

**EDQM WORKSHOP ON
ALTERNATIVES TO LEPTOSPIROSIS BATCH POTENCY TEST
26-27 January 2012**

EDQM, Strasbourg, France

ABSTRACTS

ABSTRACT 1	3
Review of 1999 International Conference on Alternatives to Animal Challenge Tests in the Batch Control of <i>Leptospira</i> Vaccines for Veterinary Use Lukas Bruckner, Institute of Virology and Immunoprophylaxis (IVI), Mittelhäusern, Switzerland	
ABSTRACT 2	4
Leptospirosis: need for multi-serovar vaccines - The occurrence in animals in Belgium and Europe. Els Goossens, Isabelle Behaeghel, CODA-CERVA, Veterinary and Agrochemical Research Centre, Brussels, Belgium	
ABSTRACT 3	5
Analysis of multiple <i>Leptospira</i> vaccine proteomes and identification of LipL32 as a potential biomarker for potency P.C. Humphryes, M.E. Weeks, A. Gielbert, G. Thomson, N.G. Coldham, Animal Health and Veterinary Laboratories Agency	
ABSTRACT 4	6
Protein profiling of inactivated <i>Leptospira</i> vaccines - an alternative to animal testing? Elisabeth Balks, Constanze Wolf, Heike Gyra	
ABSTRACT 5	7
<i>Leptospira</i> Hardjo vaccine potency testing based on ELISA alternative, OIE Leptospirosis Reference Laboratory, Veterinary Sciences Division, AFBI, Belfast, Northern Ireland Zbigniew Arent, William Ellis	
ABSTRACT 6	8
<i>Leptospira</i> Dr Eric Klaasen, Intervet	
ABSTRACT 7	10
<i>Leptospira</i> In vitro potency test - High level review of Merial activities on <i>Leptospira</i> potency testing J. Reyes and J. Lechenet	

ABSTRACT 8	11
Leptospira potency testing experience and challenges (a view from Pfizer)	
Dr Catrina Stirling, Associate Director Regulatory Affairs, Pfizer Animal Health	
ABSTRACT 9	12
Leptospira	
Dr Jérôme Denis, Imaxio	
ABSTRACT 10	13
The Biological Standardisation Programme	
Karl-Heinz Buchheit, Department of Biological Standardisation, OMCL Network and HealthCare (DBO), EDQM, Council of Europe, Strasbourg	

ABSTRACT 1

Review of 1999 International Conference on Alternatives to Animal Challenge Tests in the Batch Control of Leptospira Vaccines for Veterinary Use

Lukas Bruckner, Institute of Virology and Immunoprophylaxis (IVI), Mittelhäusern, Switzerland

In 1999 an international conference on alternatives to animal challenge tests in the batch control of *Leptospira* vaccines for veterinary use was held in Strasbourg at EDQM and co-sponsored by ECVAM.

The presentations covered the following topics:

- General aspects, such as current concepts in leptospiral immunity, the Ph.Eur. monograph, the target animal batch safety test and the hamster challenge test;
- Development of alternative methods to the hamster potency test based on direct quantification of vaccine antigen (in vitro methods) and the quantification of antibodies (serological methods).

The conference concluded with a round table discussion, where the following conclusions were drawn:

- The Ph.Eur. monograph is outdated;
- The hamster potency test has deficiencies;
- Alternative methods should be based on efficacy tests in the target species;
- A working group should be created to share the knowledge and to coordinate efforts to replace the hamster test.

References

Anonymous, Alternatives to Animal Challenge Tests in the Batch Control of *Leptospira* Vaccines for Veterinary use, Pharmeuropa Special Issue, BIO (1999) 99, 2

ABSTRACT 2

Leptospirosis: need for multi-serovar vaccines - The occurrence in animals in Belgium and Europe.

Els Goossens, Isabelle Behaeghel, CODA-CERVA, Veterinary and Agrochemical Research Centre, Brussels, Belgium

Leptospirosis, or Weil's disease or *febrile icterus*, is caused by *Leptospira spp.* Nowadays, the zoonosis is (re-)emerging in regions with a moderate climate. The USA, France, Germany and Belgium are reporting an increased number of human and animal cases and have already issued several warnings.

The presentation describes the seroreactivity of the Belgian animal populations in the Microscopic Agglutination test as performed for diagnostic purposes by the veterinary leptospirosis laboratory over the last decade. Since 2006, diagnosis of leptospirosis strikingly increased, with distinctive clinical features depending on the serovar. Domesticated animals, especially dogs and horses tend to become important as accidental hosts and as a source of direct and indirect human infection. Serogroups Australis and Pyrogenes seem to be of a significant pathogenicity in respectively dogs and horses. A new feature of leptospirosis in horses is the pulmonary syndrome and sudden death due to renal failure in foals. To a lesser extent, leptospirosis also appears in the ruminant population and is present at a low level in the Belgian pig population.

The shift in serovars from the last decades requires vigilance. It is important to study and identify the role of certain animal species as reservoir hosts. The currently available vaccines for dogs (and cattle) do not provide full protection. There is a need for vaccines containing multiple serovars, besides Icterohaemorrhagica, Canicola and Sejroe.

ABSTRACT 3

Analysis of multiple *Leptospira* vaccine proteomes and identification of LipL32 as a potential biomarker for potency

P.C. Humphries¹, M.E. Weeks², A. Gielbert¹, G. Thomson¹, N.G. Coldham^{1*}

The current vaccine batch potency test for *Leptospira interrogans* serovar Canicola (L. Canicola) utilises hamsters and has severe effects; whilst effective, a safer, cheaper, more ethical replacement is desired. The aim of this study was to analyse vaccine proteomes and identify common potential target molecules which could be used to design an *in vitro* potency test. Initial analysis of *L. Canicola* vaccines (A-E) from different manufacturers, using the Limulus amoebocyte lysate (LAL) assay and silver stained SDS-PAGE gels, indicated that LPS was not present in all vaccines. The protein contents of vaccines A-E were therefore determined by 2D-LC/MS (221±31, 9±8, 34±4, 21±5 and 34±17 proteins [mean ±1SD] found respectively). The outer membrane protein LipL32 was established to be common to all and to be present at a significantly higher ($p \leq 0.05$) relative spectral abundance in a batch of vaccine which passed the *in vivo* potency test, compared to one which had failed. Further analysis using tandem mass spectrometry and multiple reaction monitoring (MRM) revealed that the concentration of the N terminus of LipL32 was significantly lower ($p \leq 0.01$) in failed batches (n=2) of vaccine compared to passed batches (n=2); the concentration of the C terminus between the two batches was approximately the same. This *in vitro* *Leptospira* vaccine potency test, based on N terminal amino acid quantification of LipL32, shows promise but requires further detailed validation.

¹ Animal Health and Veterinary Laboratories Agency, Addlestone, New Haw, Surrey, KT15 3NB. ² UCL Institute of Child Health, 30 Guilford Street, London, WC1N 1EH.

*Correspondence: Nick Coldham, Specialist Scientific Services Department, Animal Health and Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom, KT15 3NB. Tel: +44(0)1932 357830; Fax +44(0)1932 357595; e-mail: Nick.Coldham@ahvla.gsi.gov.uk

ABSTRACT 4

Protein profiling of inactivated *Leptospira* vaccines - an alternative to animal testing?

Elisabeth Balks, Constanze Wolf, Heike Gyra

Department of Veterinary Medicine, Section Bacterial Vaccines and Immune Sera,
Paul-Ehrlich-Institut

Vaccination against Leptospirosis constitutes an important health measure in veterinary medicine. For long, batch testing of inactivated canine *Leptospira* vaccines has been performed in hamsters according to Ph. Eur. Monograph 01/2008:0447. This testing requires the use of at least 20 hamsters per batch not taking into account animals required to confirm ambiguous test results and to maintain the virulence of challenge strains. At least 8 animals will die after challenge infection. The more recent monograph 01/2008: 0447 “Bovine leptospirosis vaccine (inactivated)” advises a serologic approach.

However, improved quality control systems, new analytical methods and comprehensive post marketing surveillance increasingly promote the acceptance of non-animal approaches in batch release testing. Molecular analytical methods have been shown to be suitable in the depth characterisation of complex biological products. Protein fingerprinting based on various methods has been used for batch to batch consistency testing and demonstration of equivalence of products with clinical lots of proven safety and efficacy. Moreover, detection of immunogenic fractions of such profiles verifies the immunological functionality of product batches.

Recently, we started protein and immune profiling of inactivated leptospirosis vaccines by the use of SDS-PAGE and Western Blot. Method suitability is mainly influenced by the amount of antigen and by adjuvantation of vaccines. Characteristic and consistent profiles could be obtained for non-adjuvanted and alum-adjuvanted vaccines. Immunoreactivity of polyclonal sera raised in guinea-pigs differs markedly from reactivity of hamster, rabbit and of dog sera. Protein- and antigenic profiles might offer a global tool for thorough *in-vitro* analysis of vaccines. The strategy we plan to pursue to attune protein profiling to the needs of batch release testing is presented.

ABSTRACT 5

***Leptospira* Hardjo vaccine potency testing based on ELISA alternative**

Zbigniew Arent, William Ellis

OIE Leptospirosis Reference Laboratory, Veterinary Sciences Division, AFBI, Belfast,
Northern Ireland

At the present, the batch potency testing of *Leptospira* vaccines is performed *in vivo* by challenging hamsters or guinea pigs. Potency is measured either by the vaccine's ability to prevent the death of the hamsters when challenged or by antibody response from vaccinated guinea-pigs. An alternative to animal potency testing may be the *in vitro* quantitative assaying of protective antigens in a given vaccine batch.

As part of an investigation into this 24 monoclonal antibodies to serovar Hardjo were prepared. These included monoclones to LipL21, LipL32 , LipL41 and LPS antigens. Their ability to passively protect hamsters was assessed and 4 LPS monoclones were found to be protective. A quantitative ELISA was developed using one LPS monoclonal antibody. This worked well in dilutions of Hardjo culture inactivated by a variety of methods. Problems however arose when the assay was applied to dilutions of two commercial vaccines. The problems arose from the difficulties in consistently extracting LPS from final product i.e. after the addition of an adjuvant.

Nevertheless, the LPS- ELISA test should be considered as a practical alternative to *in vivo*-based models to measure *Leptospira* vaccine potency. LPS content in leptospiral vaccines is of particular relevance because it has been shown to elicit protective immunity against *Leptospira* serovars in animals. This ELISA test would be a simple and relatively inexpensive procedure and most of all it could contribute to progress of implementation of 3Rs (refinement, reduction, and replacement) alternatives in *Leptospira* batch vaccine potency testing.

ABSTRACT 6

Development of *in vitro* potency tests for a bivalent canine leptospirosis vaccine

Henricus L.B.M. Klaasen^{1*}, Mark van der Veen¹, Urs Bruderer², Marcus J.C.H. Molkenboer³

In order to apply vaccine quality control methods to the concept of the Three R's (Replacement, Reduction and Refinement of animal tests), and in accordance with the current specific European Pharmacopeia Monograph for Canine Leptospirosis Vaccine (Inactivated), we developed an *in vitro* batch potency test, i.e. an antigenic mass ELISA. Nobivac[®] Lepto is a vaccine containing inactivated whole cells of *Leptospira interrogans* serogroup Canicola serovar Portland-vere and *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni. The standard potency test is a hamster challenge test in which the vaccinates must show at least 80% protection against mortality (Canicola and Icteroh. challenge). Our new *in vitro* potency test replaces the animal challenge test. For development of the Canicola and Icteroh. antigen ELISA, hybridomas producing serovar-specific monoclonal antibodies (mabs) were purchased from the Royal Tropical Institute, Amsterdam, The Netherlands. *In vitro* produced mabs were tested for suitability in two capture ELISAs. In short, in the ELISAs the antigenic mass of antigen is determined by incubating serial dilutions of test samples in microtitre plates coated with a mab specific for Canicola or Icteroh. After incubation the captured antigen is quantified by using the corresponding peroxidase-labelled mabs and TMB as substrate, and measuring the absorption at 450 nm. The antigenic mass is determined by calculating the 50% binding dilution which is referred to the 50% binding dilution of the reference antigen with a defined antigenic mass. The repeatability, intermediate precision, robustness, specificity and linearity of the antigenic mass ELISAs were evaluated.

The relationship between antigenic mass and efficacy in dogs was determined by testing a 100% (=standard batch) and 25% vaccine for antigenic mass in the ELISA's and for protective immunity in 6-wk-old dogs against infection with Canicola and Icteroh. The mabs were also tested for protective effects in a passive hamster immunisation experiment, in order to demonstrate that these mabs detected immunodominant (protective) epitopes. The results were as follows. Validation of the ELISAs showed that both tests had good precision, robustness and specificity, and showed linearity in the relevant concentration range. Both mabs induced complete passive protection in hamsters. The 100% as well as the 25% vaccine induced protection in dogs against both serovars. It was concluded that these ELISAs are suitable replacements of the current hamster potency test.

MSD Animal Health, ¹Dept Microbiological R&D, ²Dept Discovery and Technology, ³Global Regulatory Affairs Immunologicals Dept; PO Box 31, 5830 AA Boxmeer, The Netherlands

ABSTRACT 7

***Leptospira In vitro* potency test**

High level review of Merial activities on *Leptospira* potency testing

J. Reyes and J. Lechenet

Expression and regulation of *Leptospira* antigens are very complex and not fully understood. Two major antigens have been identified as potential targets for *In vitro* testing (LPS and LipL32). The most advanced test is a specific ELISA measuring LPS concentration. However, the implementation of any ELISA (or other tests) will require further work to improve pertinence and robustness. Lip132 is currently being investigated as another potential target. Whichever the target antigen(s) of the *in vitro* potency test, possibly more than one *in vitro* test will have to be implemented and a strategy of replacement of the hamster potency test will have to be clearly defined.

ABSTRACT 8

Leptospira potency testing experience and challenges (a view from Pfizer)

Dr Catrina Stirling, Associate Director Regulatory Affairs, Pfizer Animal Health

Pfizer have a large number and range of vaccines for Leptospirosis globally all except one of which are released with animal potency tests. A brief range of these will be given followed by the history of our successful development and authorisation of an alternative in-vitro release test for an EU Leptospira product, Spirovac. The talk will then review other development work on alternative tests and the challenges associated with these. Finally a view will be given for the drivers to develop new tests and the barriers Pfizer view that currently exist, concluding with a view of how we see the consistency approach and step wise implementation could allow replacement of the in vivo tests for Leptospira release.

ABSTRACT 9

"Leptospirosis Batch Potency Tests on human vaccine: similarities and differences with the veterinary vaccines"

Jérôme DENIS, PhD,

Head of pharmaceutical development, IMAXIO

The vaccine SPIROLEPT against leptospirosis is one of the few vaccine available for human use, since more than 30 years.

For reasons related to the specificity of its development in the 70s, the batch release tests for the SPIROLEPT have got some differences and also similarities with those applicable for veterinary vaccines as described in the European Pharmacopoeia. A short presentation of the SPIROLEPT batch release test will be made, and a comparison with the EP method will be proposed. Some trends extracted from several SPIROLEPT batch release tests will be presented, and will support a short discussion around the kind and quality of informations that should provide an in vitro test replacing the current in-vivo based quality control methods.

Jérôme DENIS, PhD

Head of pharmaceutical development

Responsable du développement pharmaceutique

IMAXIO

181-203, avenue Jean Jaurès

69007 Lyon

tél: + 33 (0) 6 896 843 37

fax: + 33 (0) 4 376 506 17

www.imaxio.com

ABSTRACT 10

The Biological Standardisation Programme

Karl-Heinz Buchheit, Department of Biological Standardisation, OMCL Network and HealthCare (DBO), EDQM, Council of Europe, Strasbourg

The Biological Standardisation Programme (BSP) is a joint programme of the Council of Europe and the EU Commission that started in 1993. The BSP aims at i) the establishment of Biological Reference Preparations (BRPs) to be used as working standards for the tests prescribed by the European Pharmacopoeia (Ph. Eur.); ii) the standardisation of test methods, iii) the elaboration of alternative methods in application of the 3R concept (Refine, Reduce, Replace the use of animals). These goals are pursued for improving the quality control of biologicals, such as vaccines for human and veterinary use, human blood and plasma derivatives, hormones and recombinant products in close collaboration with international organisations (e.g. WHO; OIE) and national, non-European partners, particularly in the framework of ICH and VICH.

Since its start, the BSP has started work on 114 projects. Thirty-six projects were devoted to method development, including 20 projects on the validation of 3R methods. The establishment of BRPs was the aim of 78 projects.

In a number of cases it was possible to replace tests based on the use of animals by completely animal-free alternatives (e.g. establishment of *in vitro* assay for hepatitis A vaccine or Newcastle Disease vaccine). In other cases, a refinement of the test method leading to less stress for the animal and a reduction of the number of animals used for the experiments was achieved. E.g. the establishment of serological assays for diphtheria, tetanus and acellular pertussis vaccine enable to drop the much more stressful direct challenge assay and drastically reduce the number of animals used for these assays since all three components can be assayed using the serum from the same animals. Similarly it was possible to replace the direct challenge assay for erysipelas vaccine by a serological test method. In all these cases the results elaborated by the BSP convinced the Groups of Experts of the Ph. Eur. to revise the respective Ph. Eur. monographs and to include the alternative methods.

The practical work performed in the framework of the BSP thus is in line with the Council of Europe Convention on the protection of animals used for experimental and other scientific

purposes (1986), as well as the new EU Directive 2010/63 EU on the protection of animals used for scientific purposes. The BSP is an integral part of the EPAA initiative of the European Commission and industry.

All stakeholders (OMCLs, competent authorities, manufacturers) can participate in the collaborative studies performed by the BSP. Data are reported and published in an anonymised way. All generated data are published in Pharmeuropa Bio & Scientific Notes in order to support the implementation of new methods and BRPs.

The success of the BSP would not have been possible without the excellent co-operation between all the involved parties, Ph. Eur. Commission, Official Medicines Control Laboratories, manufacturers of biologicals, EU Commission and EMA.