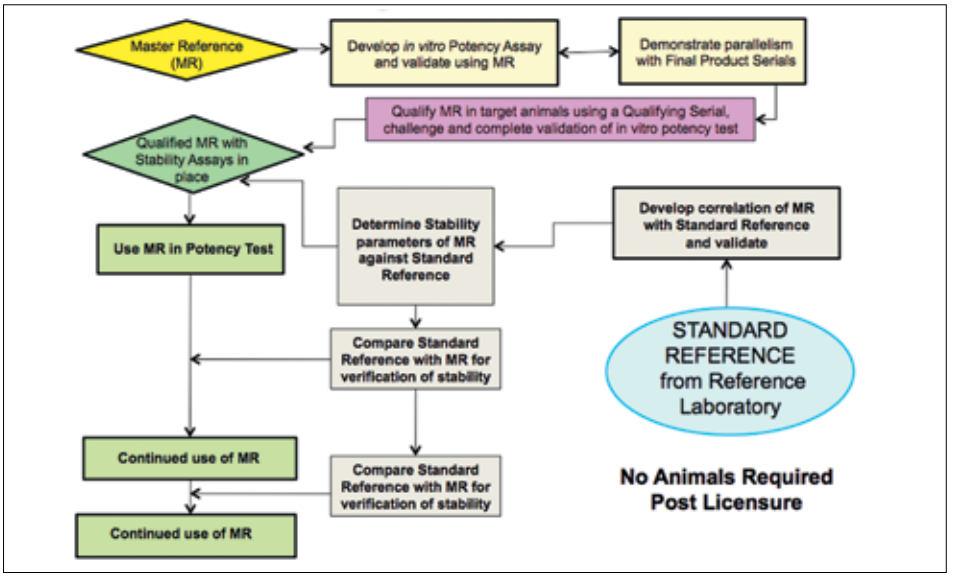

Fig. 3: Diagram of Vaccine Master Reference qualification and requalification using a Vaccine Standard Reference supplied by a Reference Laboratory.

Master Reference and the Standard Reference be established and validated with "out of specification" parameters being defined. It is proposed that the latter be defined by the CV of the assay. Once this has been defined, the assay can be used for monitoring stability of the Master Reference as well as qualifying a new Master Reference.

Use of Vaccine Standard Reference by three different companies (Fig. 4)

This shows how three different companies could use the same Standard Reference. Company A and B are using an ELISA RP whereas Company C is using an ELISA quantification test. Companies A and B have different RPs relative to the Standard Reference. Company C is measuring the mg/dose of antigen rather than an RP. All have established their own correlations and can monitor and requalify their Master References in vitro. If they find that the Master reference is trending out of specification, a company can prepare a new Master Reference and, as described above, can validate that against the Standard Reference without having to go back into animals.

There are many advantages that would be provided by developing and using worldwide Standard References to qualify, requalifiy, and monitor in vitro vaccine potency assays. These include: 1) Vaccine post-licensure animal testing could be eliminated; 2) Repeat immunogenicity testing in animals would not have to be conducted by each company; 3) Development and validation of multiple assays to monitor Master Reference stability would not have to be undertaken by each company; 4) Companies would have an economic incentive to develop and use in vitro potency assays because of the reduced testing requirements; and 5) There

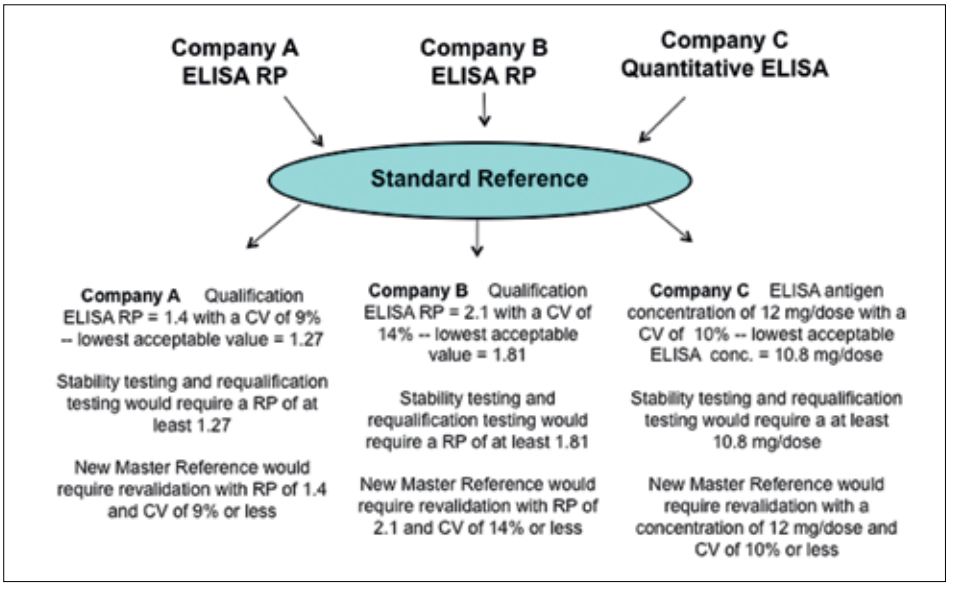

Fig. 4: Use of Vaccine Standard Reference by three different companies.

would be consistent Standard References for use by each company to compare its own Master References.

The disadvantages of this proposal are minimal and involve establishment and funding of the Standard Reference laboratories. These laboratories will have to overcome the same hurdles that each company must now individually overcome. However, it is expected that these Standard Reference laboratories will be staffed by experts in protein (antigen) chemistry and have state-of-the-art equipment available to conduct all of the necessary analyses. The result of such a program would provide a win-win scenario for animal welfare, regulatory agencies and industry.

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In Vitro Antigen Measurement and Potency Tests

Challenges Encountered During Method Development...and Lessons Learned

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Abstract: Despite significant investment and technical efforts, veterinary vaccine manufacturers continue to experience challenges with the transition from historic animal-based potency methods to in vitro potency assays. These challenges have a number of contributing factors, including an inadequate understanding of protective antigens and epitopes, a lack of ruggedness and discriminating capabilities in evolving immunologically-based methods, inconsistencies between methods used for in-process antigen measurement and finished product potency, and a lack of clear methods to characterize the finished formulation (including complex adjuvants). A lack of harmonized guidelines and consistent regulatory expectations further complicates these efforts.

There is room for optimism, however. There are numerous examples of successful in vitro potency test implementations. Titrations of modified live viral and bacterial vaccines, immune-based quantitative assays, and the recent application of direct physicochemical methods have allowed the transition from animal testing in many applications globally.

Specific challenges for assay development and implementation are discussed in the areas of 1) target antigen selection, 2) complexity of finished product formulation, 3) potency discrimination, and 4) stability-indicating relevance.

Introduction

Veterinary vaccine manufacturers continue to have a strong interest in developing in vitro testing methods for in-process antigen measurement and finished product potency testing. There are a number of professional and practical reasons for this interest, including: 1) to reduce the number of target/alternative animals used in development, production, and batch release of veterinary vaccines, 2) to improve the reproducibility of formulation activities and consistency of finished product performance, 3) to avoid the variability and financial risks associated with animal-based assays, and 4) to shorten batch-specific product testing timelines and accelerate batch release.

Despite significant investment and technical efforts, veterinary vaccine manufacturers have experienced challenges with the transition to in vitro assays. Of primary concern, there has often been an inadequate understanding of protective antigens and epitopes necessary to build an effective vaccine. Pathogenesis and virulence factors have been poorly understood and relevant antigen(s) have not been adequately identified or characterised. This is especially true with certain inactivated bacterial products. Extending this concern, there are also examples where the necessary reagents could not be developed to adequately discriminate between bacterial serovars. *Actinobacillus* bacterins for pigs have encountered this problem. In other cases, in vitro method development has been hampered by the nature of the vaccine candidate. There are cases, for example, where the dose range between the protective minimum effective dose and the maximum safety dose is less than the variability of the developed methods. Viable cell enumeration assays for some Salmonella vaccines have encountered this issue previously.

While techniques to identify and produce suitable test methods and relevant reagents have improved, many of the immunologically-based methods still suffer in terms of ruggedness and discriminating capabilities. Bridging the gap between in-process antigen quantitation and finished product potency has been difficult, as testing methods to allow antigen measurement in the presence of complex adjuvants are only now being developed and confirmed as relevant. In certain situations, the formulation of the blended finished product cannot be adequately characterised (especially in cases of combination adjuvants where component structure is relevant). Also associated with formulation challenges, antigen repartitions between aqueous and oily phases can be partial or inconsistent, especially in cases where the extraction methods are imperfect. And, there are difficulties to properly assess antigen/product stability within a formulation due to binding and/or masking of antigen epitopes. Aluminium hydroxide adjuvanted vaccines have experienced this issue previously. Finally, addressing the region-specific regulatory requirements for global vaccine development programs has been challenging, due to a lack of harmonized guidelines and consistent regulatory expectations. For example, expectations for the qualification, control, and re-qualification of potency assay reference materials vary by region. A lack of consistent guidelines can create issues when global veterinary vaccine manufacturers consider in vitro method options during vaccine development programs.

There is room for optimism, however. There are numerous examples of successful in vitro potency test implementations. Titrations of modified live viral and bacterial vaccines have led to consistently-protective products that deliver a measurable return on investment for commercial producers (and a sense of security for companion animal owners). Immune-based quantitative assays have also been highly effective when virulence factors are known and finished product formulations are well understood. ELISA and immunodiffusion assays are examples in this area. Finally, direct physicochemical assays have been developed that specifically quantify critical antigens in finished product formulations, including hemagglutination assays and high-performance chromatographic separation methods for defined subunit and synthetic antigens.

Results and Discussion

Significant technical and regulatory dialog is necessary to drive the transition from animal-based antigen measurement and finished product potency testing methodologies to in vitro assay strategies. Several examples follow that highlight the specific challenges associated with the development and approval of in vitro potency tests for global immunologicals. These examples detail the issues associated with target antigen selection, complexity of finished product formulation, potency discrimination, and stability-indicating relevance.

Target antigen selection

In vitro assays are dependent on the selection of relevant targets. In the case of modified live products, the relevance of viable titer for in-process organism quantitation and finished product testing is obvious. Historically, however, the tools have not been available to routinely develop and conduct inactivated antigen measurement and potency release where both the in-process and finished product assays utilize the same, correlated antigen measurement strategy. More often, inactivated products have been formulated based upon less-specific parameters such as total cell counts, pre-inactivation titers, or optical density measurements, whereas finished products have been tested against separate, specific parameters such as serologic response, vaccination-challenge, or relative potency against a product reference. In some cases, methods such as optical density correlate well with actual antigen content within an antigen batch. In other cases, methods do not and lead to variability in finished product antigen content and inconsistent product performance. The lack of correlation between the in-process and finished product strategies is apparent in the example illustrated in Figure 1. In this example, 20 antigen batches were evaluated for blending calculations using a conventional optical density method. Subsequently, the actual antigen content of a relevant target antigen was measured in the same batches using an enzyme-based immunoassay.

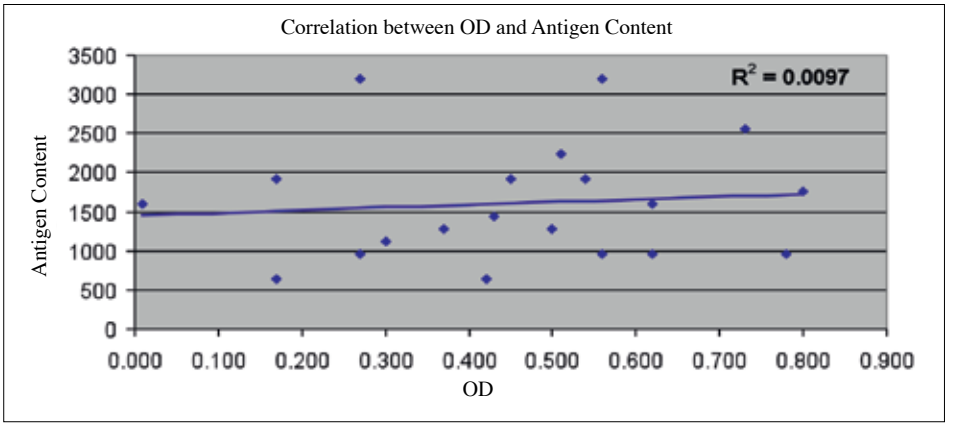

Fig. 1: An example of a correlation analysis between optical density and specific antigen content.

Clearly, there is a lack of correlation between the two methods. Use of similar approaches for bulk antigen measurement, product blending calculations, and finished product potency evaluation would be preferred and would offer benefits in terms of product consistency and performance.

In vitro assays are only as good as the specificity of the selected reagents. Due to the immunodominant nature of some bacterial antigens, the reagent production and selection process often generates reagents recognizing conserved (group-specific) antigens rather than strain specific targets. In the absence of careful (but time-consuming) screening, assays can be developed that clearly identify the potency of the product in general, but do not adequately characterize the amount of each strain contained within a multi-strain finished product. In addition, reagent development is often a balance between specificity and overall information value; in fact, high reagent specificity may actually reduce the value of the potency result in some cases (since a single monoclonal antibody will provide a very accurate reading on a very narrow window regarding the relevant protective element(s) of some vaccines). The impact of this narrow window is critical in some cases. To avoid this problem, there is sometimes value in developing an antigen-based method to measure the total amount of relevant antigen in a final formulation, plus a physicochemical assay to confirm the correct formulation between strains. Alternatively, in some cases, one must abandon in vitro approaches and pursue in vivo potency models. In this case, selection of specific serologic methods rather than challenge methods is preferred to minimize stress to the animals.

Complexity of finished product formulation

There is an ongoing effort in the animal health industry to improve the overall performance and safety profile for veterinary immunologicals. This ongoing drive to improve product performance is leading to advances in a number of relevant areas, including new innovations in adjuvant technologies. Often times, the result of this effort is the inclusion of new adjuvant components (and increasing formulation complexity). While critical for vaccine improvement initiatives, this places an additional burden on potency test development.

The need for consistent assembly of vaccine batches and the important role of the adjuvant components in the overall potency assessment has been recently highlighted by the Committee for Veterinary Medicinal Products – Immunological Working Party1. This need increases with the complexity of the final formulation. Well-defined adjuvants such as aluminum hydroxide can be adequately assessed by direct measures (such as aluminum content). Emulsion adjuvants such as oil-in-water emulsions can be assessed by physicochemical techniques such as measurement of oil content and/or particle sizing. Adjuvants using a simple blend of immuno-active molecules can be assessed by direct quantitation of the active components (and understanding of molecular stability).

Complex formulations require more sophisticated approaches within the overall potency test development program to complement in vitro antigen measurement efforts. A blend of starting material controls, qualified assembly processes, in-process controls, and finished product testing techniques is

required to confirm reproducible manufacture and predict consistent efficacious performance in the field. Often, finished product characterisation will require direct measure of active components (recognizing that extraction methods sometimes complicate stability assessments), plus suitable physicochemical methods such as particle size distributions or chromatographic profiles. An inability to characterise and monitor finished product formulation stability can lead a manufacturer to return to in vivo potency release models.

Purpose-designed separation methods (such as high performance liquid chromatography methodologies) could help in this regard by providing a reference trace for correctly-formulated vaccine. This approach requires qualification to ensure that this trace is a relevant predictor of consistent adjuvant assembly (i.e. the trace would change with inconsistencies in formulation or aging). Clearly, development of these types of characterisation is best conducted prior to pivotal clinical work, so that the vaccine batches that define the product's safety and efficacy profile can be characterised to help set future batch release specifications. An example of such an analysis is presented in Figure 2 and demonstrates how the trace for a complex formulation changes as components are added during the blending process. Combined with good manufacturing practices, this type of trace could form the basis for both release and end-of-shelf-life specifications.

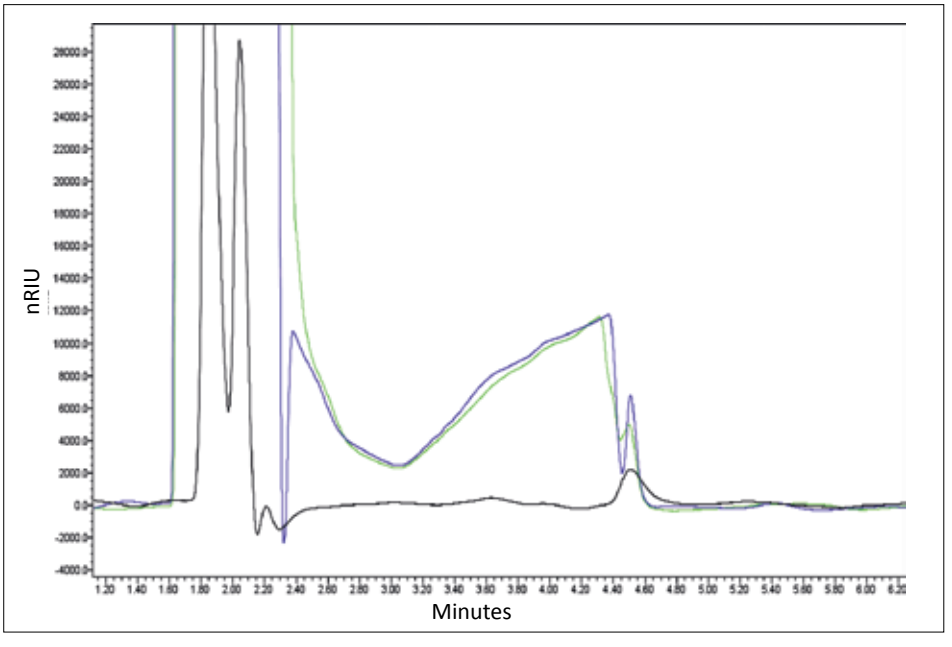

Fig. 2: An example chromatographic analysis of inactivated product assembly utilizing a four-component complex adjuvant.

Potency Discrimination

Potency assays must be able to discriminate between properly-formulated and improperly-formulated vaccine batches to ensure that only potent batches are released for commercial purposes. As such, in vivo and in vitro approaches are often co-developed and assessed during potency test development programs to determine the utility of each to assess proper formulation within a defined range of potency. Historically, animal serological responses are more variable than in vitro potency assays. This is especially true when evaluating sub-formulated (sub-potent) batches, as animal responses may be more variable in these cases, leading to broader confidence intervals and reduced discriminating ability. An illustrative example is described in Figure 3a, where multiple batches of an inactivated viral vaccine were formulated at two separate potency levels and repetitively tested using an in vivo vaccination-serological assay. The 95% confidence level for the sub-formulated batch is clearly wider than the properly-formulated batch containing a minimum efficacious dose of viral antigen (and in fact overlaps the confidence interval for this minimum potency batch).

By comparison, an in vitro potency model can provide an improved discrimination between properly-formulated and sub-formulated vaccines. Using the same approach, multiple batches of vaccine were formulated at two separate potency levels and repetitively tested using an in vitro physicochemical assay. The 95% confidence level for the sub-formulated batch is consistent with the confidence interval for a properly-formulated potent batch and confirms the ability of the in vitro model to discriminate between sub-formulated and potent formulations, without overlapping confidence intervals (Fig. 3b).

Differences in regulatory expectations in different regions also complicate method selection. In vitro methods can be developed that address the discriminating expectations for one region, but are inadequate for another. Use of in vitro methods will expand once the animal health industry and regulatory authorities reach a common understanding of acceptance criteria in this important area. Also, consistent statistical assessment criteria are needed to assess the true discriminating ability of certain assay approaches. Regulatory and industry discussions regarding statistical model preference would help here. It is clear, however, that there is always a need to ensure that perceived differences are supported by proper statistical analyses.

Stability indicating relevance

Potency assays must be adequate for batch release, but must also be adequate to monitor finished product stability over time and establish/confirm the shelf life for the product. The same is true for in-process antigen measurement methods. This requirement can be somewhat challenging when applied to the use of in vitro assays, however. Each in vitro assay strategy is biased by its approach. Antibody-based assays, for example, are driven by the quality of the antibody(ies) employed in the assay, the epitope(s) recognized, and the sensitivity of the assay to conformational antigenic changes (versus the sensitivity of the animal to the

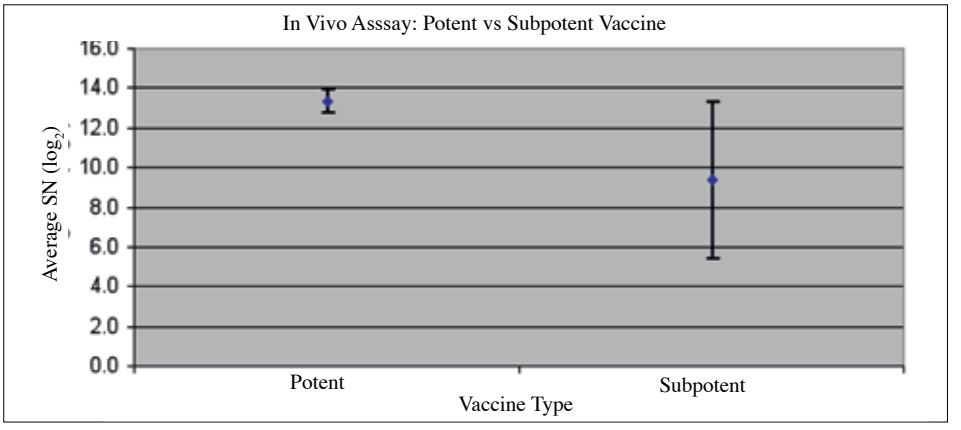

Fig. 3a: A comparison of potency assay discriminating abilities – in vivo.

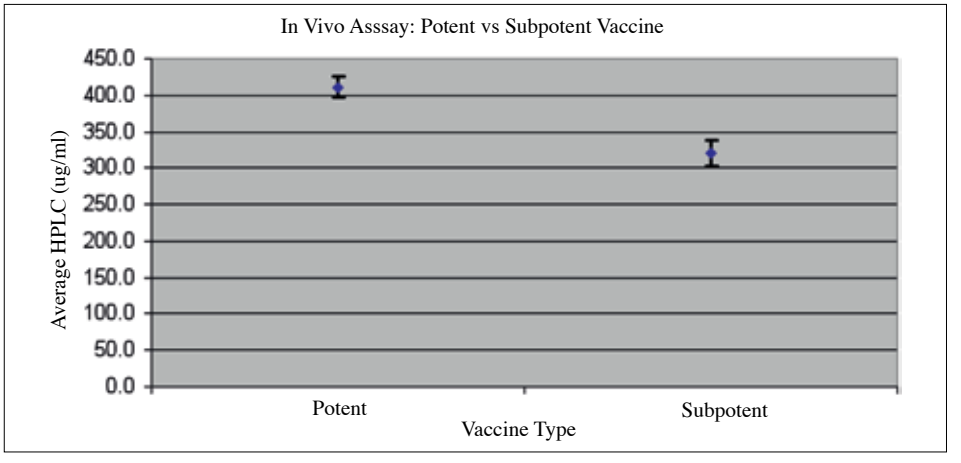

Fig. 3b: A comparison of potency assay discriminating abilities – in vitro.

same conformational changes). Physicochemical assays are driven by a separate set of parameters, including the structure and integrity of the targeted molecule.

In both cases, the relevance of the molecule is important. For example, a polymerase chain reaction (PCR) technique can be a powerful tool to estimate the total cell density within a bacterial suspension. The relevance of DNA on antigen stability can be questioned, however. Also, the physical integrity of a target antigen can be assessed using chromatographic methods (for synthetically-produced conjugate molecules, for an example). However, the intactness of the conjugate molecule may not be the most important factor regarding efficacy.

Most often, in vivo immunogenicity assays provide the best benchmark relative to stability of the finished product. Even in this case, however, problems can arise if the potency methods are poorly discriminating.

Complex vaccine formulations may compound the problems encountered with finished product stability assessments using in vitro assays. Stability can be poorly-predicted in situations where steric hindrance or masking of antigens/epitopes can occur over time with certain adsorbing adjuvants. This challenge can sometimes be offset through the development of efficient elution techniques. As such, the consistency of any extraction method is an important consideration when assessing the stability-indicating value of an in vitro method. Also, the relevance of bound versus unbound antigen must be considered in the context of product stability, especially in terms of product efficacy and safety over its shelf life.

Stability assessments are often on the critical path of veterinary vaccine development projects in the EU and other geographies. As such, the industry must assess the value of short-term predictors of vaccine stability, especially given the need to select finished product formulations and potency strategies in advance of pivotal clinical studies (thereby reducing the number of formulations – and animals – used during development programs). Accelerated stability assessments are often used in this case, but can be influenced by different types of stresses/modifications. Historically, veterinary vaccine manufacturers have found that elevated temperature studies are predictive and useful when assessing the stability of live products. The utility of this strategy for inactivated products is less predictable. An illustrative example highlights this point. The following set of figures summarizes an experiment comparing a proven in vivo potency assay to a proposed in vitro enzyme-linked immunoassay (ELISA), using both accelerated and real-time conditions. In Figure 4a, the experimental product is exposed to significant thermal stress for several defined periods of time (0, 4, and 24 hours). Product is then assessed for potency using an ELISA in vitro method as well as a host animal serology method. The results demonstrate that the ELISA method is very sensitive to elevated temperatures (and more sensitive than the animal potency model). In contrast, real time studies are summarised in Figure 4b. These results demonstrate the true correlation between the two methods when assessing properly refrigerated finished product.

Conclusions

The global veterinary vaccine industry continues to actively pursue in vitro assays and the reduction in the use of animals for in-process antigen measurement and finished product potency testing. Technical and regulatory hurdles are still present that limit the industry's progress in this area, but things are improving. Quality attributes exist that can not be assessed solely by testing; quality and consistency must be built into the product. Ultimately, progress will rely on better science, cooperation amongst the concerned parties, and a global harmonisation of regulatory requirements.

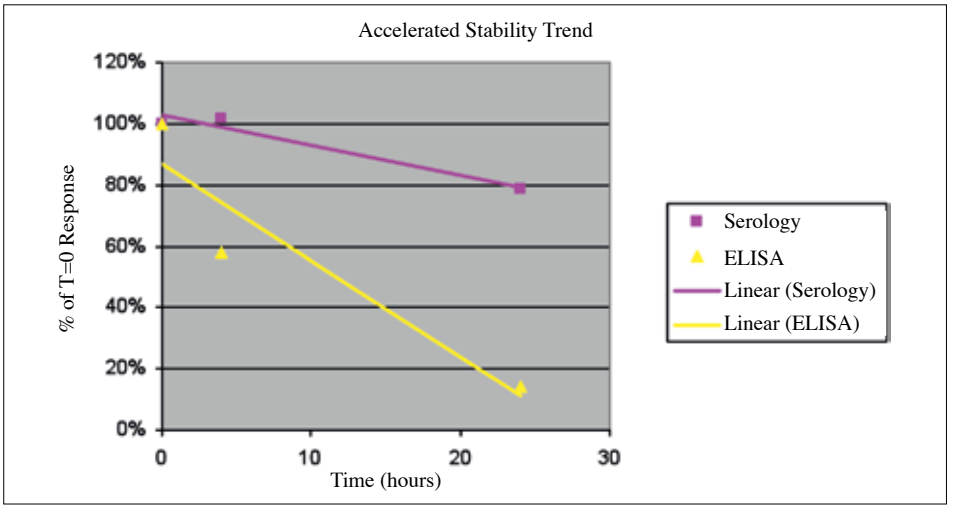

Fig. 4a: Potency assay performance using thermally-stressed product.

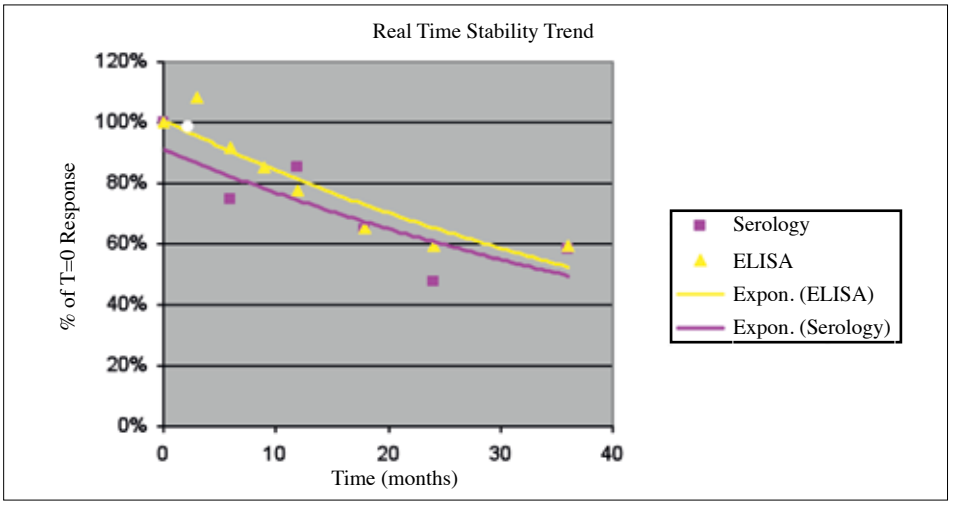

Fig. 4b: Potency assay performance using conventionally-stored product.

Reference

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In Vitro Potency Tests: Challenges Encountered During Method Development

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Key words: rabies virus, vaccine, potency test, virion integrity

Abstract: Vaccines play a key role in the control of viral diseases both in humans and in animals. In order to ensure the quality and consistency of vaccines they are extensively tested, including potency control of individual batches. In the case of vaccines against rabies the most widely used test for batch potency control is the National Institutes of Health (NIH) test. The NIH test is performed in mice leading to the consumption of thousands of animals every year. Protection against rabies after vaccination is associated with neutralizing antibodies directed against the viral glycoprotein (G). Therefore the amount of G-protein in vaccine preparations is an important parameter with regard to potency. Additionally the structural integrity of virus particles in vaccine preparations may be crucial for their immunogenicity.
The objective of our work is the development of in vitro methods to determine the potency of vaccines against rabies. The result of this ongoing project shall be an assay panel including measurement of the antigenic content as well as parameters of antigen quality in a vaccine preparation allowing a precise prediction of the potency of rabies vaccines without using animal experiments.

Introduction

Rabies represents one of the oldest known viral infections of warm-blooded animals and still causes thousands of deaths in humans worldwide every year. Rabies virus (RV), the etiologic agent, belongs to the genus Lyssavirus in the virus family Rhabdoviridae [1]. The viral genome consists of a non-segmented single-strand RNA with negative polarity, which encodes five structural proteins: N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein) and L (large protein - RNA polymerase).

The nucleoprotein associates with the genomic RNA to form the ribonucleoprotein (RNP) together with the large protein and its cofactor, the phosphoprotein. This RNP is surrounded by the matrix protein, which interacts with the viral envelope; the latter is made up of a lipid-bilayer, in which the glycoprotein is embedded as trimers (Fig. 1).

In the host the G-protein is responsible for the induction of neutralizing antibodies which confer protective immunity. The structure of the G-protein has been demonstrated to influence its immunological properties. Soluble monomeric glycoprotein was shown to be less immunogenic than virion-attached glycoprotein [2, 3]. In addition, different levels of protection were observed after immunization with viral subunits, e.g. recombinant soluble glycoprotein, recombinant or virion-derived purified glycoprotein, recombinant or virion-derived purified nucleoprotein, when compared with intact virions [2-8]. Current vaccines against rabies are usually based on cell culture derived purified virus. In contrast to veterinary vaccine preparations rabies vaccines for humans do not contain adjuvant.

The objective of this study is the development of a panel of assays that allow a precise prediction of the potency of a vaccine batch thereby avoiding the necessity of animal experiments. Along this line we aim at a correlation between antigen conformation and immunogenicity of vaccines against rabies. In this context the integrity of virions in vaccine preparations was analysed.

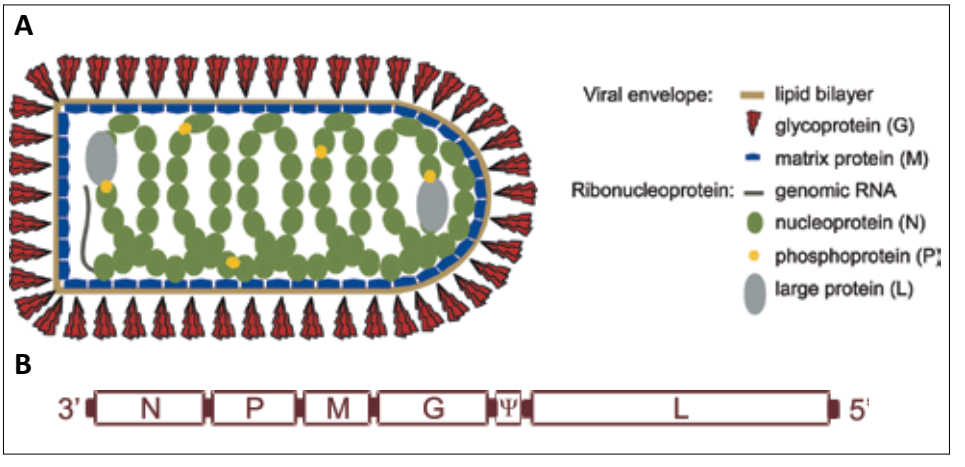

Fig. 1: Schematic presentation of a rabies virion (A) and the genome (B).

Materials and Methods

Virus and vaccine preparations

Rabies virus strain FluryLEP, freeze-dried vaccine preparation (Rabipur(TM)) and concentrated inactivated virus (a preliminary stage of vaccine production) were obtained from Novartis Vaccines (Marburg, Germany).

Rabies virus strain CVS was propagated on baby hamster kidney (BHK) cells (ATCC, CCL-10). Cell culture supernatants were inactivated by adding beta-propiolactone at a final dilution of 1:3500 (v/v). Viral suspensions were maintained under continuous circulation at 4° C for 24 hours immediately followed by an incubation of 2 hours at 37° C. Infectivity assays were performed in order to confirm complete inactivation.

Rabies virus particles were purified by ultracentrifugation at 4° C, 25000 rpm using a Beckman SW 41 Ti rotor for two hours on a continuous 0-60% (v/v) sucrose density gradient. An opaque virus band was visible at the 36% interface and recovered by puncture of the centrifugal tube. The virus band was dialysed against PBS and stored at – 20° C.

Immunization of animals and generation of antibodies

The G and N proteins of RV were expressed via recombinant baculovirus system [9].After purification of the recombinant proteins, rabbits were subcutaneously immunized using complete Freund´s adjuvant on day 0, and incomplete Freund´s adjuvant on days 14 and 56. Monospecific antisera were generated after sampling from the auricular artery. Immunization of mice was performed with recombinant N-protein using Sigma adjuvant system as well as purified complete virions without adjuvant. After three subcutaneous immunizations at 12-day intervals and one intraperitoneal immunization spleen cells were fused to SP 2/0 myeloma cells according to standard procedures and hybridomas selected by immunofluorescence against recombinant G-protein expressed on the surface of BHK cells and CVS infected BHK cells. Monoclonal antibodies (mAbs) were purified from culture supernatants and characterized by immunofluorescence, immunoblotting and neutralization tests.

Immune-electron microscopy (IEM)

Virus suspensions were adsorbed to pioloform and carbon coated glow discharged copper EM grids (400-mesh) before or after treatment with a non-ionic detergent (NP-40) by incubation on droplets (30 µl) for approximately 30 min. Grids were then blocked with 1% BSA in PBS for 15 min. After washing six times with PBS, grids were incubated with the respective dilution of the first antibodies: rabbit polyclonal antibody anti-RV N-protein and mAb anti-RV G-protein for one hour at room temperature. After washing with PBS, grids were incubated for one hour with the appropriate dilutions of goat anti-mouse antibody conjugated to 5 nm colloidal gold and goat anti-rabbit antibody conjugated to 15 nm colloidal gold (BB International). Subsequently grids were washed three times with PBS, three times with water and stained with 2% methylaminotungstate for 30 sec. Excess stain was removed and grids were air dried. For control experiments, the primary antibodies were replaced with PBS and samples treated as above. Samples were observed using a Zeiss TM 910 transmission electron microscope.

ELISA

An in-house standardized ELISA was used to measure RV N-protein. The sandwich ELISA is based on polyclonal antibodies generated against purified recombinant RV N-protein (see above). The content of nucleoprotein was measured before and after detergent treatment of samples and quantified by comparison with an in-house standard i.e. purified recombinant N-protein. Protein concentration was determined by the BCAssay method, with bovine serum albumin as standard.

Statistical analysis

The amounts of RV nucleoprotein were calculated by a regression analysis method. Results were expressed as µg/ml.

Results

Using electron microscopy, the integrity of virus particles in vaccine preparations was studied. The distribution of RV glycoprotein and nucleoprotein was examined after immune labelling of concentrated inactivated virus samples on a grid.

Bullet-shaped structures with an average length of 180 nm and an average diameter of about 75 nm are typical for intact rabies virions. Negative staining revealed the characteristic morphology of intact rabies virus particles (Fig. 2). In addition few disintegrated particles and small intact virions presumably representing defective virions were observed. Using a mAb anti-RV G-protein and a polyclonal antibody against the nucleoprotein immunogold labelling of rabies virus particles was readily observed (Fig. 3). The mAb identified the glycoprotein exclusively on the surface of virions. In contrast, reactivity of the polyclonal anti-RV nucleoprotein antibody was observed with material released from partially or completely disrupted virus particles (Fig. 3). The distribution of signals in vaccine preparations resembled the one in concentrated inactivated virus samples (data not shown). No gold particle labelling was seen in the control experiments (not shown).

The N-protein together with the genomic RNA forms the inner structure of RV particles and is therefore not accessible to antibodies in intact virions. Accordingly the nucleoprotein was not detected on intact virions as visualized by IEM (Fig. 3). In order to release the viral nucleoprotein detergent treatment was performed. IEM showed a loss of the characteristic morphology of rabies virus particles and an enhanced diffuse staining of the nucleoprotein (Fig. 4).

To allow quantification of the nucleoprotein an ELISA was used to test samples before and after detergent lysis of virions. On average there was a ten-fold increase of the amount of detectable N-protein after treatment of the samples with detergent (Fig. 5). The ELISA results suggested that up to 90% of the nucleoprotein was not accessible to antibodies but shielded in viral particles.

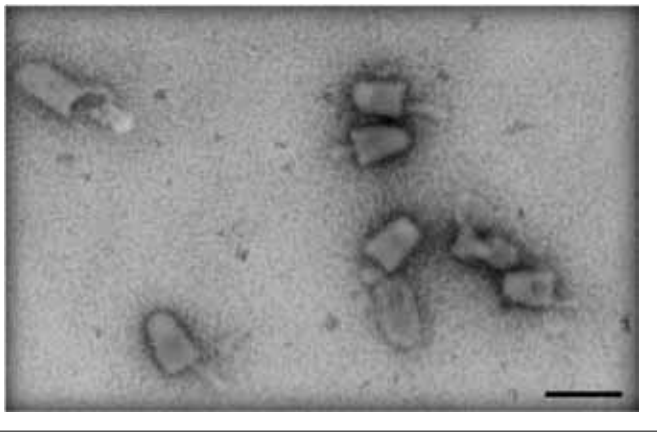

Fig. 2: Electron micrograph of rabies virus particles from a vaccine preparation. Bar: 200 nm.

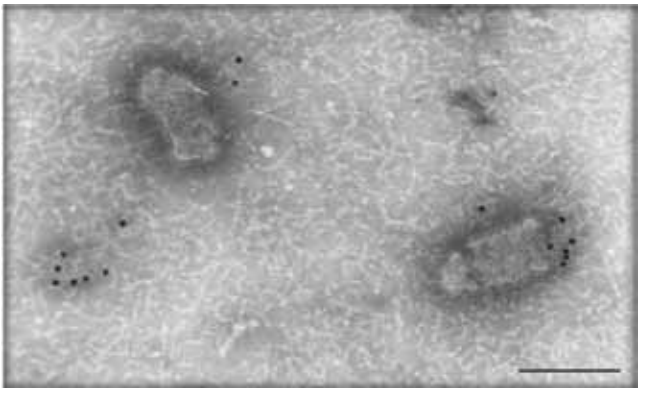

Fig. 3: Double-staining IEM of rabies virus particles. Small gold dots: mAb anti-RV G-protein, goat anti-mouse antibody conjugated with 5 nm colloidal gold; large dots: polyclonal Ab against RV N-protein, goat anti-rabbit antibody conjugated with 15 nm colloidal gold. Bar: 200 nm.

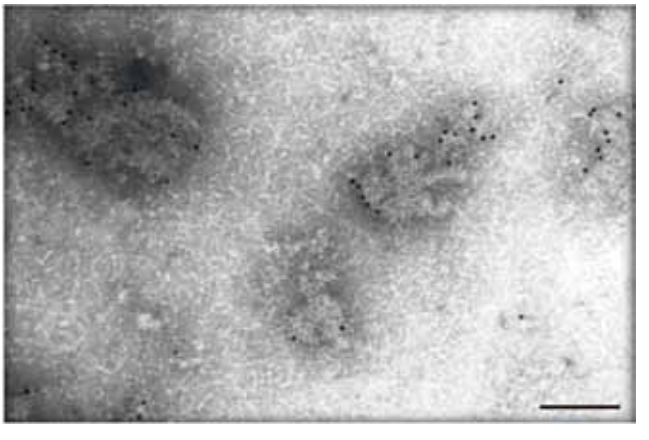

Fig. 4: Double-staining IEM of rabies virus preparations after treatment with detergent. Small gold dots: mAb anti-RV G-protein, goat anti-mouse antibody conjugated with 5 nm colloidal gold; large dots: polyclonal Ab against RV N-protein, goat anti-rabbit antibody conjugated with 15 nm colloidal gold. Bar: 200 nm.

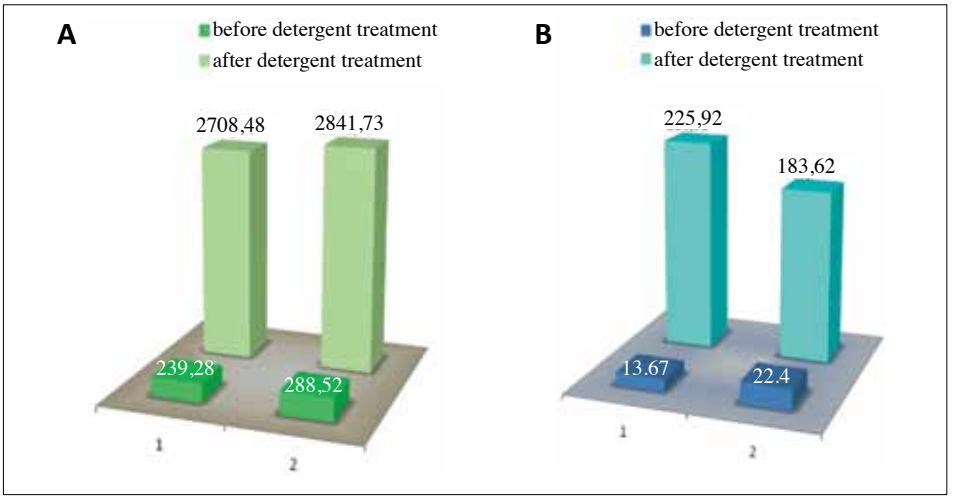

Fig. 5: Quantification of rabies virus N-protein by ELISA (µg/ml). A: concentrated and inactivated virus samples for vaccine production (1 and 2). B: non-adjuvanted vaccine samples (1 and 2).

Discussion

So far the potency of rabies vaccine preparations is mostly measured using the National Institutes of Health (NIH) test conducted in mice [10]. This assay is laborious, time-consuming and has high variability of up to 400%. Recently a serological test was established as an alternative [11]. This approach is faster and demands less animals but it still requires the use of laboratory animals and shows a considerable variability.

A true alternative to batch potency testing enabling the reduction or replacement of animal tests is the consistency approach. Applicable for vaccines that are produced under strict quality control the latter approach may involve the determination of a panel of parameters to establish a product profile. Based on product profiles consistency between each released batch and a master vaccine of proven clinical efficacy and safety is demonstrated [12]. The objective of our work is to establish a multistep protocol to demonstrate vaccine consistency focusing on three relevant aspects: (i) biochemical characterisation of the manufactured product (proteome), (ii) quantification of immunogen content (native G-protein) and (iii) assessment of antigen quality (structural integrity of virus particles). In this part of the study, we analysed the structural integrity of virions in a rabies vaccine licensed for the use in humans.

The efficacy of a vaccine, i.e. its ability to confer protection against disease, is influenced by many factors including the nature of relevant antigen(s), their dose and immunogenicity, the route of administration and the presentation of the antigen to the immune system. In the case of vaccines against rabies, several studies demonstrated that vaccines containing complete virus particles are superior in their immunogenicity when compared to subunit vaccines.

Furthermore Piza et al. [3] demonstrated that the composition of a vaccine with regard to different forms of rabies virus glycoprotein (free soluble glycoprotein, virus-attached glycoprotein) has an influence on potency. In a set of model experiments we assessed the integrity of virus particles in concentrated inactivated virus preparations and samples of a rabies vaccine for humans by IEM and by an ELISA for the quantification of the viral N-protein.

Negative staining of vaccine preparations showed the presence of intact RV particles with the characteristic bullet shaped morphology as well the presence of damaged particles. The latter is presumably due to the preparation process, which includes concentration and purification of RV, and offers an explanation for the detection of small amounts of nucleoprotein by ELISA prior to detergent treatment of samples. In order to quantify the relative amount of intact virus particles, the proportion of accessible vs. non-accessible RV nucleoprotein in vaccine preparations was taken as a parameter. It could be shown that around 90% of N-protein was not accessible for antibodies and thus probably part of virus particles.

In order to elicit a protective immune response, the relevant viral antigen(s) should be presented to the immune system in native state resembling the conformation in virions. Consequently quantification of G protein in intact virus particles should correlate well with vaccine potency. Existing ELISAs for the quantification of G-protein in vaccines against rabies are generally based on the determination of antigenic mass. Such already available tests could be integrated into a multi panel assay together with biochemical analyses and the quantification of intact virions based on measuring of the ratio of detectable nucleoprotein in non-treated vs. detergent treated vaccine preparations; the latter is expected to correlate with the amount of intact virus particles. This assay panel would offer a precise picture of vaccine consistency based on parameters relevant for efficacy.

Furthermore we plan to improve the quantification of viral glycoprotein by development of a detection system based on virus neutralizing mAbs that recognize only the native form of the G-protein and show a broad reactivity with different vaccine virus strains.

Besides the amount and quality of the G-protein as the main antigen additional factors may play a role in the efficacy of vaccines against rabies; e.g. the viral genomic RNA preserved in virions may modulate the immune response. Comprehensive biochemical analyses of vaccines including the integrity of the viral RNA will be helpful to study such parameters. The final multi step protocol should be the basis for the complete replacement of animal experiments in batch potency control of vaccines against rabies.

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Successful Development and Validation of an in Vitro Replacement Assay for Leptospira Vaccine Potency Tests

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Key words: leptospira vaccine; potency; replacement alternative

Abstract: The standard requirement for serial release potency testing of Leptospira bacterins in the United States is the hamster vaccination challenge test. It is a test that uses a large number of animals experiencing pain or distress, takes weeks to conduct, can be expensive and requires that laboratory personnel handle a viable zoonotic pathogen. In an effort to address these concerns, the United States Department of Agriculture (USDA) developed an in vitro method for potency testing of four Leptospira serovars. This enzyme-linked immunosorbent assay (ELISA) was subsequently validated in the target species. USDA informed their biologics licensees, permittees and applicants of the availability of reference bacterins and the regulatory acceptance regarding this alternative test method in notices issued in 2007 and 2009. This presentation describes how the initial research and subsequent development and validation work were accomplished.

Introduction

Leptospira bacteria are comprised of several species, some of which are saprophytic organisms living on dead or decaying organic matter in pond water. L. interrogans, however, is a pathogenic, disease-producing organism. Diagnosing leptospirosis is made difficult due to the fact the pathogenic leptospires are not readily distinguishable on the basis of morphology, biochemical, or culture characteristics. Instead they are identified by their distinctive antigenic properties that can be demonstrated serologically using the microscopic agglutination test. The serovar provides the basis for classification of these bacteria and there are currently over 220 identified pathogenic serovars.

Leptospirosis is a zoonotic disease. Leptospires occur naturally in a wide variety of feral and domestic mammals. The natural, or maintenance hosts include rats, raccoons, dogs, cattle and sheep. The organisms colonize the kidneys of these animal hosts and are shed in urine. The zoonotic concern arises when incidental hosts (humans) are exposed to the leptospires, usually due to occupational or recreational activities involving direct contact with infected urine, or contact with water or soil contaminated with infected urine.

Clinical symptoms are variable and depend on whether the patient is a natural host or an incidental host. They may range from inapparent infections to "chronic phase" signs seen in the natural host (kidney and liver damage, abortion and stillbirths) to "acute phase" signs seen in an incidental host (flu-like illnesses, hemolytic anemia, hemoglobinuria, jaundice). Other factors contributing to the variation in signs and symptoms include the dose and route of exposure, and immune status of the host.

The primary means of disease control is through animal immunization. Adequate immunity prevents infection in the natural hosts and minimizes environmental shedding of the organism. Immunity is generally humoral, but there is a cell-mediated component. Immunity is measured by a strong and rapid antibody (agglutination) response, and is life-long but serovar-specific.

The Virus-Serum-Toxin Act [1] passed by Congress in 1913, prohibits manufacturers from shipping or delivering any worthless, contaminated, dangerous or harmful vaccine product. This Act is enforced by the United States Department of Agriculture (USDA) Center for Veterinary Biologics, whose oversight ensures that veterinary vaccines on the market are pure, safe, potent and efficacious.

Background

The current potency tests for leptospira vaccines are referenced in the United States Code of Federal Regulations (CFR), Title 9, Part 113, Sections 101 through 104 [2]. They are hamster vaccination challenge assays. Some hamsters are injected with the test serial and others are held as non-vaccinated controls. All are later exposed to virulent challenge bacteria. A minimum of 80% of vaccinates must survive and 80% of controls must die in order to have a valid, satisfactory result. This test accounts for the vast majority of hamsters reported as experiencing pain or distress in research in the United States [3].

There are several disadvantages to this test, including that it requires a large numbers of hamsters and is expensive; it is time consuming (>5 weeks per test) and labor intensive; and it exposes laboratory personnel to viable pathogenic organisms.

The in vitro test [4] is an enzyme-linked immunosorbent assay (ELISA) that utilizes monoclonal antibodies in a sandwich format. Polyclonal rabbit serum is coated to the bottom of the wells in a microtiter plate. Test serial bacterin is added to some wells; reference bacterin to others. The mouse monoclonal detecting antibody is added next, followed by an anti-mouse immunoglobulin conjugated to an enzyme. When the color substrate is added as the last step, any wells that have successfully bound the antigen from the bacterins will turn color. Those

where the antibodies have been washed away (i.e., there is no bacterin antigen present) will be clear. The difference in color concentration between the test serial wells and the reference bacterin wells is used to determine the relative potency of the test serial.The advantages of this serial release test method is that it measures a relevant antigen, there are no hamsters involved; it is less expensive (in 2001, the hamster test was estimated to cost $640; the ELISA test $2); and personnel are not exposed to a human pathogen.

Test Development

USDA interest in this area began in the early 1990s, with the discovery of an antigen on the leptospire considered relevant to the immunological response in the animal [5] Monoclonal antibodies to that antigen were produced and these provided passive protection in hamsters against infection. During the same time-frame, manufacturers were developing in vitro tests for other products and in 1998, USDA published guidance regarding their regulatory acceptance of these types of assays [6].

By 2000, relevant antigens had been identified for all four Leptospira serovars, and the test method developed to the point that supplemental assay methods were published for manufacturers to review.

In 2002, USDA published a memo clarifying the requirements necessary for a firm to receive an exemption to the requirement for testing leptospiral bacterins for potency in hamsters [7]. The manufacturer would be expected to qualify a reference bacterin in host animals, and correlate the host animal protective dose to its protective end-point in hamsters. The costs involved with conducting these qualification assays were not encouraging to the firms. As the primary goal for the USDA Center for Veterinary Biologics at the time was to "significantly refine, replace and reduce animal testing of veterinary biologics" [8], the decision was made to support the development of a USDA reference bacterin. Funding was secured, in the amount of $250,000 to begin validating references for two serovars used in canine products. A contract was issued to produce L. canicola and L. icterohaemorrhagiae bulk fluids for ELISA reference bacterins.

In 2003, an additional $500,000 was allocated by the USDA for efficacy testing of all four serovars in dogs and swine. Unfortunately, it was discovered that the challenge cultures had exceeded 100 hamster passages, and there was a concern these had become too attenuated to be effective in the host animals. Qualification assays were undertaken on new challenge cultures to evaluate their ability to induce clinical disease in dogs, swine and calves.

In 2004, the challenge culture media studies were completed and work was begun on qualifying pathogenicity of challenge material in host animals. At the same time, it was determined the amount of ascites fluid monoclonal antibodies remaining was inadequate to continue comparative ELISA testing. A decision was made to replace these antibodies with bioreactor-produced monoclonal antibodies. The new antibodies had to undergo both in vitro equivalency studies and passive protection studies in hamsters.

The first validation studies were initiated in 2005. A contract was awarded to Michigan State University to conduct the dog efficacy trials, and these were completed in 2006, followed by the swine efficacy trials. Both studies supported satisfactory results for the reference bacterins, in both hamsters and host animals. USDA guidelines on the qualification of leptospiral reference bacterins were published in 2007 and 2009 [9].

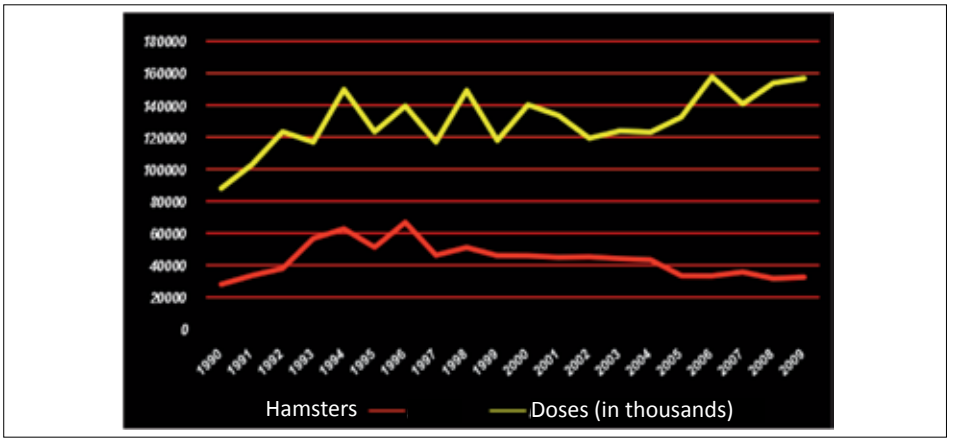
Conclusions

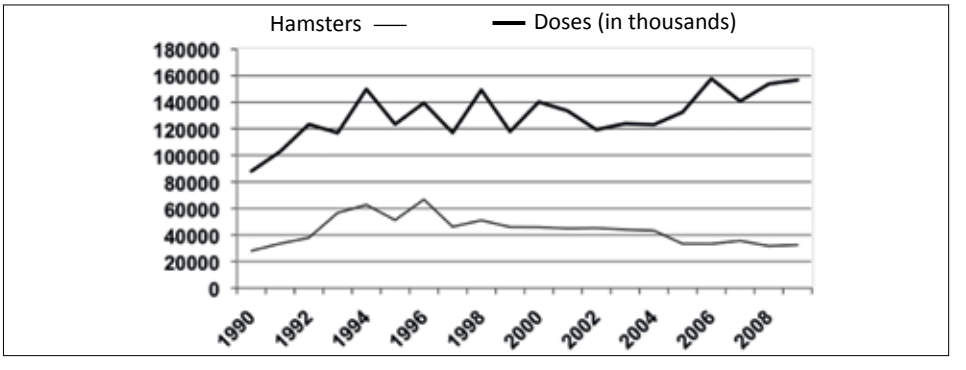
As shown in this timeline, it has taken the USDA approximately 19 years to develop the ELISA tests. The estimated cost, including personnel, materials, and contracts, is approximately $2 million. In retrospect, some of the time spent producing and requalifying test components could have been done in parallel. This would have lessened both the time involved and the total amount spent.

In return for the investment, there are now four in vitro tests that have been validated to replace tests that use a large number of animals experiencing pain and distress, and require laboratory workers to work with pathogenic organisms.

One remaining hurdle is the fact that each manufacturer completes their product using different components (e.g., adjuvants, preservatives). Not only may these interfere with the ELISA, the reference bacterins developed by USDA may not work for each product. However, the validation process also supported a laboratory animal method by which manufacturers may qualify their own reference bacterin. While this will not totally eliminate the use of all hamsters, the numbers needed should decrease and this, in fact, may already be occurring (Figs 1 and 2).

During the 1990s, the number of doses of leptospira bacterin produced increased from around 80 million annually to 130 million per year. During this same period, the number of hamsters used in research and reported as experiencing pain or distress paralleled the number of doses produced from year-to-year. The hamster numbers stabilized after 2000, even as the number of doses produced continued to rise. In fact, a decrease in the numbers of hamsters used was observed in 2005 after issuance of a notice stating humane endpoints will be acceptable unless USDA identifies a compelling reason why the proposed endpoint would not be appropriate. Encouraging the use of more sensitive endpoints, and developing in vitro assays and reference vaccines are just some of the ways USDA continues to support the tenets of replacement, reduction and refinement in veterinary biologics testing.





Figs. 1, 2: Comparison of the number of doses of Leptospiral bacterins produced in the United States to the number of hamsters reported used in painful/distressful research, teaching or testing.

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AlphaLISA® Assays to Improve the Vaccine Development Process

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Key words: homogenous assay biomarker serum immunogenicity

Abstract: Testing vaccines involves expensive animal models and extensive in vitro characterization. Techniques such as ELISA and ELISPOT are traditionally used to measure immunogenicity, assess the potency of recombinant vaccines and detect the presence of biological contaminants. However, these time-proven techniques suffer from technical limitations affecting the overall vaccine development process. Limitations include: consumption of large volumes of biological sample (eg. plasma), high variability, and limited dynamic range. Furthermore, ELISA and ELISPOT involve a multitude of blocking and wash steps which limit their automatability. AlphaLISA® technology is an exceptionally sensitive non-wash immunoassay platform which alleviates all the aforementioned drawbacks, allowing one to improve biologics development processes. Examples of how AlphaLISA® assays can be used to assess the potency of vaccines will be presented.

Introduction

Approaches to minimize animal use in vaccine development and manufacturing are of increasing interest for both technical and ethical reasons. While the in vivo challenge model for potency determination continues to be a standard technique, a number of alternative methodologies have been outlined that will ultimately serve to reduce, replace or refine animal models in this application area. As reviewed recently by Hendriksen [1], these approaches include the use of quantitative serological readouts in place of qualitative biological endpoints (ie. disability or death); the application of in vitro immunogenicity models to recapitulate the functionally relevant components of the immune response; and application of the consistency approach to in-process and final lot release testing using sensitive analytical tools that can evaluate quality of antigen preparations throughout the manufacturing process.

Using such approaches to advance the 3R concept will require the availability of reliable, sensitive, specific, simple and validatable assay technologies for the measurement of dilute analytes in a variety of biological matrices. ELISA is the most widely used detection platform for the quantification of analytes in such

samples, however the relative insensitivity and narrow dynamic range of this method often requires testing large volumes of sample in a wide dilution series to ensure useful data can be obtained. Furthermore, the need for extensive wash steps introduces measurement error and makes this technology difficult to adapt to automation. The AlphaLISA® homogenous assay platform has been specifically designed as an ELISA-alternative that addresses these shortcomings and should be of significant value in the effort to develop in vitro potency tests for vaccines.

Principle of AlphaLISA® Technology

AlphaLISA® (Amplified Luminescent Proximity Homogeneous Assay) is a next generation bead-based technology related to PerkinElmer's AlphaScreen® platform utilizing a unique luminescent oxygen-channeling chemistry [2]. In the sandwich assay format (Fig. 1), a protein or peptide analyte is captured between a specific biotinylated antibody bound to streptavidin-coated donor beads and a second antibody (against a non-overlapping epitope) covalently conjugated to AlphaLISA® acceptor beads. Bridging of the two antibodies with the analyte brings donor and acceptor beads into close proximity. Laser irradiation of donor beads at 680 nm activates a photosensitizer which results in generation of unstable singlet oxygen. The singlet oxygen decomposes with a half-life of 4 μsec, allowing this intermediate to diffuse approximately 200 nm prior to decay. Acceptor beads that have been brought within this proximity limit by virtue of analyte bridging, interact with the singlet oxygen thereby triggering a cascade of chemical and energy transfer events that result in a sharp and intense chemiluminescent emission at 615 nm. Heme has a broad and intense absorbance spectrum up to 600 nm, therefore signal emission above this wavelength minimizes the inner filter effects of hemoglobin, making AlphaLISA® technology particularly suitable for measurement of analytes in serum and plasma.

AlphaLISA® assays are simple and fast to develop, as well as miniaturizable and automatable for increased laboratory productivity. These homogeneous assays eliminate the need for multiple washes to separate bound from unbound assay components, and the resulting mix-and-measure protocols significantly reduce hands-on and total assay times compared to ELISA assays (Fig. 2). Moreover, increased sensitivities can be obtained with AlphaLISA® technology due to the amplified signal resulting from multiple singlet oxygen molecules generated by each Donor bead, which are then capable of triggering activation of nearby Acceptor beads.

As a result of these characteristics, AlphaLISA® technology enables the rapid quantitation of analytes from a large number of samples in a highly miniaturizable and automatable assay format with no loss of sensitivity. This feature is crucial when quantitative detection of analytes must be performed on precious pre-clinical or clinical samples available in a limited quantity. AlphaLISA® assays are highly robust when using sample volumes as low as 1 μl in total assay volumes of 10 μl.

Assays can be performed routinely in 96- or 384-well microplates. In addition, fully validated AlphaLISA® protocols are available for PerkinElmer's JANUS® automated workstation to automate various types of AlphaLISA® assays in an efficient and highly reproducible automated format.

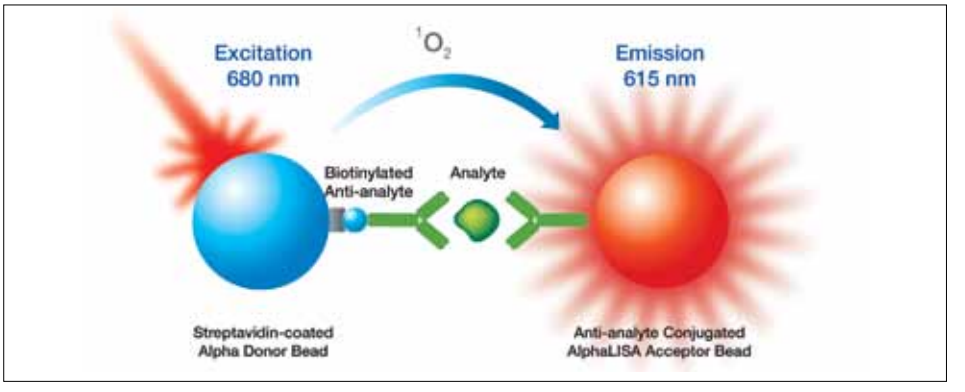

Fig. 1: Principle of the AlphaLISA® technology.

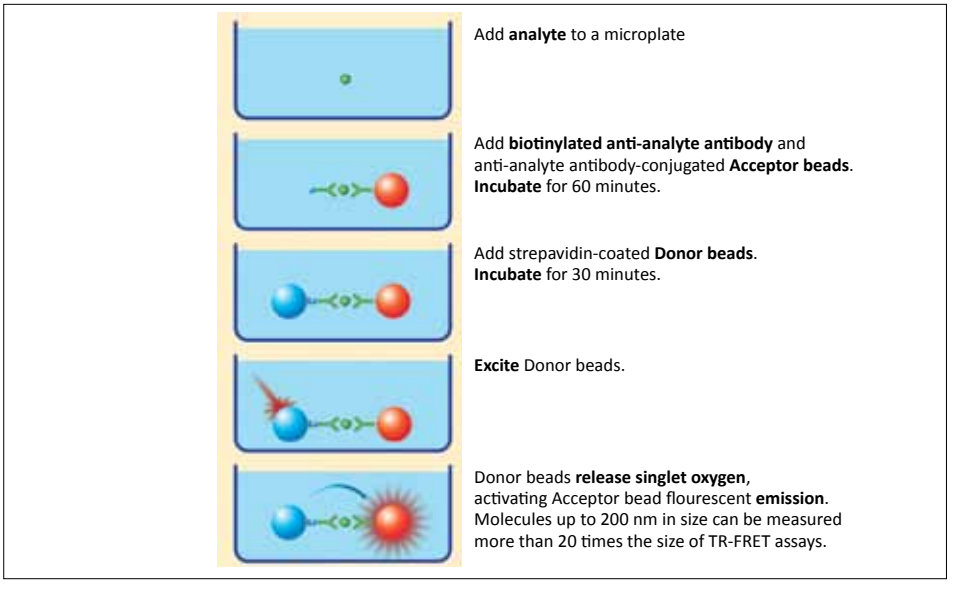

Fig. 2: AlphaLISA assay protocol.

Applications of AlphaLISA® Technology to Vaccine Development and Manufacturing

Numerous assay formats have been developed with AlphaLISA® technology for such applications as (1) detection and quantification of molecular targets in a variety of sample backgrounds, including biomarkers in serum and plasma, intracellular and membrane-bound proteins in cell lysates, or secreted proteins in cell supernatants; (2) measurement of protein-protein interactions, including large multi-subunit complexes; (3) measurement of various enzyme activities, in particular those leading to post-translational protein modifications such as phosphorylation and epigenetic modifications (4) detection and titration of full-sized viral phage particles (5) biotechnology applications for antibody screening and process analytics – ie. for purity assessment of biotherapeutics, and (6) immunogenicity assays for quantitation of anti-drug antibodies in pre-clinical and clinical studies. Many of these assay formats will have significant utility for application toward in vitro potency testing of vaccines.

PerkinElmer offers a variety of generic immunoaffinity and fusion-tag Toolbox beads that can be used for development of user-configurable assays. In addition, pre-validated kits for quantitation of over 100 secreted or cell associated biomarkers specific to immune cell activation, inflammation, neurodegeneration, oncology, metabolism and bioprocessing applications are presently available. Performance of these kits can be exemplified through recent studies which made direct comparisons between AlphaLISA® and ELISA assays to measure human insulin in plasma samples [3]. Assay sensitivity in the sub-picomolar range was observed in conjunction with a 4.3-log dynamic range for detection, allowing measurement over a wide range of analyte concentrations without the need for extensive dilution series. The assay also presented excellent intra- and inter-assay precision and was unaffected by plasma and serum matrices. Further, AlphaLISA® assay detected 15-fold lower levels of analyte than ELISA, while utilizing one fifth the sample volume – and provided a two-fold wider assay range with similar precision levels. The authors of this case study concluded that AlphaLISA® technology provided a valuable ELISA alternative, and cited the homogeneous format as a major enhancement for automated protocols.

Crisino et al [4] have also demonstrated the usefulness of AlphaLISA® assay for quantification of a biologic MIMETIBODY™ construct (MMB) in serum from three animal species (cynomologous monkey, rat, and mouse). The assay employed a double sandwich format with non-competing anti-idiotype mouse monoclonal antibodies to the MMB as capture and detection reagents in the same two-step, homogeneous assay. Observed sensitivity in cynomologous monkey serum was 40 ng/mL, while the rat and mouse serum assays gave similar sensitivities at 80 ng/mL. The methods used for all three species had similar dynamic ranges of approximately 2.5-logs. Inter-assay precision, as well as minimum required dilution and control diluent were similar for all three matrices, suggesting that the development of methods from species-to-species should be straight forward and efficient.

Validation of in vitro immune cell activation models also represents a useful opportunity to reduce animal use for vaccine development, and simple quantitation of secreted cytokines in such assays would be a distinct asset. Along these lines, Rodriguez-Suarez and colleagues [5] have described an "all-in-one-well" assay model where AlphaLISA® reagents are added directly to the cell culture to measure cytokine levels in a single step. In this published model, IL-1β secretion was directly measured following LPS stimulation of the monocytic leukemia cell line, THP-1. The presence of cells in this direct assay did not interfere with performance (robust quantitation was reported for the range of 3.3 – 2,300 pg/mL IL-1β), thereby eliminating the need to isolate cell-free supernatant for analysis. Additional studies have now demonstrated the suitability of the AlphaLISA® "all-in-one-well" approach for quantitation of TNF-α, IL-6, and IL-8 in cell assay models.

Summary

As a sensitive, robust, and simple assay platform, AlphaLISA® technology provides numerous opportunities to satisfy multiple approaches of the 3R concept in vaccine development and manufacturing. This technology can be characterized as a homogeneous, no-wash bridging assay exhibiting extremely high sensitivity and wide dynamic range for analyte quantitation in varied biological matrices and cell culture media. Such versatility makes AlphaLISA® technology of value for implementation in alternative potency assays. Possible applications include measurement of vaccine specific antibodies in serum of immunized animals, measurement of immune cell activation biomarkers for in vitro immunogenicity models, and in-process/final lot consistency testing for vaccine product quality or for measurement of process contaminants.

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SESSION IV

Consistency as an Alternative to Potency

Chairpersons: *Paul Midtlyng*
William Stokes

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Consistency as Tool to Support in Vitro Batch Potency Testing in GMP Production

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Abstract: There is great interest in the veterinary vaccine field to move away from in vivo release tests for vaccines to reduce cost and testing time, improve consistency and of course the 3Rs (reduce, refine, replace). A brief overview of Good Manufacturing Practice (GMP) and the consistency approach is discussed below and an overview of how manufacturers can use the consistency approach and GMP controls along with statistical analysis of processes at each stage of the production process (starting materials, antigen and finished product) to build in quality and reduce the need for in vivo finished product tests. A final summary and outline of some challenges we will face in moving this approach forward is covered in conclusion.

Introduction

It is in the interests of all stakeholders in the veterinary vaccine field to move away from in vivo release tests for vaccines for a number of reasons; cost and testing time, inconsistency of in vivo tests, inconsistency of product with poor control systems and of course the 3Rs (reduce, refine, replace).

The introduction of GMP in the EU along with ever evolving legislation and technology has changed the nature of manufacturing control and finished vaccine testing. There is now much greater focus on building in quality throughout the production process from starting materials through to finished product with the ultimate aim of improving quality and reducing testing required on the finished product.

The GMP regulations recognise that veterinary biological manufacture is unique in the range of different antigens produced; the need for low cost of goods and therefore the need for good control on the manufacturing process to ensure a consistent, safe and efficacious product is produced. GMP quality controls along with legislative requirements and a consistency approach all build quality and consistency into products at all stages of manufacture which should reduce the need for in vivo finished product potency testing for vaccines to confirm efficacy.

Human vaccine manufacturers are already using in-depth antigen measurement and characterisation and although much of this is not viable for the veterinary sector we can learn from this approach and move towards better characterisation of our vaccines to improve consistency of product and address the 3Rs.

The following discussion reflects how this could be applied to the veterinary sector and why this should give us more consistent, better controlled and ultimately more reliably safe and efficacious products.

Discussion

There are two elements to building consistency for veterinary vaccines, GMP and the consistency approach in manufacturing. GMP requires strict controls on all areas of manufacturing from equipment validation to training and control of staff and above all ensuring high quality sterile products are produced. The consistency approach has been defined as follows: *The consistency approach is a concept which includes the strict application of GMP rules and guidelines, process validation and in-process and final product tests and is aimed at verifying if a manufacturing process produces final batches which are consistent with one that fulfils all the criteria of Quality, Safety and Efficacy as defined in the marketing authorisation, ultimately resulting in replacement of routinely used in vivo tests.* The combination of the two can be used by vaccine manufacturers to provide data to authorities to support a consistent process and define a set a of test parameters that reflect consistent product which should no longer require in vivo potency release testing.

In developing a consistency approach manufacturers control starting materials, the antigen production process, finishing of product and the release testing for finished product and it is possible develop a clear understanding of consistent product.

When looking at antigen production, traditionally data are gathered from a number of production runs to establish appropriate ranges for each control parameter. Now and moving forward manufacturers are looking towards statistical design for process validation experiments at lab scale and the use of control charts to define a consistent process before scale-up. This type of analysis provides increased confidence in the data and therefore confidence in process robustness and consistency in line with GMP and consistency approach and increased confidence for authorities that a consistent and therefore safe and efficacious product can always be produced without the need to demonstrate potency in vivo.

Following on from the antigen production a similar approach can be used to develop a set of appropriate process and test parameters for finished product that enable characterisation of a product that is equivalent to one shown to be safe and efficacious in clinical trials. Many current in vivo potency tests are far from consistent and tell us little about the consistency and efficacy of the product other than the fact that it works in the test system used. The challenge for all manufacturers with regards potency testing is to find a suitable set of parameters that will define and reflect product efficacy. This is easier for some antigens than

others, for some viruses that are small and the protective antigens well defined while for larger viruses and bacteria this is much more difficult. There are significant benefits to all stakeholders in being able to do this though.

The Rabies challenge potency test is of particular concern; this test has European Pharmacopeia defined test confidence limits of 25-400% and is poorly validated. Data in Table 1 shows that duplicate results from the same batch vary widely. In addition Rabies is one of those small viruses with a well defined protective antigen, the Glycoprotein (GP) data in Table 1 also shows results for GP in each batch and it is clear from the numbers and analysis of these that there is no correlation between the GP content and either potency test result, nor any correlation between the two potency test results. Rabies therefore with it's well defined protective antigen, long production history and inconsistent in vivo potency test is an ideal candidate for taking steps towards in vitro release backed up by GMP and a consistency approach.

Summary

To summarise, GMP requires extensive control and monitoring of processes, monitoring of all aspects of a product's production allows us to build a picture of a consistent process. In addition statistical analysis of processes can be used to build-in consistency. Combining these approaches enables a manufacturer to have a clear picture of a consistent, safe and efficacious vaccine. In order to move this forward there are some next steps which are needed to facilitate this. Manufacturers should look to use of consistency approach and GMP to support in vitro testing for new products and this is already starting. Authorities in the EU are very open to this approach. The use of consistency approach in conjunction with GMP to move from in vivo to in vitro finished product tests for existing products is a bigger challenge as there are significant costs associated with this and no framework in which to do it. Industry and licensing authorities will need to work together to define a framework under which this could happen.

Table 1: Example of Rabies potency test data and Glycoprotein content.

Batch Number	GP	Potency at Manufacturer	Potency OMCL
7	18.44	3.29	17.0
8	20.64	10.8	18.9
9	19.52	6.04	2.07
10	22.67	4.88	52.4
11	28.61	6.67	4.6
12	33.72	6.29	13.3
13	22.97	3.42	11.6

However there are already examples of where this has happened in a 3Rs context with the target animal safety test removal, where defined requirements to remove a release test have been established. This gives us hope that this can also happen for potency tests.

Finally it should be should be kept in mind that there are significant challenges to overcome in using GMP and the consistency approach to move from in vivo to in vitro release tests. Finding suitable and relevant in vitro antigen targets to assess finished product consistency is perhaps the biggest hurdle and will require co-operation between industry and academia to do this in the most effective manor. Development of assays that work on finished product is also a challenge where many adjuvants can interfere with tests which would normally be used such as ELISAs. Adjuvant characterisation for new and complex adjuvants is also a problem as often although you can characterise these prior to blending, once blended they often form complex interactions which are difficult to quantify and qualify. And of course this will ultimately not work for everything, every antigen and process is different and it will require investment of both time and money and some sort of definition of what data are going to be required to support this approach.

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Consistency as an Alternative to Potency Testing

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Key words: consistency, batch potency, multi-criteria in vitro testing, reference vaccine, abandonment of target species challenge

Introduction

The consistency approach in the quality control of vaccines has been repeatedly discussed at several workshops. It was widely agreed that the level of quality control throughout the complete manufacturing process could be enhanced by a bundle of process- and product-specific tests. This would create a promising tool to facilitate the implementation of in vitro methods instead of continuing in vivo tests, in particular as regards some very demanding specific batch safety and batch potency trials in laboratory animals. In the future, vaccine manufacturing including refined process- and product-specific testing methods could result in the production of more consistent vaccine batches with similar characteristics as batches demonstrated to be safe and efficacious in the target species.

Current Status

Conventionally produced vaccines are often not well characterised. This applies in particular to products which have been established in the market for years, sometimes for decades. Here, in many cases, laboratory animals are extensively used in the quality control of finished product testing, with a focus on inactivated vaccines. There is general agreement on the difficulty to closer characterise classical inactivated vaccines in order to obtain valid parameters for batch release including non-animal potency tests.

In contrast, the use of new molecular technologies for the production of modern vaccines such as HPV and polysaccharide conjugate vaccines led to well characterised and highly purified products, in particular for human use. For those vaccines batch release is mainly based on in vitro methods.

Notwithstanding, producers and regulators are confronted with particular immunological veterinary medicinal products that are already manufactured by using new technologies such as bacterial and viral expression systems. Even though these products are per se well characterised and highly purified, the batch potency test consists either of an in vivo test or an in vitro test including a reference vaccine which needs to be renewed from time to time. Depending on the type of vaccine, this may also imply a challenge in the target species.

Particular Situation and Problem

For one of those innovative products a complex in vitro batch potency test was established during the licensing procedure, requiring for batch release the inclusion of and comparison with the reference vaccine batch which had been demonstrated to be efficacious in the target species. During the ongoing life cycle of this vaccine, several "standard" vaccine batches were established for this in vitro test. Each new standard vaccine was compared in vitro to its predecessor. Over time, a shift became evident which necessitated the generation of a new reference vaccine batch including a challenge in the target species. One may assume that any target species required is generally available in veterinary medicine and therefore there is no major obstacle to conduct challenge tests whenever it is considered necessary. This would also apply for the establishment of a new reference vaccine. However, ethical concerns arise easier when pet animals are affected. Moreover, they increase when the value of the batch potency test in use turns out to be questionable with regard to its capability to detect defective batches. This was the situation the marketing authorisation holder (MAH) and the competent authority (CA) were confronted with.

Idea, Procedure and Outcome

Thus, the value of an established in vitro test method for batch release for which a new reference vaccine was required, including a challenge trial in

companion animals was questioned. Furthermore, since no surrogate such as a known protective antibody level was available, there was also no alternative batch potency test. Based on this the MAH decided not to proceed with the establishment of a new reference vaccine but to implement a multi-criteria batch potency test focussing on consistency.

The MAH analysed the production process and assessed several manufacturing steps including the stage of adjuvant adsorption in view of potential critical incidences which could result in a negative impact on the active substance. The approach to look for valid criteria for the batch potency test caused the MAH to create a scenario which simulated the potential risks of deterioration effects. The influence of artificially derogated vaccine batch samples on the ability of each of the chosen control tests to detect defective batches was investigated. The originally agreed batch potency test was included. At the end of the various treatments, the products obtained were tested and compared with the same batch that had not been subjected to any treatment. It could be clearly demonstrated that the impact on the tested vaccine sample varied depending on the type of treatment. Using the multi-criteria batch potency test system, defective products will definitively be detected even if the original single batch potency test is misleadingly passed. For this product, antigen characteristics relevant for the multi-criteria batch potency test depend on the quantification of the protein content, the confirmation of the identity and purity by immunotransfer Western blotting technique using a specific monoclonal antibody (Mab) and the SDS PAGE electrophoresis technique followed by a densitometric analysis.

There was/were

i) no detection of batch failure by the original batch potency test in the case of out of specification (OOS) results of another of the control tests,
ii) no detection of batch failure by the original batch potency test alone,
iii) OOS results of at least two other control tests where the original batch potency test also failed.

Conclusion and Follow-Up

In conclusion, the combined analysis of SDS PAGE electrophoresis, Western blot profiles and protein content was found to be more suitable in detecting inconsistent batches than the original batch potency test. Consequently, the intention to establish a new reference vaccine including a challenge in the target species could be abandoned. The multi-criteria batch potency test guarantees:

i) the identification and proof of immunogenicity by Western Blot analysis using a specific Mab which recognises the presence of the relevant immuno-protective epitope,
ii) a defined level of purity by conducting SDS-PAGE and densitometric analysis,
iii) the determination of sufficient protein content.

The methods have been validated in compliance with VICH Guidelines GL1 and GL2.

An additional study was successfully performed to verify that the multi-criteria batch potency approach does not only ensure the consistency and activity of vaccine batches at the beginning of their shelf life but also to detect sub-standard or sub-potent batches after expiration.

With regard to the efficacy of the vaccine, the relationship between the multi-criteria batch potency test and the efficacy in the target species has been demonstrated by the MAH. Several vaccine batches, for which efficacy had been demonstrated by challenges previous to the consideration to implement the multi-criteria batch potency test, were chosen as correlates indicating that manufacturing and related testing methods result in the production of consistent vaccine batches with similar characteristics as the batches demonstrated to be safe and efficacious in the target species.

Furthermore, based on the product characteristics of a properly defined and well controlled recombinant product, sub-potent or sub-standard batches are most likely caused by negative impacts during the manufacturing process, e.g. by protein damaging effects or under-loading of the product. Such effects will be safely detected by the multi-criteria batch potency test. The final product testing is then completed by a set of further tests relevant for consistency.

Finally, general acceptance of this approach was achieved throughout the EU member states.

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Human Adenovirus-Vectored Foot-and-Mouth Disease Vaccines: Establishment of a Vaccine Product Profile Through in Vitro Testing

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Key words: FMD, adenovirus, vaccine, potency, consistency

Abstract: Next generation, foot-and-mouth disease (FMD) molecular vaccines based on replication deficient human adenovirus serotype 5 viral vectored delivery of FMD capsid genes (AdFMD) are being developed by the United States Dept. of Homeland Security and industry partners. The strategic goal of this program is to develop AdFMD licensed vaccines for the USA National Veterinary Stockpile for use, if needed, as emergency response tools during an FMD outbreak. This vaccine platform provides a unique opportunity to develop a set of in vitro analytical parameters to generate an AdFMD vaccine product profile to replace the current lot release test for traditional, inactivated FMD vaccines that requires FMDV challenge in livestock. The possibility of an indirect FMD vaccine potency test based on a serological alternative was initially investigated for a lead vaccine candidate, Adt.A24. Results show that serum virus neutralization (SVN) based serology testing for Adt.A24 vaccine lot release is not feasible, at least not in the context of vaccine potency assessment at one week post-vaccination. Thus, an in vitro infectious titer assay (tissue culture infectious dose 50, $TCID_{50}$) which measures FMD infectious (protein expression) titer was established. Pre-validation results show acceptable assay variability and linearity and these data support further studies to validate the $TCID_{50}$ assay as a potential potency release test. In addition, a quantitative physiochemical assay (HPLC) and three immunochemical assays (Fluorescent Focus-Forming Unit (FFU); tissue culture expression dose 50 ($TCED_{50}$); Western blot) were developed for potential use as in vitro assays to monitor AdFMD vaccine lot-to-lot consistency and other potential applications. These results demonstrate the feasibility of using a traditional modified-live vaccine virus infectivity assay in combination with a set of physiochemical and immunochemical tests to build a vaccine product profile that will ensure the each AdFMD vaccine lot released is similar to a reference vaccine of proven clinical safety and efficacy.

Introduction

The development of new, first-in-class veterinary vaccines provides strong ethical and scientific rationale to explore the feasibility of using in vitro methods rather than in vivo animal test methods for routine lot-release testing. By definition, many of the next generation molecular veterinary vaccines will be produced using manufacturing technologies established for human vaccines that embed consistency in production, including good manufacturing practice (GMP) and quality assurance (QA). In-process product monitoring systems for increased product standardization and improved product characterization can be used to augment, or perhaps even replace results generated by a single potency release assay that relies on animal testing.

Foot-and-mouth disease (FMD) is arguably the single most important veterinary disease with respect to global impact on animal health, animal productivity and international trade in livestock and livestock products. All currently licensed FMD vaccines are based on inactivated whole-virus suspension cell culture preparations and produced using manufacturing methods that have remained largely unchanged since initially described nearly 50 years ago [1]. Potency lot release testing of inactivated FMD vaccines is detailed in the World Organization for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [2]. Briefly, two test methods are described, both of which require live FMDV challenge and use of target host animals (e.g. minimum of 17 or 18 animals) in an approximately one month test. The 50% protective dose (PD_{50}) test has low in vivo repeatability and reproducibility, inherent statistical variability and does not permit useful discrimination between vaccine lots of normal ($3PD_{50}$) and emergency ($6PD_{50}$) potency based on outcome of a single release trial [3]. In contrast, the protection against generalization (PG) test possesses acceptable vaccine accordance (VACC) and concordance (VCON) compared to the PD_{50} test [4], but fails to provide an estimate of how many protective doses are in the vaccine serial – an important value needed for emergency FMD vaccine banks. The European Pharmacopoeia (Ph. Eur.) does accept FMD vaccine batch potency testing using an in vitro serology test based on serum virus neutralizing (SVN) antibody titers, provided there a statistical correlation between antibody response and vaccination-protection in the target host is established. However, validation for every FMD vaccine antigen (>20) will be likely necessary and the serology test still requires the use of animals and their intrinsic, biological response variation.

Selection of a replication-deficient human adenovirus serotype 5 vectored molecular vaccine platform for next generation FMD vaccines (AdFMD) [5] and the subsequent development of AdFMD serotype- and subtype-specific vaccine products will require the establishment and validation of a potency release assay for potency. The AdFMD vaccine development program provides an excellent opportunity to replace the current animal test methods for FMD vaccine lot release. The present study provides results on the initial evaluation of modified-live virus infectivity assays and new physiochemical and immunological tests to begin to define a vaccine product profile for AdFMD vaccines.

Materials and Methods

Construction and production of Adt.A24.11D

A replication deficient adenovirus vector, Adt.A24.11D (Adt.A24), was constructed that contains an expression cassette comprised of the FMDV A24 (strain Cruzeiro) P1-2A structural capsid and A12 3Cpro – coding regions. The expression cassette is under the control of a cytomegalovirus (CMV) immediate early promoter inserted into the E1 region of the human adenovirus C, serotype 5 genome containing deletions in the E1, E3 and E4 regions. The AdFAST procedure for adenovirus vector construction was followed. An adenovirus vector plasmid containing the FMDV coding regions was constructed, confirmed by restriction enzyme analysis, linearized with PacI and transfected into a replication – permissive cell line (M2A) that was constructed by transfection of 293-ORF6 cells [6] with a tetracycline repressor (TetR) expression cassette using standard procedures. The adenovector lysate from transfected cells was serially passaged to expand the titer and volume to produce a high titer stock of Adt.A24. The stock was confirmed by PCR analysis and then used to generate a Pre-Master Seed in M2A cells. Following the vaccine outline of production, expansion stocks were produced. An Adt.A24 working reference vaccine was used for in vivo tests and for in vitro method development. Three experimental AdFMD vaccine candidates based on other FMDV serotypes or subtypes (AdFMD1, AdFMD2, and AdFMD3) were also constructed and evaluated in vivo and in vitro.

In vivo test

The Adt.A24 working reference vaccine virus was evaluated for efficacy in cattle using the OIE recommended experimental homologous virus challenge route, challenge dose and generalized disease scoring system. Cattle received a single 2 ml intramuscular vaccine dose, challenged with virulent FMDV A24 Cruzeiro seven days later and clinically scored for FMD generalized disease on 3, 7, 10 and 14 days post-challenge. Pre-immunization (day zero) and post-immunization/pre-challenge serum samples (day seven) were collected and serum virus neutralization (SVN) titers to FMDV A24 Cruzeiro determined using standard methods. Two independent trials using Adt. A24 were conducted. Cattle efficacy studies using AdFMD1, AdFMD2 and AdFM3 were similarly conducted.

In vitro tests

Infectious titer assay - tissue culture infectious dose 50 ($TCID_{50}$).

This assay measures both Adt.A24 infectious titer (e.g., FMDV capsid protein expression) and adenoviral vector titer. Briefly, permissive cells (293-ORF6) were plated in collagen-coated microtitration plates and incubated overnight. Ten-fold dilutions of Adt.A24 (four replicates per dilution) were inoculated onto the cells and removed after one hour incubation. Cells were re-fed with $ZnCl_2$ induction medium to stimulate protein expression and were allowed to incubate for 48 hours. Following incubation, cells were fixed with methanol and stained for FMDV capsid expression with a mouse monoclonal anti-FMD antibody [7] followed by a goat anti-mouse Cy3 conjugate, or stained for adenovirus protein expression using a FITC conjugated goat anti-hexon polyclonal antibody. Plates were read for specific fluorescence and the Spearman-Kärber tissue culture infectious dose 50 ($TCID_{50}$)/ml titers were calculated for FMDV capsid and adenovirus. For intra-assay variability, five replicates of the reference virus were assayed in a single experiment. For inter-assay variability, six assays were run over a two month time period.

Physiochemical assay - particle unit (PU) quantification

The concentration of adenovector vaccine particle units (PU)/ml was measured by anion exchange (POROS® D50 resin) HPLC (Agilent with Chemstation software) using UV detection (260 nm). The PU/ml concentration was determined by interpolation of the peak area of the test sample against a standard reference curve. Included in every test run was a continuing suitability sample to verify absence of drift in the test run.

Immunochemical tests

Fluorescent Focus-Forming Unit (FFU) Assay

To determine the adenoviral infectious titer, 293-ORF6 indicator cells were infected with various dilutions of test article and adenoviral DNA-binding protein was detected 24 hours after infection by immunocytochemistry. Fluorescent Focus Forming Units (FFU)/mL were determined by counting positive cells under fluorescent microscopy and applying a dilution factor and a microscope constant.

Whole Cell ELISA - Tissue Culture Expression Dose 50 ($TCED_{50}$) Assay

This assay measures FMDV infectious titer (e.g., FMDV capsid protein expression) in adenovirus replication non-permissive cells. Briefly, 293 cells were plated in collagen-coated 96-well plates and incubated overnight. Two-fold dilutions (1:400 to 1:409,600) of AdFMD1, AdFMD2, or AdFMD3 vaccine virus (four replicates per dilution) were inoculated onto the cells and removed after 24 hours incubation. Cells were fixed with cold methanol (-20° C), incubated for one hour with blocking buffer (PBS + 5% non-fat milk + 0.05% Triton-X 100), washed (PBS + 0.05% Triton X-100), incubated with a mouse anti-FMDV monoclonal antibody [7] followed by incubation with a goat anti-mouse HRP conjugate and subsequent substrate development. Positive control wells contained a known concentration of binary ethylinamine (BEI) inactivated FMDV A24 Cruzerio. Plates were read at 450 nm and the specific optical density in each well was transformed into a categorical variable based on a cut-off value equivalent to twice the background mean. The estimated FMDV infectious titer, expressed as $TCED_{50}$/ml, was calculated by applying the Spearman-Kärber formula and its equivalence in PU established according to the previous determined PU/ml titer of the AdFMD sample.

Western Blot Assay

An in vitro assay was developed to monitor Adt.A24 capsid protein expression in adenovirus replication non-permissive cells. Briefly, HEK-293 cells were infected with Adt.A24 vector, cell monolayers were harvested and dilutions of the cell lysate (neat, 1/16, 1/32) were separated by SDS-PAGE, and transferred to PVDF membrane. Membranes were probed using a monoclonal anti-FMDV antibody (F1412SA) specific for VP0 (p36-38) capsid precursor and the VP2 fragment (p26-28) of VP2 [7].

Results

The feasibility of developing an in vitro serology-based release test as an alternative to the current in vivo lot release test requiring FMDV challenge was initially investigated. Cattle were immunized with Adt.A24 and individual post-vaccination/pre-challenge SVN titers (day seven post-vaccination) were determined (positive titer = SVN >0.6 $\log_{10}$). Results were compared to clinical disease outcome (protected = no pedal lesions; susceptible = one or more pedal lesions) in each vaccinate following IDL challenge at one week post-vaccination.

Results from two independent Adt.A24 vaccine efficacy trials show that approximately 76% (19/25) of immunized cattle with negative SVN titers at time of challenge were subsequently protected against FMD generalized disease (Table 1). Similar percentages of SVN negative, protected cattle were also observed using other AdFMD vaccine candidates (data not shown).

An Adt.A24 modified-live virus infectivity assay was developed to measure FMDV infectious (protein expression) titer and adenovirus infectious titer and then evaluated for assay performance in a series of pilot experiments. Results show that Adt.A24 working reference FMDV infectious titers ranged from 6.50 – 7.00 $TCID_{50}$ and the adenovirus infectious titers ranged from 7.25 to 7.75 $TCID_{50}$ with low intra-assay variability (Table 2). Results further demonstrate low inter-assay variability for both Adt.A24 FMD infectious titer and adenovirus infectious titer (Table 3). Adt.A24 working reference FMDV titers ranged from 6.50 to 7.25 $TCID_{50}$ and adenovirus titers ranged from 7.00 to 7.75 $TCID_{50}$. Results for assay linearity show that both FMDV titers and adenovirus titers decreased with each two-fold sample dilution (Table 4). Collectively, results from these three Adt.A24 infectivity assay experiments indicate that the mean adenoviral infectious titer is approximately 0.8 log10 higher than the mean FMDV infectious titer.

An in vitro, physiochemical anion-exchange HPLC (AE-HPLC) assay was developed to quantify Adt.A24 adenoviral particle concentration. Results from two independent test runs on five different run dates in two different laboratories demonstrate that the HPLC assay provides a very precise measure of particle concentration (Table 5).

A series of three, independent immunochemical tests were also developed and evaluated for potential use as vaccine in-process and/or serial lot release tests. Adt.A24 and AdFMD vaccines with an established level of in vivo efficacy in the target host were used for assay development.

The first test, the fluorescent focus-forming unit (FFU) assay, was designed to measure adenovirus DNA-binding protein expression following Adt.A24 infection in a replication permissive cell line. Results demonstrate a highly consistent PU: FFU ratio using three different Adt.A24 experimental lots (Table 6).

The second in vitro test is based on a whole cell ELISA principal, in which the FMD infectious titer (expressed as $TCED_{50}$) was calculated based on expression of the FMDV VP0 capsid precursor and VP2 processed capsid following AdFMD vaccine virus infection in a replication non-permissive cell line. Results demonstrate a highly consistent $TCED_{50}$ titer and associated PU:$TCED_{50}$ ratio using three different AdFMD vaccine constructs (Table 6).

A Western blot assay was evaluated as a third immunochemical assay and was based on semi-quantitative measurement of Adt.A24 FMDV capsid expression using serial dilutions of a Adt.A24 infected cell lysate prepared from a replication-permissive cell line. Results demonstrate a relatively consistent pattern of FMDV A24 VP0 and VP2 capsid expression across three dilutions of cell lysate prepared from three different lots of Adt.A24 vaccine (Fig. 1).

Table 1: Day seven post-vaccination SVN titers in Adt.A24 vaccinated cattle and protection outcome following FMDV A24 Cruzerio direct challenge at one week post-vaccination.

Study	Group Size (N)	# SVN Negative [a]	# SVN Negative/Protected
1	30	20	14
2	10	5	5
Total	40	25	19

[a] SVN titer ≤ 0.6 $\log_{10}$

Table 2: In Vitro Adt.A24 Infectivity Assay – Intra-assay Variability.

Working Reference Replicate	FMD Titer ($TCID_{50}$)	Adenovirus Titer ($TCID_{50}$)
1	6.75	7.25
2	6.50	7.75
3	7.00	7.50
4	6.75	7.75
5	6.50	7.25
Mean	6.70	7.50
Standard Deviation	0.21	0.25

Table 3: In Vitro Adt.A24 Infectivity Assay – Inter-assay Variability.

Working Reference Replicate	Assay #	FMD Titer ($TCID_{50}$)	Adenovirus Titer ($TCID_{50}$)
1	1	6.75	7.00
2		7.00	7.25
1	2	7.00	7.25
1	3	6.75	7.25
2		6.50	7.75
3		7.00	7.50
4		6.75	7.75
5		6.50	7.25
1	4	7.25	7.25
1	5	6.50	7.50
2		6.75	7.75
1	6	7.00	7.50
Mean		6.81	7.42
Standard Deviation		0.24	0.25

Table 4: In Vitro Adt.A24 Infectivity Assay – Linearity.

Working Reference Dilution	FMD Titer ($TCID_{50}$)	Adenovirus Titer ($TCID_{50}$)
Neat	7.00	7.50
1:5	6.00	7.00
1:10	5.75	6.25
1:20	5.50	6.50
1:40	5.50	6.00

Table 5: In Vitro Adt.A24 Physiochemical Assay – AE-HPLC Quantification of Adenovirus Particles.

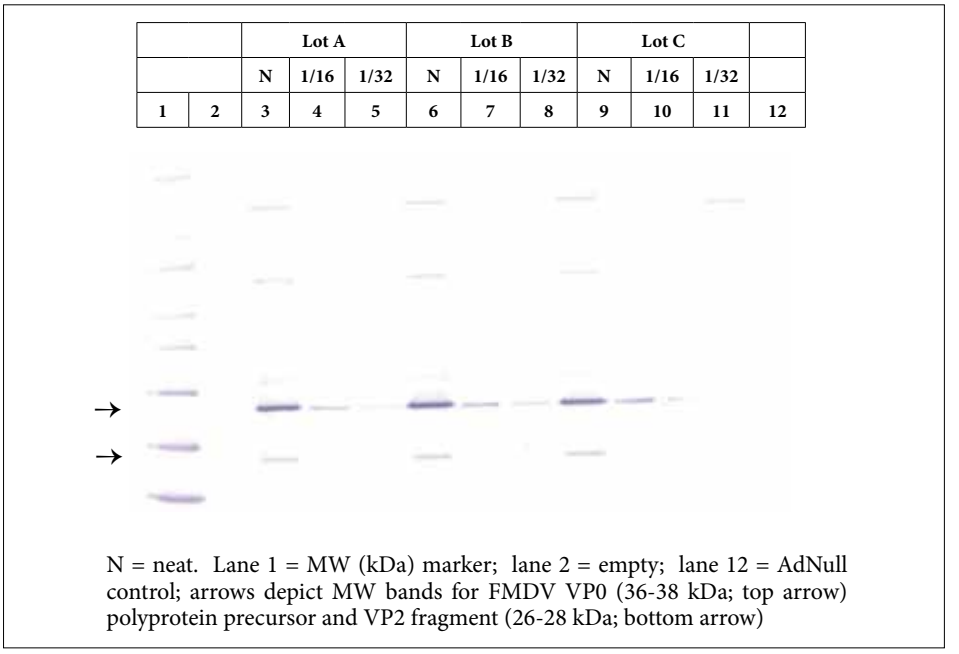
Run Date	Laboratory	Run 1[a]	Run 2[a]	Average[a]
Month 1/Day 1	A	2.27	2.26	2.27
Month 1/Day 2	A	2.35	2.35	2.35
Month 1/Day 3	A	2.37	2.37	2.37
Month 2/Day 1	B	2.48	2.51	2.50
Month 2/Day 2	A	2.37	2.34	2.36
Month 3/Day 1	B	2.28	2.37	2.33
Month 3/Day 2	A	2.36	2.33	2.35
			Overall Mean	2.36
			Standard Deviation	0.069
			% Correlation of Variance	2.94%
			Maximum Fold Difference (Max/Min)	1.36

[a] Particle unit per ml (PU/ml) x 10^{10}

Table 6: In Vitro Infectivity Assays – FFU and $TCED_{50}$.

Vaccine	Lot #[a]	PU/ml[b]	FFU/ml[c]	PU:FFU	Pass/ Fail[d]	$TCED_{50}$/ml[e]	PU:$TCED_{50}$[e]
Adt.A24	1	8.0	5.5	14.5	Pass	Not tested	Not tested
Adt.A24	2	12.0	8.0	15.0	Pass		
Adt.A24	3	2.5	1.5	16.7	Pass		
AdFMD1	1	69.9	48.1	14.5	Pass	5.8	6.1
AdFMD2	1	151.6	209	7.3	Pass	6.5	5.7
AdFMD3	1	125.4	95.8	13.1	Pass	5.8	6.3

[a] All lots tested demonstrated >80% efficacy in the cattle IDL challenge model.
[b] PU/ml x 10^{10}
[c] FFU/ml x 10^{9}
[d] Based on PU:FFU specification of ≤30:1.
[e] $\log_{10}$



N = neat. Lane 1 = MW (kDa) marker; lane 2 = empty; lane 12 = AdNull control; arrows depict MW bands for FMDV VP0 (36-38 kDa; top arrow) polyprotein precursor and VP2 fragment (26-28 kDa; bottom arrow)

Fig. 1: In Vitro Adt.A24 Immunochemical Assay – FMDV A24 VP0 and VP2 Capsid Expression in Adt.A24 Infected Cell Lyastes.

Discussion

The development of new veterinary vaccines based on recombinant technologies which also address the 3Rs (reduce, refine, replace) animal concept offers an extraordinary opportunity for industry and regulatory agencies to collaborate in issuance of a new set of guidelines for veterinary molecular vaccine lot release testing. The capacity to manufacture recombinant veterinary vaccines under cGMP within a strong QA environment strengthens the concept that multiple, in vitro assays can be used together to demonstrate a high level of vaccine lot-to-lot consistency, and thus replace the traditional paradigm of lot release based on a single animal test in the target host or in a surrogate animal model. In the case of the AdFMD vaccine platform for the development of FMD emergency vaccines, this could translate into regulatory acceptance of in vitro release test(s) for vaccine serials that would obviate the current requirement for lot release testing in cattle requiring virulent FMDV challenge (e.g., PD_{50} and PG tests).

One of the critical operational requirements for the USA National Veterinary Stockpile is that emergency use FMD vaccines confer early onset of protection in a significant percentage of treated animals. There is general acceptance of a serology-based (ELISA) readout for inactivated FMD vaccines in South America as an in vitro alternative to the PG potency test [8]. Similarly, replacement of the test in the Ph. Eur. by indirect potency assessment of inactivated FMD vaccines using SVN

and ELISA formats is scientifically achievable [9]. To determine the feasibility of an indirect serology assay for AdFMD vaccine lot release, post-vaccination/pre-challenge FMDV A24-specific SVN responses in AdA24 vaccinated cattle were determined and analyzed in the context of the FMDV experimental challenge outcome (protected or susceptible). Results clearly demonstrate that an indirect, SVN-based serology test for AdA24 vaccine lot release is not feasible using serum samples collected at one week post-vaccination. This result is not unexpected, since current inactivated FMD vaccines also induce non-detectable or highly variable SVN titers at one week post-vaccination. For this reason, blood samples used for indirect serology testing are typically collected at a later post-vaccination/pre-challenge time points (e.g. 21 [9] or 60 [8] days post-vaccination).

Since the replication-deficient AdFMD platform is considered a modified-live vaccine virus, an infectious titer assay may be useful as a release assay for potency. In the present study, an Adt.A24 infectious titer (FMDV A24-specific protein expression) assay was developed. Although full validation studies are still required, pre-validation assay results for assay variability and linearity are encouraging. Based on adenovector molecular design for FMD vaccine candidates, in theory the Adt.A24 FMDV infectious titer and the adenovirus infectious titer should be identical. However, infectious titer values are known to be highly dependent on the assay test conditions, including the relative levels of target antigen expression and the affinity of the immunological reagents. Results show that the FMDV infectious (expression) titer is consistently at least 0.5 to 0.8 log10 lower than the adenovirus infectious titer. The FMDV infectious titer assay uses a non-conjugated mouse monoclonal antibody specific for a linear epitope on the FMDV VP0 polyprotein and VP2 processed capsid. In contrast, the adenovirus infectious titer assay employs a directly conjugated anti-adenovirus hexon polyclonal antibody. Results to date suggest that the FMDV infectious titer assay ($TCID_{50}$) is less sensitive than the adenovirus infectious titer method. Future studies using anti-FMDV A24 Cruzeiro polyclonal antibody will be used to try and increase FMDV infectious titer assay sensitivity.

Use of in vitro physiochemical methods for determination of adenovirus particle concentration and vaccine dosing in human is supported by theUnited States Food and Drug Administration (FDA) [10]. In particular, AE-HPLC methods have been well described [11, 12] and can provide a highly precise measure of particle concentration [13]. Results from the present study demonstrate the feasibility of using an AE-HPLC relative method assay to quantify AdFMD adenoviral particles. During vaccine manufacturing, AE-HPLC could be used as an in-process assay to monitor AdFMD vaccine yields. An additional potential application for the AE-HPLC is preparation of an AdFMD particle concentration standard curve using a working reference with a defined minimum protective dose from a target host immunogenicity study. Assuming the vaccine serial PU dilution series is parallel to the working reference internal standard curve, the vaccine serial particle concentration could be calculated by interpolation from the internal standard curve. Preliminary results also indicate that the PU assay can be used for assessment of long-term vaccine stability (data not shown).

To demonstrate that adenovector virus physical particles have biological activity, an infectious titer assay is often employed. In addition to the FMDV $TCID_{50}$ assay described above, two additional infectious titer assays were developed. The FFU and $TCED_{50}$ assays, based on the detection of an adenovirus DNA binding protein or and FMDV capsid protein, respectively, were also used to generate adenovirus and FMDV infectious titer values. Data provided herein are consistent with the fact that the infectious titer concentrations are almost always lower than the PU concentration. One possible explanation for this observation is assay efficiency, since most adenovector particles do not reach a target cell in the assay [14]. In this regard, a PU to infectious titer ratio (e.g., PU/FFU, PU/$TCED_{50}$) could be set and used as a pass/fail specification for vaccine release as well as used to monitor vaccine serial lot-to-lot consistency. For example, FDA regulated adenovector vaccine must have a PU/IU ratio less than 30:1.

A semi-quantitative Western blot assay was also developed to track FMDV VP0 polyprotein precursor and processed VP2 capsid expression. Tracking VP0 expression and processing is important and has been previously shown to be required for AdFMD vaccine efficacy [15]. Similar to the other infectious, immunochemical-based assays developed for AdFMD vaccine vectors, the Western blot assay offers the potential for use in vaccine lot release, monitoring manufacturing consistency, and long-term vaccine stability studies.

Conclusions

The development of multiple in vitro tests for next generation, AdFMD-based molecular vaccines that may eliminate the current regulatory requirement for FMD vaccine release testing in FMDV challenged livestock was investigated. This described approach is based on recognition of a fundamental shift from traditional vaccine lot uniqueness toward a new paradigm of molecular vaccine lot-to-lot consistency. Results demonstrate that it should be possible to set a vaccine product profile for each AdFMD vaccine that is underpinned by an established set of infectivity, physiochemical and immunochemical tests. Collectively, these in vitro tests can be used to ensure the each vaccine lot released is similar to the AdFMD reference vaccine of proven clinical safety and efficacy.

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Potential Application of the Consistency Approach for Vaccine Potency Testing

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Key words: consistency approach; vaccine; potency

Abstract: The Consistency Approach offers the possibility of reducing the number of animals used for a potency test. However, it is critical to assess the effect that such reduction may have on assay performance. Consistency of production, sometimes referred to as consistency of manufacture or manufacturing, is an old concept implicit in regulation, which aims to ensure the uninterrupted release of safe and effective products. Consistency of manufacture can be described in terms of process capability, or the ability of a process to produce output within specification limits. For example, the standard method for potency testing of inactivated rabies vaccines is a multiple-dilution vaccination challenge test in mice that gives a quantitative, although highly variable estimate. On the other hand, a single-dilution test that does not give a quantitative estimate, but rather shows if the vaccine meets the specification has been proposed. This simplified test can lead to a considerable reduction in the number of animals used. However, traditional indices of process capability assume that the output population (potency values) is normally distributed, which clearly is not the case for the simplified approach. Appropriate computation of capability indices for the latter case will require special statistical considerations.

Consistency of production, sometimes referred to as consistency of manufacture or manufacturing, is an old concept implicit in vaccine regulation, which aims to ensure the uninterrupted release of safe and effective products [1-3]. In addition to this regulatory practice, some animal bioassays, such as the potency measurement in the final product, were given extra weight for many years, in the hope that the results of these tests would unequivocally reflect product efficacy in the target species [4]. Due to the absence or scarcity of suitable in-process tests for vaccine characterization, this reliance on final lot testing was excessive, and somewhat equivalent to an attempt to re-licensing the vaccine when each new batch was up for release.

The predictive capacity of the selected bioassay was also expected to be adequate for use in licensing new products on the basis of this final product testing, with minimal additional clinical studies.

However, a better understanding of the limitations of animal testing in predicting vaccine efficacy, improvements in manufacturing and testing procedures, adoption of comprehensive quality systems, and concerns regarding the expansive and sometimes not-well justified use of animals, has forced both manufacturers and regulatory authorities to re-examine the value of animal tests in vaccine characterization [4].

In May of 2006, the European Centre for Validation of Alternative Methods (ECVAM) convened a workshop in Ispra, Italy on the Consistency Approach and its potential to reduce animal tests in the quality control of vaccines [5]. This quality control philosophy, recently expanded in a second workshop in Brussels, Belgium [6], offers the opportunity to use perfected analytical tools, in the context of fully functional quality systems, to guarantee consistency in both production and testing. The Consistency Approach assures that any batch manufactured post-licensure possesses similar characteristics to those batches shown, as part of the licensure or registration process, to be safe and effective in the target population.

Adoption of the Consistency Approach in general may lead to a reduction in animal use: a narrow set of animal tests performed on each final batch, with potentially limited predictive power with regards to vaccine behavior on the target population, may be replaced by a battery of meaningful tests with enhanced capacity to measure equivalence with batches of proven safety and efficacy.

Application of the Consistency Approach to a particular test design may also be conducive to a reduction or refinement of animal use. For example, once it has been established by testing that a high quality product is consistently manufactured, and that a required animal test consistently displays desirable characteristics [e.g. linearity of a response relative to dose), a test entailing the use of many animals or relying on end points that may cause more than transient pain or distress may be replaced by a simplified and more humane method. This could be done without compromising the information that the original procedure provides. For instance, the early assay recommended by the Expert Committee on Biological Standardization of the WHO for the potency testing of diphtheria toxoid [7] consisted of an active protection test that required more than 100 guinea pigs per test. Although the use of such design is still recommended for new vaccines registration and variations that involve critical manufacturing changes, modifications where the assessment of protection circumvents lethal challenge and is replaced by non-lethal versions or serology (Refinement) have been introduced. Moreover, the recommended multi-dose, parallel line potency estimation has been replaced by a single-point limit test for routine release purposes (Reduction).

In the field of biologics for veterinary use, an important reduction in the use of mice might be achieved through the introduction of alternative methods for the potency testing of inactivated rabies vaccines, in the context of the Consistency Approach.

Variants of a multi-dilution vaccination challenge test in mice, known as the NIH test, are used by manufacturers and regulatory authorities to measure vaccine potency, in relative terms to a reference vaccine, before the release of each batch or serial [8-9]. The NIH test has a number of disadvantages, including the fact that it involves a lethal challenge, consumes a large number of animals, is highly variable, and requires the handling of live rabies virus. The need for a more humane and precise potency test has been evident for many years.

In 1982 Aubert and Blancou [10] proposed a simplification of the NIH potency test that involves the use of a single dilution of the test and of the reference vaccines, once experience has been gained with the performance of the multi-dilution design in a given laboratory. Inactivated rabies vaccines for veterinary use can be screened for potency using the simplified test. They would either be subject to a subsequent multi-dilution test, if they fail to meet the potency requirement, or accepted without further testing, if the vaccine shows a potency well above the specification. Although this test design does not give a precise value of potency, but rather is limited to showing if a vaccine satisfies the minimal requirement, its adoption can lead to a considerable reduction in the number of animals used.

France implemented this approach in the late 1990's, and an ECVAM workshop recommended in 2003 its extended introduction for the batch potency testing of veterinary rabies vaccines by National Control Authorities [11]. As a matter of fact, although European Pharmacopeia Monograph 07/2010:0451 for Rabies Vaccine (Inactivated) for Veterinary Use [12] describes a variant of the NIH test to estimate the potency of a batch, it includes not only the possibility of using an alternative, non-lethal endpoint to assess protection in the regular potency test, but also an outline of a single-point serological batch potency test to be used under certain circumstances.

When a test is targeted for simplification in the spirit of the 3Rs, attention should be paid to its performance as an analytical tool. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has defined validation of a test as the assessment of its reliability and relevance for its intended purpose [13]. Through validation a test's intra- and inter-laboratory reproducibility (reliability) and its ability to measure the effect it is designed or proposed for (relevance), are determined.

The contribution of a result to the establishment of consistency, in the context of the Consistency Approach, should be examined in the light of two elements: the consistency of the result (e.g. potency) with the specification, and the consistency of outcomes obtained in different lots over time. The specification set for the potency or efficay-indicating parameter takes into account not only the assay reliability, but also the predictive power of the measurand and the outcomes of the test on lots used in clinical studies performed in support of licensure (relevance). Consistency of manufacturing (process variability) and test reliability (assay variability) also have an impact on the marketing of lots that not only meet the specification, but do so consistently over time.

Validation protocols for refinement and replacement methods are more often concerned with establishing the relevance of the alternative chosen (e.g. serology

as a substitute for active protection). When reduction in animal number is involved, however, maintenance of assay reliability is the challenge to confront. The adoption of a single-dilution testing strategy for potency testing of inactivated veterinary rabies vaccines constitutes an example of the latter situation.

Control charts are used by manufacturers to monitor if a process is in control, with variation coming only from sources common to the process (including testing). The most typical kind of chart used to track and trend potency results is a Levey-Jennings chart (similar to Shewart's individual controls chart), which originated in clinical laboratory practice [14]. In this chart, run or test number is plotted on the X-axis. As in any other control chart, a Levy-Jennings chart contains a center line that represents the mean value for the in-control process. Two other horizontal lines, called the upper control limit (UCL) and the lower control limit (LCL), are shown on each side of the mean. These control limits are chosen so that almost all of the data points will fall within them, as long as the process remains in control. To draw the control limits, one, two and sometimes three long-term (i.e., population) estimates of σ (standard deviation) are used [15].

Process Capability studies are useful for describing how consistent a process is in terms of making a product that meets a specification [16]. Process capability indices use both the process variability and the test specifications to make a statement in this regard. A Capability Index, lower, estimates process capability for specifications that consist of a lower limit only (for example, potency for rabies vaccine). However, calculation of this index assumes that the process output measurand is approximately normally distributed. If that is not the case, capability analysis demands the use of a Box-Cox transformation of the process output [17], or the use of capability indices applicable to non-normal distributions [18]. In this regard, the potency outcomes from a simplified, single-dilution test of an inactivated rabies vaccine for veterinary use pose a challenge for use in process control: since they are expressed as pass or fail, these potency outcomes constitute an example of a nominal scale, which is obviously not normally distributed.

In conclusion, some Reduction strategies may diminish the information a test can provide, due to a change in the level of measurement [19] of the outcome (e.g., from ratio to nominal). This reduction in information may require an expansion of the expression of potency to a combination of efficay-indicating characteristics, each of which may have a weight based on relevance and reliability assigned.

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Appropriateness of in Vitro Potency Tests as a Measure of Vaccine or Reference Stability

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Key words: vaccine, potency, stability

Abstract: A proposed definition of a stability indicating assay is "a validated quantitative analytical procedure that can detect changes over time in the pertinent properties of the product" (Federal Register/Vol. 75 No. 180/ Friday, September 17, 2010 / Proposed Rules). In vaccines intended for veterinary usage, the potency assay has traditionally been used as a measure of stability. Some potency assays may be acceptable as stability indicating assays, whereas other potency assay will not meet the criteria for stability indicating assays. For example, an ELISA potency test may or may not detect degradation products depending on the specificity of the antisera. With time, the ELISA may overestimate the antigen as partial degradation occurs or if an aggregated or particulate antigen dissociates. Specific assays parameters and attributes that are required for a potency assay to be indicative of serial or reference stability are discussed.

Introduction

Testing of veterinary vaccines and vaccine references for stability and potency utilize both in vivo and in vitro testing. In order to reduce in vivo testing, in vitro tests need to be developed for potency and stability testing of vaccines and vaccine references. In vitro stability testing is readily applied to a more purified reference or vaccine. An in vitro approach is more easily achieved with DNA vaccines and vaccines made from pathogens where the predominant protective antigen has been identified and characterized. The in vitro approach for stability is difficult for pathogens that have multiple antigens that contribute to the protective immune response.

Stability for veterinary vaccines has traditionally been tested using the potency assay. However, in the United States, a set of revised rules for stability testing of veterinary vaccines has been proposed [1]. The proposed rules indicate that a stability indicating assay will be required. The stability indicating assay will be "a validated quantitative analytical procedure that can detect changes over time in the pertinent properties of the product" [1].

The proposed changes to stability testing in the United States represent a challenge to manufacturers of veterinary vaccines. Stability programs will need to be implemented that preferably will not increase in vivo testing and will be cost effective. If potency assays are developed that have stability indicating properties, this will reduce the number of quantitative assays that need to be developed and validated.

Stability programs utilize protocols with defined storage conditions, defined intervals of testing, a defined number of lots and samples per lot, and the appropriate assays that detect changes in identity, purity and potency.

Identity assays for stability programs demonstrate that the antigen has not degraded; this approach is difficult if the degradation product is not stable or if it is present in a complex matrix. In a complex matrix, identity assays for stability may utilize a specific antibody in Enzyme-Linked Immunosorbent Assays (ELISA) or Western blots.

Purity in stability programs may be indicated by changes in color, pH, or precipitates, or the appearance of visible microbial growth. In veterinary vaccines, this does not typically include re-testing for purity.

A number of types of potency assays are used in the veterinary vaccine industry. For live vaccines, potency is determined by viability or infectivity that may be measured by a number of different types of assays that include determination of colony forming units, infectious doses, plaque forming units, and viability stains. These types of potency assays will not be discussed in this text, as these assays are readily adapted as stability indicating assays.

Potency of inactivated vaccines is often determined by an in vivo immune response, ELISA or in vitro bioactivity. The following text discusses only the use of in vitro assays and the appropriateness of using ELISA and in vitro bioactivity potency assays as stability indicating assays.

Materials and Methods

New Castle Disease Virus hemagglutinin ELISA

Newcastle Disease virus (NDV) hemagglutinin protein (HN) was produced in tobacco plant cells [2]. The HN was inactivated by heating at 90° C for 30 minutes.

Potency was determined by ELISA. The ELISA assay was performed as described previously [2] using two formats. In ELISA #1 and ELISA #2, modifications of the procedure for antibody coating and detector antibody were used. Bioactivity of HN was determined by HA assay [2]. Truncated HN antigen was detected using SDS PAGE and Western blots.

SDS PAGE and Western blots

The Novex X-cell II mini-gel apparatus was used for gel electrophoresis. The SDS PAGE gels were run at a constant 200 Volts for 40 minutes using two gels per apparatus. Invitrogen gels (4-12% gradient, 1.0 mm, 12 wells per gel) and Invitrogen running buffers, sample buffers, and molecular weight markers were used. Gels were stained for 20 minutes with Coomassie-methanol-acetic acid stain and destained using a methanol-acetic acid solution for four hours with one change of destain. An image of the destained gel was captured using GeneSnap software using optimal focus and lens distance. The GeneSnap image was directly transferred to GeneTools. For band analysis, Rolling Disk integration was used to define the background threshold, and automatic background reduction was used.

For Western blots, proteins were transferred from the gel to a nitrocellulose membrane. Invitrogen NuPage transfer buffer was used for the transfer for 80 minutes at a constant 30 Volts. After transfer, membranes were rinsed with deionized water, dried and stored refrigerated for 12 to 16 hours. Membranes were equilibrated to room temperature and rehydrated for five minutes with deionized water. Membranes were incubated with antiserum, followed by incubation with a detector antibody. Invitrogen's Western Breeze Chromogen Detection Kit was used.

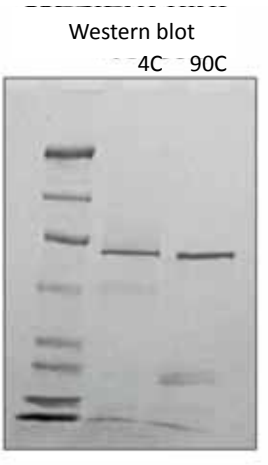
Swine influenza HA bioactivity

Swine Influenza Virus (SIV) strain (A/IOWA-73) was grown in Maden Darby Canine Kidney cells. A stock of virus was produced and assayed for infectivity and HA activity. The stock had 8.00 $CCID_{50}$/mL and 2560 HA units/mL. A portion of the stock was inactivated using binary ethyleneimine (BEI). The untreated and BEI-inactivated stocks were aliquoted, and samples were stored at 4, 23, 37, and 41° C. Samples were collected at 0, 7, 14 days, and 1, 3, 3.5, and 6 months in storage. Bioactivity was assessed by HA assay; the wobble in the HA assay is ± one two-fold dilution.

Results

New Castle Disease Virus hemagglutinin ELISA

In order for an ELISA to be stability indicating, the ELISA should not detect denatured or inactive antigen. Denatured HN does not hemagglutinate or induce a protective immune response upon vaccination. The ELISA results for NDV HN in Figure 1 indicate that the expected amount of HN was detected only when native HN was tested. The HN that was subjected to heat, inactivating the HA bioactivity, was not detected in the ELISA. Denaturation of HN by heating did not degrade the protein as observed in the Western blot in Figure 1. The Western blot demonstrates that the HN was not degraded based on the size and amount of HN observed, yet the denatured HN was not detected in the ELISA. The HN ELISA with supporting data from the Western blot exemplify desired features of stability indicating assays.



ELISA

Expected ng/ml	Native form ng/ml 4° C	Denatured ng/ml (90° C 30 min)
10.00	10.01	0.00
8.00	7.97	0.00
6.00	5.87	0.00
3.75	3.87	0.00
2.00	1.94	0.00
.75	0.65	0.00
0.25	0.24	0.02

Fig. 1: Effect of denaturation of Newcastle Disease virus HN protein on ELISA quantitation.

The native form of HN has HA activity and elicits a protective immune response in chickens, while a truncated form of HN has reduced HA titer. The two forms of the HN antigen were purified and assayed with two ELISA assays. The ELISA #1 detected both forms of the HN antigen to an equal extent, while ELISA #2 differentiated between the native and truncated forms of HN (Fig. 2). ELISA #1 would not be acceptable as a stability indicating assay, while ELISA #2 appears to be stability indicating.

SDS PAGE and Western blots

In order to use SDS PAGE gels and Western blots as part of a stability program, the gels and blots must be run in a reproducible manner. To assess the reproducibility of gels and Western blots, the amount of protein within a band was quantitated. Numerous parameters for running gels and blots, staining, destaining, and gel imaging were investigated to determine the effects of varying the parameters on gel reproducibility.

Using the methods described on the previous page, the following parameters were defined and held constant in order for gels and blots to be reproducible: acrylamide percentage and gradient type, the sample volume, run time and voltage, transfer procedure and membrane type, blocker and blocking procedure, staining and destaining procedure.

The following imaging parameters needed to be defined and remain constant in order for the quantitation to be reproducible: focus, zoom, iris setting, intensity of light, and the analysis program. The area of analysis, (i.e., the area defined around the band) was not a critical parameter.

The methods described on the previous page were used to run SDS PAGE and Western blots in order to assess the reproducibility of the methods using quantitation. The amount of protein was calculated using standards run on the

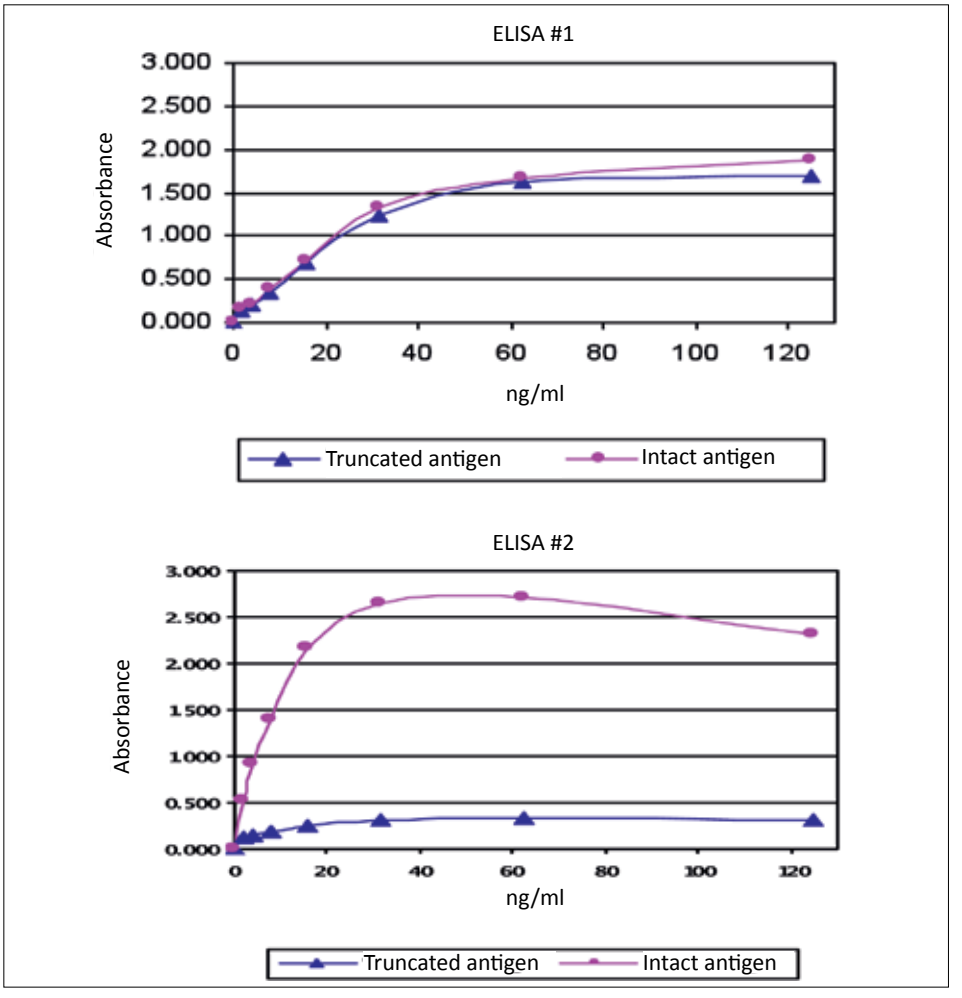
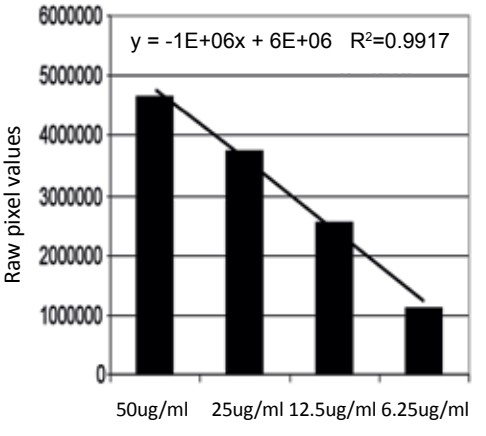


Fig. 2: Comparison of detection of intact and truncated HN protein of NDV using modified ELISA.

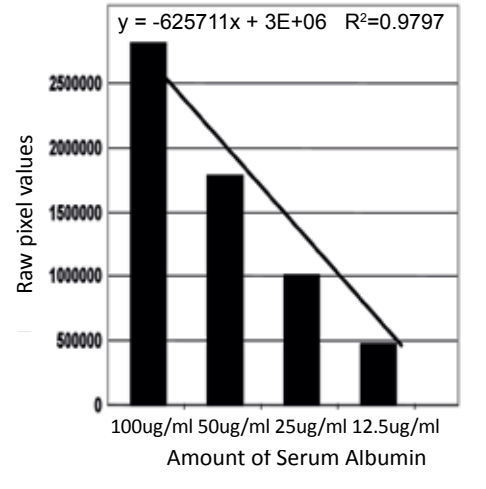
same gel or blot, the pixel values averaged, and the coefficient of variation (CV) was determined. Figure 3 demonstrates the intra-gel variation for quantitation of IgG using SDS PAGE, and Figure 4 demonstrates inter-gel variation for quantitation of bovine serum albumin (BSA) using SDS PAGE. In Figures 3 and 4, the CVs were less than 10% for IgG and BSA. The samples run on the gels were purified proteins that gave sharp bands. When analyzing bands on SDS PAGE using different proteins that were in a complex matrix, the CVs were typically under 15% (data not shown). When analyzing bands on Western blots, the CVs were typically under 20%, and chemilluminescence detection was more reproducible than colorimetric assays (data not shown).



% CV (within gel)

IgG (ug/mL)	Pierce Blocker	Invitrogen Blocker
50	4	7
25	4	4
12.5	3	5
12.25	6	8

Fig. 3: Intra-gel variation of IgG quantitation using SDS PAGE.



BSA (ug/mL)	% CV (between gels) N=4
100	4.8
50	4.1
25	3.4
12.5	5.6

Fig. 4: Inter-gel variation of protein quantitation using SDS PAGE.

Swine Influenza HA Bioactivity

Vaccine stability may be assessed using an in vitro bioactivity assay. The stability of the Hemagglutinin protein (HP) of SIV was determined using HA assays. Figure 5 demonstrates the HA activity of untreated SIV under refrigerated storage and accelerated stability storage conditions. The HA activity was stable for six months under refrigeration or at room temperature, but bioactivity decreased at the higher temperatures used for accelerated stability conditions.

Figure 6 demonstrates that inactivated SIV was stable for six months under refrigeration; activity decreased by six months for SIV stored at room temperature. Bioactivity decreased at the higher temperatures used for accelerated stability conditions.

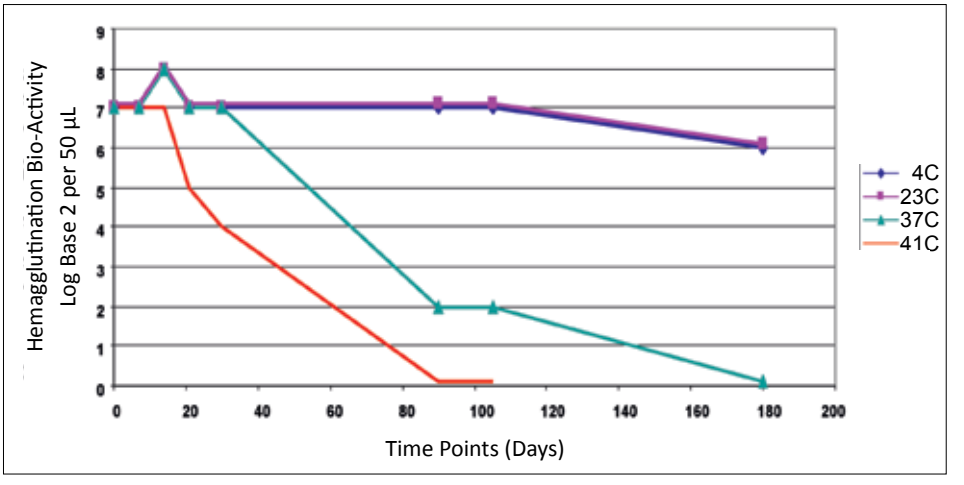


Fig. 5: Stability of swine influenza potency determined by HA bioactivity of native virus.

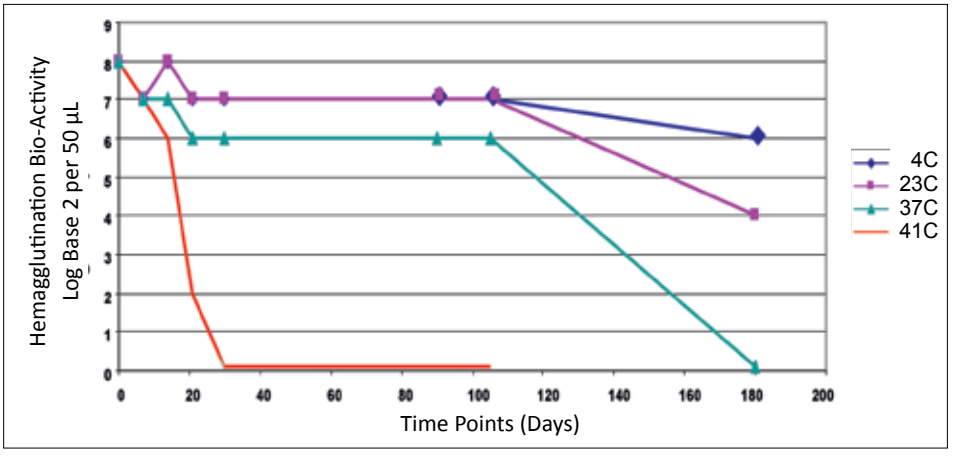


Fig. 6: Stability of swine influenza potency determined by HA bioactivity of inactivated virus.

Discussion

Stability for veterinary vaccines has traditionally been determined using the potency assay. In the near future, stability may need to be determined using a validated quantitative stability indicating assay. The development of a potency assay that has stability indicating properties would be beneficial in that it would reduce the number of assays for a specific vaccine that need to be developed and validated. The potency assay may need to be coupled with another type of assay, such as Western blots, in order to demonstrate stability of the vaccine or reference.

For antigens such as HN of NDV or HP of SIV, in vitro assays for potency and stability are more readily developed due to the identification and characterization of protective antigens, the availability of a range of reagents for detection of the antigens, and the in vitro bioactivity of the protective antigens. Vaccines for diseases caused by pathogens that are not as well defined as NDV or SIV present more challenges for the development of in vitro assays. The development of in vitro stability indicating assays for references and vaccines will aid in reducing the use of in vivo assays by veterinary vaccine manufacturers.

Acknowledgements

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2 United States Patent Application Publication, No. US2006/0222664 A1, Oct 5, 2006.

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Workshop Summary

The participants of the meeting on **Potency Testing of Veterinary Vaccines: The Way From in Vivo to in Vitro** organized by the Paul-Ehrlich-Institut (PEI), the International Alliance for Biological Standardization (IABS) and the European Directorate for the Quality of Medicines & HealthCare (EDQM), held in Langen, Germany, 1-3 December 2010 summarize the meeting as follows:

Conclusions:

The purpose of the meeting was to present the current scientific status on the development and application of 3R alternatives (replacement, reduction, refinement) to the in vivo potency testing of vaccines for veterinary use to and by manufacturers, researchers, regulators and assessors.

The goal of the unified efforts is to reduce in vivo tests, which are historically developed and most of which are not validated according to the current state of the art. In case an in vivo test cannot be avoided, efforts should be made to reduce the number of animals and to minimize potentially induced pain and distress to the lowest level possible. Although the main focus of the meeting was on post licensing routine batch release testing, the alternative methods called for might be extended to other phases of product development.

The scientific data presented and discussed at the meeting provided a number of possible solutions to revise the currently used in vivo tests by direct replacement by an equivalent in vitro test or by introduction of consistency testing. The consistency approach is intended to include a set of biological and physico-chemical tests, performed as in process and final product control, which shall ensure that all produced batches are of the same quality as the batches proven to be safe and efficacious during licensing. This test panel needs to be defined product (group) by product (group) and has to reflect the specific manufacturing conditions.

It must be stressed in this context that the batch potency test is a manufacturing test to ensure consistent quality of final product batches and not an additional efficacy test.

The number of appropriate in vitro or consistency test systems currently approved or ready for approval is small. Further development is necessary.

Improvements in the quality and consistency of vaccine production are reached by the use of defined starting materials, standardised production steps and precise specifications for in process and final product control test results. This elaborated approach supports the replacement of the long ago established in vivo tests by in vitro tests of different nature (e.g. GMP, qualified production).

Wherever possible, the post licensing routine batch release testing should consist of an appropriate set of in vitro testing. In vivo tests should only be included when inevitable and should strictly follow the 3R principles.

In vivo potency tests described in the current legal provisions are often created on historical gained data and/or empirical experience. Although they hitherto have served the purpose well, many of these cannot be documented according to current requirements for test validations. Direct correlation between in vivo and in vitro tests is therefore often not possible.

Replacement of in vivo by in vitro testing faces two distinct scenarios:

- Replacement of in vivo testing by in vitro for well established products being on the market for a long time.
- Avoiding the use of in vivo testing and development of in vitro tests whenever possible.

The costs needed to establish in vitro tests should not block the research efforts, and investments into standardised reagents, reference preparations and scientific methods by the industrial, regulatory and scientific communities should be encouraged.

Roadblocks

- "Comfort" with current test—it seems to work well (vaccine failures are rare).
- Public health impacts—concerns over consequences that would be associated with release of sub-potent veterinary products. Current conventional products were licensed using the established in vivo test
- "Conventional wisdom": validation would require a new assay to be compared against host animal challenge (which is costly and includes the use of live animals)
- Regulators want a single format for all manufacturers
- Manufacturers at present tend not to share assay development progress with each other. Intentions to share some information were communicated during the meeting
- Unilateral acceptance by one regulatory body will not incentivize the industry—acceptance of a new approach must be global
- Current industrial and regulatory dogma calls for a single assay to determine product potency
- Manufacturers are reluctant to invest in an alternative test without assurance of regulatory acceptance
- Regulators are reluctant to assure acceptance in the absence of data.

Conclusion Summary

Replacing more in vitro tests by in vitro assays will require:

- A fresh approach to the way we think about implementation of a new potency test. The currently requested direct link between potency and efficacy needs replacement by consistency proof
- A close partnership between regulatory bodies and the regulated industry
- Consideration of assay sets for purposes of evaluating potency/quality/consistency
- Decisions about acceptance of alternatives and choice of alternatives to in vivo tests case by case (agent by agent)
- Consideration for the use of new and emerging technology.

Recommendations:

As the market for vaccines for veterinary use is a global one, requirements for testing should be harmonised: one world – one health. The following points would be of great help in this context to save human and financial resources as well as to avoid manifold suffering in animals:

- The development and international availability of standard methods and reference materials (test kits, reagents and reference sera) for the in vitro quality control of veterinary biologicals is urgently needed
- In collaborative jointly agreed work, all stakeholders should be willing to share their data
- New developed tests should immediately be subject to collaborative studies, involving manufacturers and Official Medicinal Control Laboratories (OMCLs) to speed up the implementation of these approaches in the legal provision. As a prerequisite for a collaborative study, pre-validations and feasibility assessments are needed
- The legal provisions should facilitate the replacement of in vivo tests by in vitro tests and/or consistency testing
- Regulators and assessors should take a proactive approach to encourage and accept tests or test panels to reduce, refine and replace the use of animals in quality control of veterinary biologicals
- Scientific advice how to proceed with the replacement of in vivo by in vitro testing should be provided by regulators and assessors. This replacement could follow a stepwise and pragmatic procedure: e.g. replacement of challenges by serology, introduction of in vitro tests as in process tests and after a sufficient number of batches tested in parallel (in vivo and in vitro) making a decision if the in vivo tests can be replaced by consistency testing
- The validated tests or test systems used for the final product testing of vaccines for veterinary use should justify compliance with the batches used for the safety and efficacy tests which are provided for licensing purpose
- Provided in vivo potency tests are regarded as un-waivable, clear indication has to be made where these tests need to be used, if any

- In most cases a direct relation between the immune response / immune mechanisms and the in vitro tests is not known. This fact should not hinder the replacement of in vivo tests by in vitro tests. Nevertheless, research that clarifies the relationship between immune response and the detection of parameters to be targets for in vitro tests needs further efforts and support
- A roadmap for the future steps to be undertaken with respect to already licensed and new vaccines should be established.

The possible scenario for a road map could be as follows:

- Strict time schedule, controlled by the coordinators
- Actors: - Regulators to harmonise requirements in the context of 3R - one world-one health
  - Industry to present applications for the replacement of in vivo tests by in vitro tests/consistency testing.
- Priorities: Antigens, where sufficient data are supposed to be already available:
  Clostridia
  Leptospira
  Rabies
  FMD
  Newcastle
- Coordinators: EDQM/ USDA/EMA
  ECVAM/NICCVAM
  IFAH global

Recommendation Summary

- Consideration for allowing assay panels to evaluate potency with appropriate quality indicators
- Enhance collaboration in an international dimension (e.g. VICH)
- Collaboration between regulators and manufacturers (e.g. EDQM, EPAA platform)
- **Creative thinking! The time is ripe for changes.**

Workshop Presentations not in This Publication

Session I

1 Report from IABS and OIE meeting in Buenos Aires February 2010. "Practical alternatives to reduce animal testing in quality control of veterinary biologicals in the Americas" Rodolfo Bellinzoni, Biogenesis Bago

2 Testing of Vaccines against Rabies: Replacement of challenge by serology: collaborative study. Catherine Milne, EDQM

3 Testing of Vaccines against Rabies: Replacement of challenge by in vitro methods: comparison of test methods (RFFIT serology and in vitro). Fabrizio de Mattia, Intervet/SPAH

Session II

4 An antibody ELISA for potency testing of furunculosis (Aeromonas salmonicida subsp. Salmonicida) vaccines for Atlantic salmon: validation against experimental challenge tests. Anne Berit Romstad and Paul J. Midtlyng, NVH

5 The quantitative ELISA for inactivated Newcastle antigen: Experience report from a manufacturer. Fabrizio de Mattia, Interviet/SPAH. (Published as The influence of the inactivating agent on the antigen content of inactivated Newcastle disease vaccines assessed by the in vitro potency test. HJM Jagt et al., Biologicals 2010; 38:128-134)

Session III

6 Successful Development and Validation of in vitro replacement assays for veterinary vaccine potency tests: Lessons learned, a manufacturer's point of view. Jean-Cassien de Foucauld, CEVA.

7 Summary of the conclusions and recommendations of the EPAA/ECVAN workshop on the consistency approach for quality control of vaccines in Brussels, January 2010. Marlies Halder / ECVAM. This has been published as: The consistency approach for quality control of vaccines - a strategy to improve quality control and implement 3Rs. F. de Mattia et al, Biologicals, 2011;39/1:59-65.

Session IV

8 An in vitro antigen measure to replace an in vivo test, a proposal for a road map. Gergely Hamar, CEVA

9 Cost analysis for in vitro potency testing, validation, and re-qualification. Justification for use of in vitro systems. Marc Lee, Boehringer-Ingelheim.

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134 Potency Testing of Veterinary Vaccines for Animals: The Way From in Vivo to in Vitro

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The International Scientific Workshop on Potency Testing of Veterinary Vaccines for Animals: The Way From in Vivo to in Vitro, was held in Langen, Germany in December 2010.

Before being placed on the market, inactivated vaccines are predominantly tested in vivo, mainly in laboratory animals. In recent years substantial efforts have been made either to modify these animal tests in order to reduce the number of required animals and the stress imposed on them (Refinement) or to completely replace these experiments by in vitro tests. The acceptance of these tests differs considerably among vaccine manufacturers and licensing authorities. It is thus understandable that vaccine manufacturers hesitate to adopt the new test methods.

This international scientific workshop aimed to promote further progress. It was attended by more than 130 participants from industry, academia and regulatory authorities from some 22 countries around the world.

The current state of knowledge of replacement of in vivo tests was recapitulated, including examples of its successful implementation as well as still existing hurdles. Advantages and disadvantages of existing replacement approaches were presented and discussed. Proposals for new ways to ensure the quality of veterinary immunological medicinal products (IVMPs) were made. The hurdles to be overcome whenever the 3Rs should be implemented in the potency testing of inactivated veterinary vaccines were identified and discussed and a proposal for a road map for future steps towards in vitro testing was made.

KARGER