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Short paper

Development of *Leptospira in vitro* potency assays: EU/industry experience and perspectives

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ABSTRACT

Nobivac® Lepto (MSD Animal Health) is a non-adjuvanted canine leptospirosis vaccine containing inactivated whole cells of *Leptospira interrogans* serogroup Canicola serovar Portlandvere and *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni. The current standard *in vivo* potency test is a hamster challenge test associated with major drawbacks such as animal suffering and poor reproducibility. Here, the quantification of antigenic mass by ELISA as a new *in vitro* potency test is described, supporting the 3Rs concept (replacement, reduction, and refinement of animal tests) and in accordance with European Pharmacopoeia Monograph 0447 (Canine Leptospirosis Vaccine [Inactivated]). The two corresponding sandwich ELISAs are based on monoclonal antibodies specific for immunodominant leptospiral lipopolysaccharide epitopes. Protection in passive immunization experiments demonstrate that these monoclonal antibodies recognize key protective antigens in currently licensed human and veterinary whole cell *Leptospira* vaccines. The high precision and robustness renders the two ELISAs much more reliable correlates of potency in dogs than the hamster potency test. The recent approval of these assays for a new canine leptospirosis vaccine is an important contribution to the 3Rs in quality control testing of *Leptospira* vaccines.

1. Introduction

Leptospirosis is a re-emerging global zoonosis with a disease spectrum in humans and animals ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality [1]. Vaccination is widely used to control leptospirosis in dogs and livestock. To the authors' knowledge, all licensed veterinary leptospirosis vaccines are based on chemically or physically (for example with heat) inactivated whole cells of *Leptospira interrogans* (*sensu lato*). Immunity induced by natural leptospiral infection and vaccination is largely humoral and relatively serovar specific [1]. Cell-mediated immunity in cattle has been demonstrated to be involved in the immunological response to vaccination [2]. Complete protection from renal infection with serovar Hardjo in calves was achieved after intravenous injection with serum from calves vaccinated with inactivated whole cells, demonstrating the dominant role of humoral immunity (Klaasen, unpublished results). There is a general agreement [3,4] that serovar-specific leptospiral lipopolysaccharide (LPS) epitopes are responsible for immunity against leptospiral infection in humans and animals. While the relative importance of humoral versus cell-mediated immunity in infection with or vaccination against serovar Hardjo in cattle is still under debate, in several studies evidence has been reported for the central role of LPS epitopes in infected or immunized cattle as well [5–8]. These findings are consistent with oligosaccharide epitopes of the leptospiral LPS being the key protective antigens in currently licensed human and veterinary whole cell *Leptospira* vaccines, including the bovine vaccines.

According to the European Pharmacopoeia (Ph. Eur.) monograph for inactivated canine leptospirosis vaccines (Monograph 0447), the standard batch potency test consists of hamster vaccination followed by *Leptospira* challenge. Virtually the same test is described in the Code of Federal Regulations (United States Department of Agriculture, Center for Veterinary Biologics), Title 9, Animals and Animal Products, Part 113 – Standard Requirements (9 CFR). This potency test has been used for decades in batch release testing for leptospirosis vaccines licensed in Europe and the United States. However, this test has the following important drawbacks: (1) animal

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suffering; (2) poor reproducibility [9]; (3) human biosafety risks; (4) relatively high costs; (5) relatively long test running time (7–8 weeks); and (6) invalid in case of challenge strains that are not hamster-lethal, like all strains of serovar Hardjo [10]. Because significant pain and distress are involved and a large number of animals are used for this test, *Leptospira* vaccines are included in a priority list of vaccines that most urgently require the application of the 3Rs concept (refinement, reduction and replacement of animal tests) [11]. Twenty years ago, for the first time, studies describing an *in vitro* batch potency test (an enzyme-linked immunosorbent assay [ELISA]) for *Leptospira* vaccines were published [12]. Seven years later, in 1999, in Strasbourg, France, the "International Conference on Alternatives to Animal Challenge Tests in the Batch Control of *Leptospira* Vaccines for Veterinary Use" [13] was held, with the following conclusions: (1) the Ph. Eur. monograph is outdated; (2) the hamster potency test has deficiencies; (3) alternative methods should be based on efficacy tests in the target species; and (4) a working group should be created to share the knowledge and to coordinate efforts to replace the hamster test. Since 2005, Ph. Eur. Monograph 0447 options for alternative batch potency tests include *in vitro* tests for vaccines without adjuvant and serological (non-challenge) animal tests for vaccines with or without adjuvant. An "EDQM Workshop on Alternatives to the Leptospirosis Batch Potency Test" took place in Strasbourg in January 2012, with the aim to share information and experiences on the recent advances with regard to alternative methods for the leptospirosis batch potency test [14]. Part of the work described in this paper was presented at this EDQM workshop.

This publication describes the development and validation of two ELISAs for quantification of protective LPS epitopes in a commercial bivalent canine leptospirosis vaccine (Nobivac® Lepto), in order to replace the current hamster potency test. This is a non-adjuvanted vaccine containing inactivated whole cells of *Leptospira interrogans* serogroup Canicola serovar Portlandvere and *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni. Because the LPS epitopes are the key protective antigens in this type of vaccine, an assay for quantification of these epitopes fully complies with the requirement in Ph. Eur. Monograph 0447. That requirement states that the components that are quantified in a suitable alternative method function as "indicators of protection." One assay is specific for the Canicola antigen and the other specific for the Icterohaemorrhagiae antigen of the vaccine. Supporting animal studies needed for the acceptation of these new *in vitro* potency tests by European regulatory authorities are described in this paper also.

2. Materials and methods

2.1. Selection of monoclonal antibodies

Based on general knowledge of ELISAs and the *Leptospira* antigenic mass ELISAs developed by Ruby and coworkers [12], antigen-specific screening ELISAs were used to test monoclonal antibodies (mAbs) purchased from the Royal Tropical Institute, WHO/FAO/OIE and National Leptospirosis Reference Centre, KIT Biomedical Research, Amsterdam, The Netherlands. In this reference laboratory (henceforth the KIT) is a large collection of anti-*Leptospira* hybridomas and mAbs which are used for diagnostic purposes, such as serovar identification of clinical (human or animal) isolates [15–19]. With the microscopic agglutination test (MAT), the agglutination titers of all mAbs against the relevant serovars were determined. The results are described in the KIT Biomedical Research list of "Monoclonal antibodies for easy and rapid typing" [19]. From this list, the serogroup Canicola mAbs and serogroup Icterohaemorrhagiae mAbs with the highest agglutination titers against the serovars to which the two vaccine strains of Nobivac® Lepto belong, serovar Portlandvere and serovar Copenhageni, were selected and tested for sensitivity and specificity in screening experiments with indirect ELISAs.

2.2. Assay development

2.2.1. Hybridomas, mAbs; production, purification and conjugation

The hybridomas producing mAbs F152C11 and F12C3 were purchased from the KIT. Hybridomas and mAbs had been produced by the KIT as described by Korver et al. [17]. MAb F152C11 originated from mice immunized with live leptospires of *L. interrogans* serovar Canicola strain Hond Utrecht IV. The isotype of mAb F152C11 is IgM. MAb F12C3 originated from mice immunized with live leptospires of *L. interrogans* serovar Copenhageni strain Wijnberg. The isotype of mAb F12C3 is IgG2A. In the present studies, production of hybridoma cells and production of mAbs from hybridoma culture supernatant were optimized using conventional *in vitro* production techniques [20]. Briefly, mAbs were purified by sequential precipitation, first with caprylic acid followed by ammonium sulfate using standard procedures [21]. Purified mAbs were conjugated according to a previously described method [22]. The carbohydrate moieties of the horseradish peroxidase were oxidized with sodium periodate after which the formed aldehyde groups reacted with the free amino groups of the immunoglobulin preparations.

2.2.2. Reference standards and internal standard

A reference standard (one for the Canicola assay and another one for the Icterohaemorrhagiae assay) consisted of production antigen that was formulated as monovalent vaccine at standard antigen concentration of Nobivac Lepto vaccine. The Canicola production antigen was prepared from a vaccine strain belonging to serogroup Canicola serovar Portlandvere, and the Icterohaemorrhagiae production antigen was prepared from a vaccine strain belonging to serogroup Icterohaemorrhagiae serovar Copenhageni. An arbitrary antigen concentration of 1000 ELISA Units/mL (U/mL) was attributed to each reference standard. The reference standards were used as references against which the concentration (in U/mL) of test samples was calculated. A representative batch of Nobivac Lepto vaccine was used as internal standard. The internal standard was used to assess the validity of each individual ELISA test, using sequential analysis of the results (in U/mL) of the internal standard. Test samples consisted of either antigen samples or vaccine samples.

2.2.3. ELISA format

Concentrations of preparations containing one or both serovars were determined by sandwich ELISAs utilizing monoclonal antibodies with the corresponding specificity. For both serovars, the same corresponding monoclonal antibody was used for capture and conjugated as detector antibody. Optimal concentrations of capture and detection antibodies were determined by checkerboard titrations.

Capture antibodies, diluted in carbonate buffer pH 9.6, were coated in polystyrene flat-bottom microtiter plates (Greiner, Frickenhausen, Germany) and incubated overnight at 2–8 °C. Remaining binding sites of microtiter plates were quenched by an incubation of 1% (w/v) skim milk diluted in PBS in antibody-coated wells for 1 h at 37 °C. After quenching and between all subsequent incubations, plates were washed with an ELISA washer (Skatron Skan Washer 300, type 12201, Skanstacker, Lier, Norway) using PBS containing 0.05% (v/v) Tween 20. References and test samples, serially diluted in PBS containing Tween 20 (0.05%; v/v) and skim milk (1%; w/v), were added, followed by incubation for 1 h at 37°.