# International Workshop on Alternative methods for Leptospira Vaccine Potency Testing: State of the Science and the Way Forward,

19-21 September 2012, Ames, Iowa, USA

## Development of *Leptospira In vitro* Potency Assays: EU/Industry Experience and Perspectives

**Eric Klaasen et al.**

**Merck Animal Health,**
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# *Structure presentation*

**History and rationale**

**Development *in vitro* potency tests**

**Test validation *in vitro* potency tests**

**Additional, supportive studies**

- **Passive protection in hamsters**
- **Dose-response in dogs**

**Adjuvanted vaccines**

**General conclusions**

**Alternative *in vivo* potency tests**

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## Present potency test (EU/USA):

- Hamster challenge test according to EP Monograph 0447
- Disadvantages:
  - challenge causes animal suffering
  - poor reproducibility
  - relatively high costs
  - relatively long running time
  - zoonotic risks for lab workers
- 9CFR: almost identical test



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# History and rationale

## Alternatives described in EP Monograph 0447:

- ***In vitro*** **potency test (vaccines without adjuvant)**
- ***In vivo*** **potency test using serology (adjuvant)**

## Potency test existing non-adjuvanted bivalent canine leptospirosis vaccine:

- ***in vitro*** **potency test = antigenic mass ELISA**
- **one test for Canicola, one test for Icterohaemorrhagiae**

## (Peer reviewed) publications describing comparable assays:

- **Ruby et al, Biologicals 1992, 20, 259-266 (sv. Pomona)**
- **Ruby et al, Dev Biol Stand 1996, 86, 341 (sv. Canicola)**



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# *Development* in vitro *potency tests*

## Choice of assay

- **Based on type of vaccine (whole cell bacterin)**
- **Based on abundance and immunodominance of LPS**
- **Capturing and detecting antibodies: mabs against LPS epitopes**
- **Hybridomas and mabs from Royal Tropical Institute (KIT)* :**

  ***WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis, A'dam**

  - **Canicola: F152C11 (strain Hond Utrecht IV)**
  - **Icteroh. : F12C3 (strain Wijnberg)**
  - **mabs based on agglutination patterns KIT, papers KIT and in-house pre-testing**

## Hybridomas purchased from KIT

- **Use of mabs within KIT: primarily for diagnostic purposes**
- **Condition purchase: only for internal QC purposes**
- **Routine method Merck AH, Boxmeer, used for upscaled production of mabs**



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# *Development* in vitro *potency tests*

**Leptospira and Leptospirosis, S. Faine, CRC Press, 1994, p. 166:**

2. **Nature and Immunochemical Basis of Specificity**

***a. Immunoglobulins Conferring Specific Resistance***

The effects of antibody in conferring resistance are similar, for a given serovar, regardless of the species of animal from which the immunoglobulins are derived, or of the immunoglobulin class. Murine monoclonals produced by immunizing with whole leptospires, or subcellular fractions or chemical derivatives most usually react with epitopes of the LPS, visualized as a recognizable band in PAGE. They thus afford a means of identifying protective epitopes of LPS if the monoclonal protects passively against challenge infection. Specificity can be assessed to the same order of accuracy as the specificity of the epitopes of the monoclonals.[31,32]

***b. Antigenic and Immunochemical Basis of Specific Resistance***

Protective epitopes are part of the PS of LPS.[32,85,95,96] Immunization with LPS and its PS from serovar *copenhageni* protected guinea pigs or hamsters from challenge infection, and LPS from serovar *hardjo* and *pomona* were immunogenic, although the PS was less effective than that from *copenhageni*. The PS, or an oligosaccharide fraction from it, became an effective antigen when it was conjugated to diphtheria toxoid, stimulating specific immunoglobulins in mice.[97] Differences between serovars may be related to differences in genospecies rather than serovar.

**Immunochemical structure and specificity.** The reacting groups can be followed to relatively small oligosaccharides composed of 3-4 sugars. The determinants of all the epitopes are unknown, except that in the PS of the LPS of serovar *hardjo* two of them are mannosamine, and mannose-6-phosphate. The detailed structure and linkages are still elusive, but phosphate linkage is important.[98,99] Since serological activity and immunity are also serovar-specific, it is clear that these epitopes also comprise the basis for serological classification and serovar differences.



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# *Development* in vitro *potency tests*

## Relevant examples from literature re: monoclonal antibodies against *Leptospira* serovars:

1) Z Bakt Hyg A 1985, 259, 498-506: mabs serogroup Sejroe serovars, against antigens of carbohydrate nature;
2) Z Bakt Hyg A 1987, 266, 412-421: mabs serogroup Pomona serovars, against antigens of carbohydrate nature;
3) Isr J Vet Med 1988, 44, 15-18: mabs serogroup Icteroh. serovars;
4) J Gen Microbiol 1989, 135, 73-78: serovar Copenhageni (serogroup Icteroh.), protective mab F12C3 ↔ carbohydrate epitope;

5) J Med Microbiol 1987, 23, 1-7: opsonic mabs serogroup Sejroe serovars, against LPS antigens;
6) Z Bakt Hyg A 1990, 272, 328-336: opsonic and protective mabs serogroup Icteroh. serovars, against LPS antigens.
7) Arend & Ellis (EDQM Workshop on Alternatives to Leptospirosis Batch Potency Test, 26-27 Jan 2012, Strasbourg): anti-LPS-mabs were protective.

**References 1-4: Royal Tropical Institute (KIT), WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis, A'dam**



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# *Development* in vitro *potency tests*

**Steps taken prior to selection of mabs and type of assay:**

1) **Preparation/selection of materials for: reference standards (monovalent antigen) and internal standards (bivalent vaccine)**
   - **selection homologous and heterologous ref. standards**

2) **Pre-testing of ascites (KIT) in ELISA's**

3) **Selection of mabs/hybridomas (KIT), purchase, small-scale production mabs, purification and conjugation of mabs**

4) **Start optimisation large-scale production mabs**

5) **Start development ELISA's with:**
   - **several batches of the same mab**
   - **homologous and heterologous standards and samples**
   - **several types of plates**
   - **several buffers**
   - **etc.**



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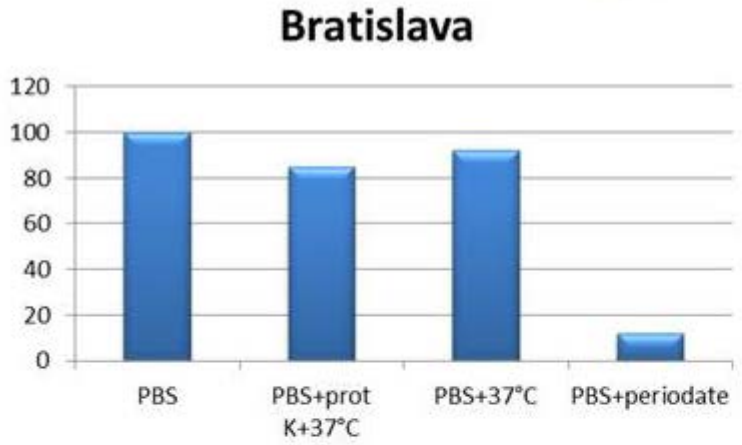
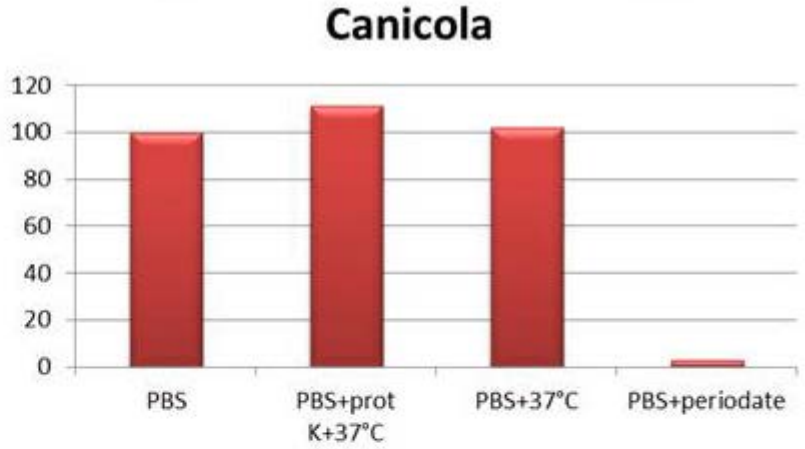
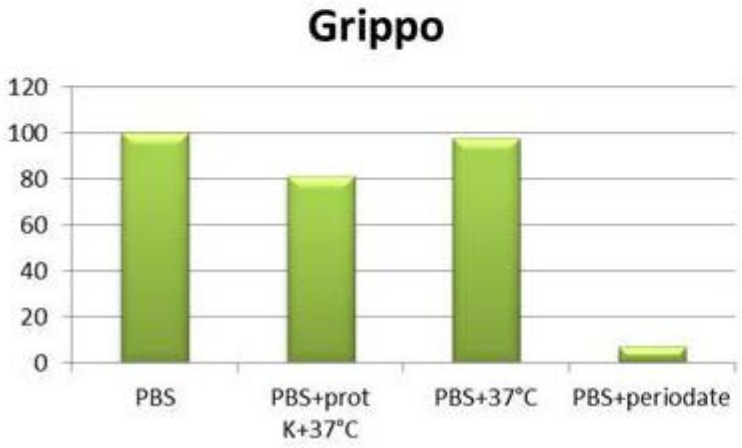
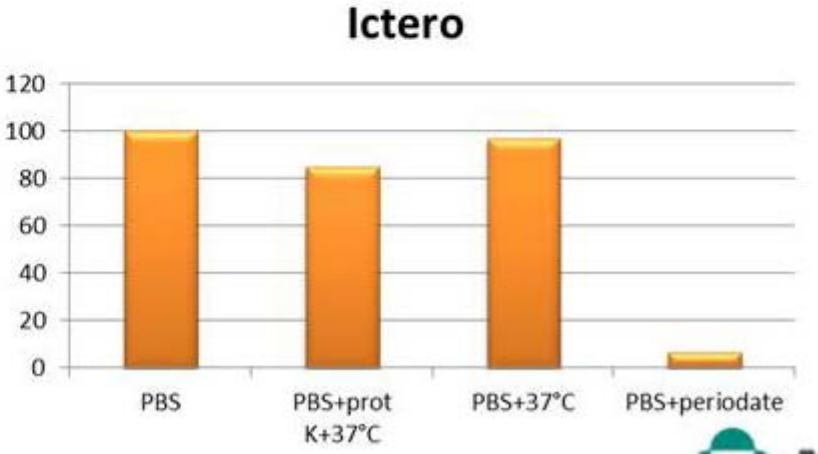
# *Development* in vitro *potency tests*

**Effects of proteinase K and periodate on measured antigenic mass of four different *Leptospira* antigens (in % of ag mass untreated antigen)**



# *Development* in vitro *potency tests*

**After pre-testing, following protocol chosen for antigen ELISA Canicola and Icterohaemorhagiae:**

- **Coating plates with capture mab**
- **Post coating: blocking buffer**
- **Washing cycle (ELISA washer)**
- **Blocking buffer and then:**
  - **reference standard (1000 U/ml)**
  - **internal standard**
  - **test samples (antigens/vaccines)**
- **Washing cycle (ELISA washer)**
- **Detecting mab = conjugated mab**
- **Washing cycle (ELISA washer)**
- **Substrate = tetramethyl benzidine**
- **4N $H_2SO_4$**
- **Absorption at 450 nm and calculation of antigenic mass in U/ml relative to reference standard**



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# *Development* in vitro *potency tests*

## Calculation of Units/ml

- **Dilution corresponding to 50% relative binding (RB) determines the concentration of a sample**
- **50% RB = (Bmax – Bmin) : 2**
- **Values of test samples calculated against those of reference standard (defined conc: 1000 U/ml), and expressed in U/ml**

## Test optimisation

## Preliminary test validation

- **Stability testing of standards**
- **Robustness testing**



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# *Test validation* in vitro *potency tests*

**Complete test validation (results coefficients of variation):**

- **Repeatibility (within-assay variability):**
  - **Can, 11.0%; Ict, 8.2%**
- **Intermediate precision (inter-assay variability):**
  - **Can, 15.2%; Ict, 11.1%**
- **Robustness**
  - **Can and Ict, satisfactory**
- **Specificity (test heterologous antigens against ref. stand.)**
  - **Can and Ict, satisfactory**
  - **NB: ELISA measures correctly low ag conc. (20%) of one serovar in presence of 100% ag conc. of 5 other serovars**
- **Linearity**
  - **Can and Ict, linear range between 800% and 6.25% of corresponding ref.standards**



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# *Supportive studies: passive protection in hamsters*

## Experimental set-up and results

| N per group | Treatments | | Number of dead animals |
|---|---|---|---|
| | IP injection mab | IP challenge* | |
| 5 | 0.5 ml F152C11 | Sv. Can. strain Moulton | 0 |
| 5 | None | | 5 |
| 5 | 0.5 ml F12C3 | Sv. Ict. strain Verdun | 0 |
| 5 | none | | 5 |
| 5 | 0.5 ml F12C3 | Sv. Cop. strain CF1 | 0 |
| 5 | none | | 5 |

* 1.0 ml containing 1 x $10^9$ cells/ml

**Conclusion: these mabs are directed against immunodominant epitopes of the resp. strains.**



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# *Supportive studies: dose-response in dogs*

## Experimental set-up

| N per group | 2 vaccinations, 6 and 10 wks of age | Challenge*, 14 wks of age |
|---|---|---|
| 6 | Full ag strength (100%) | Sv. Canicola strain Moulton ($5 \times 10^8$ cells/ml) |
| 6 | 25% | |
| 6 | None | |
| 6 | Full ag strength (100%) | Sv. Copenhageni strain CF1 ($1 \times 10^9$ cells/ml) |
| 6 | 25% | |
| 6 | None | |

* 2 ml IP and 0.25 ml instilled into right and left conjunctival sac



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# *Supportive studies: dose-response in dogs*

## Results clinical symptoms, thrombocytes, blood and urine cultures, histopathology

| Vacc. | Chall. | Stat. significant differences: | | | % dogs with clinical signs | % dogs with nephritis |
|---|---|---|---|---|---|---|
| | | Thromb (day 3) | Blood cultures | Urine cultures | | |
| 100% | Can | A | A | A | 0 | 0 |
| 25% | | A | A | A | 0 | 0 |
| Control | | B | B | B | 100 | 100 |
| 100% | Cop | A | A | A | 25* | 0 |
| 25% | | A | A | A | 16* | 0 |
| Control | | A | A | B | 75* | 50 |

* Dogs chall. with Cop. only had pale conjunctivae.



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## Conclusions:

- **Full strength (100%) and 25% vaccine induced protection in dogs from infection with Canicola and Icterohaemorrhagiae**
- **ELISA antigenic mass values of 25% vaccine are comfortably below the future minimum release values* for each component**

***(based on statistical analysis of ELISA ag mass values of "x" standard vaccine batches)**



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# *Adjuvanted vaccines*

- **Preliminary experiments Merck AH, Boxmeer:**
  - ***Leptospira*/rabies combi vaccine with Alum. adjuvant: no interference of adjuvant with ELISA;**
  - ***Leptospira* vaccine with saponine adjuvant: idem**
- **Successful ag extraction methods described in literature:**
  - **Proc Vaccinol 2011, 5, 175-183: ag extraction from inactivated ND vaccine adjuvanted with mineral oils**
  - **Proc Vaccinol 2011, 5, 60-83:**
    - *** Priority to adjuvants for which separation methods already exist**
    - *** Consider potency test bulk material, prior to addition adjuvant (GMP!)**



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# General conclusions

- **Immunodominant protective epitopes detected and quantified;**
- **Compliance with general requirements of validity: precision, robustness, specificity and linearity.**
- **(artificial) 25% ag strength batches will be protective in dogs, but be rejected with the future *in vitro* potency test.**
- **ELISAs not affected by Al adjuvant.**



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# *Alternative in vivo potency tests*

- **Goddard *et al*. (J Biol Stand 1986, 14, 337-344): based on close correlation with protection in calves, serological test guinea pigs proposed;**

- **Ebert E (Pharmaeuropa Bio 1999, 102e10, 17-24): serological test guinea pigs proposed;**

- **Merck AH, Boxmeer: serological test rabbit differentiated 20% from 100% vaccine.**



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