Artigo científico

# Canine anti-leptospira bacterins commercialized in Brazil: a challenge made with indigenous strains of serovars Canicola and Copenhageni

Wilsilene Aparecida Silva Coelho [1]
Silvio Arruda Vasconcellos [1]
Zenaide Maria de Morais [1]
Cassia Yumi Ikuta [1]
Amane Paldes Gonçales [1]
Gisele Oliveira de Souza [1]
Cristina Corsi Dib [1]
Francisco Rafael Martins Soto [2]

**Abstract.** It was performed a comparative potency evaluation of canine anti-leptospira vaccines commercialized in Brazil, using for the challenge Canicola and Copenhageni leptospira indigenous strains isolated in Brazil. Nine polyvalent commercial bacterins to be used in dogs were were identified by letters A, B, C, D, E, F, G, H and I and compared. Challenge was made using strains L1-130 and LO4, respectively from Copenhageni and Canicola serovars, typified by the monoclonal antibodies technique. The adopted protocol was in agreement to American technical standards. Challenge infective dose for serovar Copenhageni was lower to the threshold established by the technical report and for Copenhageni serovar it was 10,000. Animals were observed during 21 consecutive days, and those which died of leptospirosis were counted. At the end of this period, survivors were euthanized with carbon dioxide and necropsied to collect kidneys and to perform culture to control leptospira kidney infection. Of the nine vaccines evaluated, seven were rejected for both serovars and two were approved against clinical disease and kidney infection for Canicola LO4, however they were only effective against clinical disease for Copenhageni L1-130serovar. Manufacturers laboratories of canine anti-leptospira bacterins commercialized in Brazil need to review the quality of their products regarding disease and infection protection against Canicola and Copenhageni serovars.
**Keywords**: Potency, Dogs, Animal Leptospirosis, Vaccine, Hamsters.

[1] University of São Paulo, Department of Preventive Veterinary Medicine, São Paulo, Brazil

[2] Federal Institute of São Paulo, *campus* São Roque. Correspondence to: Caixa Postal 34 – Ibiúna, SP - CEP 18150-000; e-mail: chicosoto34@gmail.com

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## 1 Introduction

Canine leptospirosis immunity, especially the humoral kind, is serovar-specific and in minor degree it is serogroup specific (CHO et. al, 1992). Vaccines currently used to prevent canine leptospirosis have whole inactivated bacteria which induce immunity through the bacteria opsonization, resulting in the presentation of membrane antigens (lipopolysaccharide and external membrane proteins; GREENE, SCHULTZ, 2006). Other vaccines constituted of membrane protein antigens and of subunits are also being investigated (HAGIWARA, 2003).

The anti-leptospirosis vaccines worldwide used for canine immunization are bacterins produced with strains of Icterohaemorrhagiae and Canicola serovars, which are the most prevalent in dogs worldwide (HAGIWARA, 2003). However, in the last few years in some countries, other serovars have been isolated of domestic dogs due to their contact with wild or synanthropic reservoirs or with environment contaminated with urine of such animals (FREITAS *et al.,* 2004). This fact has justified the inclusion of new serovars in bacterin production for use in dogs. The increased number of serovars also increases the possibility of undesired side effects such as hypersensitivity reactions (HAGIWARA, 2003). Vaccines against canine leptospirosis should include only those serovars prevalent in the region where they are to be used (RODRIGUES, 2008).

In Brazil, there are about ten commercial brands of vaccines against canine leptospirosis produced with reference serovars isolated abroad, some of them including up to six different serovars.

The objective of this study was to comparatively evaluate the potency of canine anti-leptospira vaccines commercialized in Brazil, using a challenge with indigenous leptospira strains of serovars Canicola and Copenhageni, according to vaccine brand and serovar, and also for disease protection and against the renal carrier state among challenge survivors with leptospira strains isolated in Brazil.

## 2 Materials and methods

A total of 350 young male hamsters (*Mesocricetus auratus*), weighing between 60 and 100 g were used. During the experiment animals were distributed and maintained in propylene cages, and groups weight were balanced in order to homogenize them. Cages were filled with wood shavings and animals received tap water and commercial feed pellets *ad libitum.* Nine polyvalent canine anti-leptospirosis commercialized in Brazil were used and identified respectively by letters A, B, C, D, E, F, G, H and I, and the unvaccinated control group was only challenged with Canicola $LO_4$ and Copenhageni $L_1$-130 serovars. The strain Copenhageni $L_1$-130 was obtained from Fiocruz (KO *et al.*,1999; NASCIMENTO *et al.*, 2004) sample and the $LO_4$ Canicola serovar came from Universidade Estadual de Londrina – Paraná (FREITAS *et al.*, 2004). Both strains were typified with monoclonal antibodies produced by the Royal Tropical Institute - Amsterdam, Netherland. $LO_4$ strain challenge inoculum was an infected hamster hepatic tissue suspension. Suspension was prepared in EMJH[1] liquid culture media at a 1:10 (weigh/volume) proportion. There were made ten fold serial dilutions from $10^{-5}$ to $10^{-19}$. Titration was performed using 80 hamsters divided into groups of five. Each group was inoculated with one of the dilutions and a volume of 200µl/hamster, intraperitoneal route. Animals were observed during 21 days and lethal dose (LD 50) was calculated according to Reed and Müench method (REED, MUENCH, 1938). The challenge dilution was $10^{-6}$.

$L_1$-130 strain challenge inoculum was an EMJH culture with 15 days after first inoculation. There were made ten fold serial dilutions from $10^{-3}$ to $10^{-16}$. Titration was performed using 75 hamsters divided into groups of five. Each group was inoculated with one of the dilutions and a volume of 200µl/hamster, intraperitoneal route. Animals were observed during

[1] Ellinghausen-McCullough-Jonhson-Harris (DIFCO-Detroit, EUA).

21 days and lethal dose (LD 50) was calculated according to Reed and Müench method (REED, MUENCH, 1938). The challenge dilution was $10^{-4}$.

The potency test was performed according to the American *Code Federal Regulation* (CODE OF FEDERAL REGULATIONS. ANIMALS AND ANIMAL PRODUCTS, 2006), hamster challenge.

Vaccine was diluted at 1:80 of the manufacturer recommended dose for dogs.

Hamsters were distributed in 20 groups of ten animals. Each group received subcutaneously a dose of 0.25mL of the respective bacterin challenge inoculum. After 14 days all animals were challenged with Copenhageni $L_1$-130 and Canicola $LO_4$ serovars live cultures through intraperitoneal route.

Animals were daily observed during 21 days, counting those that died by leptospirosis characterized as: loss of weight, natural orifices bleeding, jaundice, hepato and splenomegaly as well as petechiae and pulmonary suffusions. At the end of this period, survivors were euthanized with carbon dioxide inhalation ($CO_2$ chamber), and necropsied to collect kidneys and to perform culture to control leptospira kidney infection.

After 21 days of leptospira infection (d.a.i), hamsters were anesthetized in a $CO_2$ chamber, blood was collected for MSA and then they were euthanized through anesthesia overdose. Animals were necropsied and their kidneys were aseptically collected, mashed and then buffered saline solution was added to obtain an initial dilution of $10^{-1}$, from which two ten fold dilutions were prepared ($10^{-2}$, $10^{-3}$). A hundred microliter of each dilution were seeded in bakelite lids tubes containing 5.0 ml of semi solid Fletcher media (MYERS, 1985), two tubes for each dilution, and they were incubated at 28-30°C during six weeks, weekly observed (FAVERO *et al.*, 1997) to verify the subsurface leptospira growth ring (Dinger zone) (MYERS, 1985) and then the presence of leptospira was confirmed in dark field microscopy.

Results obtained from hamsters were analyzed by the criteria of the Potency Test for bacterins following the American technical standards, proposed by the *Code Federal Regulation* (CODE OF FEDERAL REGULATIONS. ANIMALS AND ANIMAL PRODUCTS, 2006).

## 3 Results

The results obtained according to the methodology used are described below:

Titration of infectious challenge inoculums: during the 21 days period after inoculation, animal deaths were observed at $10^{-5}$ to $10^{-10}$ dilutions for Canicola $LO_4$ serovar and $10^{-3}$ to $10^{-9}$ for Copenhageni $L_1$ 130 serovar (Table 1).

Protection against the disease: results showed at Figure 1 refer to the rate of hamster that survived to challenges with Canicola $LO_4$ serovar and with the strain $L_1$-130 of Copenhageni serovar. For infectious challenge inoculum control group there was one survivor among ten inoculated animals with Canicola $LO_4$ serovar and two survivors among ten inoculated animals with Copenhageni $L_1$-130. For vaccinated groups challenged with Canicola $LO_4$ serovar, survival rates were 10/10 for vaccine A, 0/10 for vaccine B, 9/10 for vaccine C, 0/10 for vaccine D, 0/10 for vaccine E, 3/10 for vaccine F, 7/10 for vaccine G, 5/10 for vaccine H and 0/10 for vaccine I and for vaccinated groups challenged with Copenhageni $L_1$-130 serovar, survival rates were 8/10 for vaccine A, 3/10 for vaccine B, 8/10 for vaccine C, 4/10 for

vaccine D, 3/10 for vaccine E, 6/10 for vaccine F, 6/10 for vaccine G, 6/10 for vaccine H and 4/10 for vaccine I. According to the approval criteria adopted of a maximum of two leptospirosis deaths among ten challenged animals, only the bacterins A and C were approved. Bacterins F, G and H still could be re-tested to verify if the number of deaths would be a maximum of five in twenty challenged animals for each bacterin.

**Table 1**. Hamsters infected with pathogenic leptospira strains according to infectious inoculum dilution, strain of leptospira and leptospirosis death rate.

| | Strain | |
|---|---|---|
| Dilution | $LO_4$ – Canicola serovar | L1-130, Copenhageni serovar |
| $10^{-3}$ | ... | 4/5* |
| $10^{-4}$ | ... | 8/10 |
| $10^{-5}$ | 5/5 | 0/5 |
| $10^{-6}$ | 9/10 | 1/5 |
| $10^{-7}$ | 5/5 | 2/5 |
| $10^{-8}$ | 5/5 | 1/5 |
| $10^{-9}$ | 5/5 | 1/5 |
| $10^{-10}$ | 3/5 | 0/5 |
| $10^{-11}$ | 0/5 | ... |
| $10^{-12}$ | 0/5 | ... |

* Number of deaths by leptospirosis/number of inoculated animals, ... = not done. Challenge dilution was $LO_4 = 10^{-6}$, $L_1130 = 10^{-4}$, LD 50 titration $LO_4 = 10^{-10}$ , $L_1130 = 10^{-4,7}$, number of LD 50 effectively used = $LO_4$ = 10.000 , $L_1130$= 5,4.

Figure 1 shows the vaccines performance against challenge inoculums in relation to survival animal rate that were challenged with $LO_4$ and $L_1$ 130 strains.

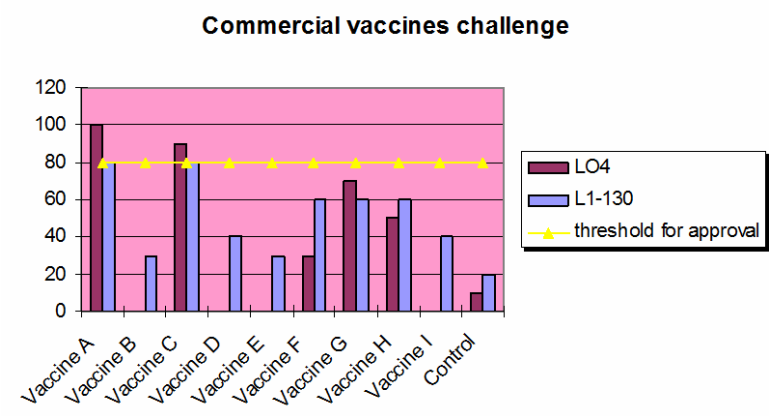


**Figure 1.** Vaccines performance against challenge inoculums in relation to survival animal rate that were challenged with $LO_4$ and $L_1$ 130 strains.

Table 2 shows survival hamsters' proportion to a challenge with $LO_4$ and $L_1$ 130 strains and vaccinated with commercial bacterins that presented renal carrier state, confirmed by kidney tissue leptospira isolation in Fletcher semi-solid culture media.

**Table 2.** Proportion of hamsters immunized with canine anti-leptospirosis bacterins that survived to a challenge with pathogenic leptospiras and characterized as kidney carriers at 21 days after challenge according to bacterin identification code and challenge strain used.

| Vaccine | Canicola serovar, $LO_4$ strain | Copenhageni serovar, $L_1$-130 strain |
|---|---|---|
| A | 0/10* | 5/8 |
| B | ... | 3/3 |
| C | 1/9 | 3/8 |
| D | ... | 4/4 |
| E | ... | 3/3 |
| F | 1/3 | 5/6 |
| G | 0/7 | 5/6 |
| H | 0/5 | 5/6 |
| I | ... | 4/4 |
| U** | 0/1 | 2/2 |

... = not performed, * number of animals with positive leptospira kidney culture/number of challenge survivors; ** control (unvaccinated).

Figure 2 simultaneously shows the proportions of hamsters that survived to the challenge and the percentage of survivors characterized as leptospira kidney carriers for challenges performed with $LO_4$ strain Canicola serovar.

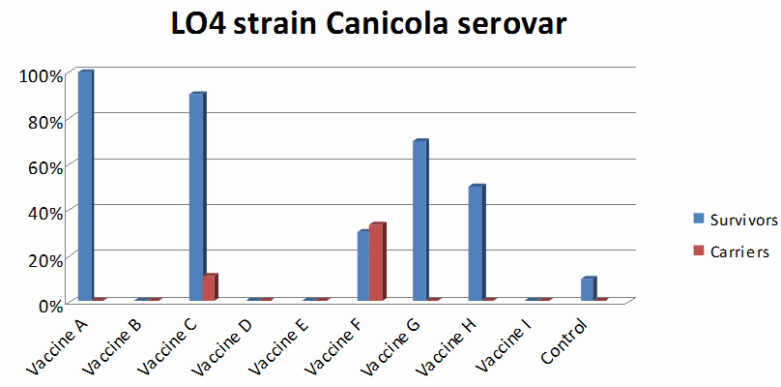


**Figure 2.** Proportion of hamsters immunized with canine anti-leptospirosis bacterins that survived the challenge and characterized as kidney carriers at 21 days after challenge according to bacterin identification code.

Figure 3 simultaneously shows the proportions of hamsters that survived to the challenge and the percentage of survivors characterized as leptospira kidney carriers for challenges performed with Copenhageni $L_1$-130 serovar.

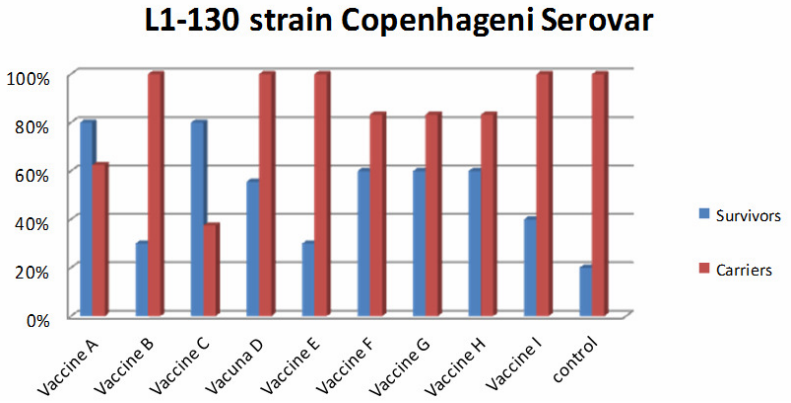


**Figure 3.** Proportion of hamsters immunized with canine anti-leptospirosis bacterins that survived the challenge and characterized as kidney carriers at 21 days after challenge according to bacterin identification code.

## 4 Discussion

Different results were observed for the nine vaccines evaluated, considering the number of challenge survivors as well as the number of survivors characterized as leptospira kidney carriers.

Anti-leptospirosis potency test recommended by the Ministry of Agriculture of the United States of America (UNITED STATES, DEPARTMENT OF AGRICULTURE, ANIMAL PLANT HEALTH INSPECTION SERVICE, VETERINARY SERVICES LABORATORIES, 1977) validates the vaccine when the proportion of leptospirosis deaths in the control unvaccinated group is equal or higher than 8/10 and in the vaccinated group this proportion is not superior than 2/10 or 5/20.

Challenge tests results with the serovars Canicola $LO_4$ strain and Copenhageni $L_1$-130 strain in vaccinated hamster with commercial anti leptospira bacterins A and C are in agreement with parameters required and these vaccines were approved according to the international evaluation criteria (UNITED STATES, DEPARTMENT OF AGRICULTURE, ANIMAL PLANT HEALTH INSPECTION SERVICE, VETERINARY SERVICES LABORATORIES, 1977) which are 80% of surviving animals for the bacterin dilution recommended for hamsters. The other vaccines were not approved, as they did not present the expected protection against the serovars that they were challenged.

The proportion of vaccinated hamsters that survived to the challenge with Copenhageni serovar $L_1$-130 strain and characterized as kidney carriers at the 21$^{st}$ d.a.i showed that protection conferred by the two approved vaccines (A and C) was not satisfactory, because even though the challenged was performed with half of the lowest value established by CFR, there was a high number of leptospira kidney carriers among survivors. In fact, for Copenhageni serovar $L_1$-130 strain the vaccines responses were not satisfactory, as none of the tested vaccines protected against infection with this serovar. Considering disease protection there was a better result for vaccines A and C. However, for vaccine A among eight survivors five presented kidney colonization, for vaccine C among eight survivors three presented kidney colonization and for the others vaccines all survivors became kidney carriers. Considering

Canicola serovar $LO_4$ when it was used the LD50 upper threshold of recommended range (10 to 10.000) the results were satisfactory since vaccines A and C had positive results for challenge and also avoided kidney infection. With vaccine A none of ten survivors presented kidney colonization and with vaccine C only one among nine survivors presented kidney colonization. Vaccines A and C were approved at the challenge test, provided protection against clinical disease, death and kidney infection for Canicola serovar $LO_4$, but for Copenhageni serovar $L_1$-130, they provided protection against clinical disease and death but not against kidney infection.

It is noteworthy that both approved vaccines (A and C) didn't have Copenhageni serovar in their formula and the possible protection against Copenhageni serovar $L_1$-130 clinical disease and death may be due to cross protection with the Icterohaemorrahagiae serovar that is included in both formulas. Similar result was obtained by Tabata (TABATA *et al.*, 2002) that found cross protection among members of Sejroe serogroup. Cross reactions between serovars of the same or different serogroups are usual in natural infections or during post-vaccination period (GREENE, SCHULTZ, 2006).

In relation to the results for challenges made with $LO_4$ strain Canicola serovar, it was possible to verify that although bacterins A and C are imported and manufactured with reference strains of this serovar, they conferred good protection against disease and infection for this serovar but for the other seven brands of vaccine tested, including national and imported ones, the protection was not satisfactory.

Considering the results of partial protection observed for challenges made with $L_1$-130 strain Copenhageni serovar in animals immunized with imported bacterins A and C and produced with reference strains of Icterohaemorrhagiae serovar, it has to be considered that Cho *et al.*, (1992) verified that surface lipid of Icterohaemorrhagiae serovar have an exclusive specificity and that the Copenhageni serovar have some antigenic components that are absent in Icterohaemorrhagiae serovar (ARIMITSU *et al.*, 1980). Thus, based in these differences dogs immunized with vaccines that don't contain Copenhageni serovar in their formula may be infected by it (RODRIGUES, 2008).

The low performance of canine anti-leptospirosis vaccines commercialized in Brazil verified in this study showed that canine leptospirosis prevention is compromised and that may have consequences in animal health as well as in veterinary public health, since dog may be an important source of infection for human and animals.

Under the conditions of the present study it is concluded that from nine canine anti-leptospirosis vaccines brands commercialized in Brazil and submitted to a potency test with challenge in hamsters and indigenous strains of Canicola and Copenhageni serovars, only two were approved.

Despite the protection conferred by two imported canine anti-leptospirosis bacterins, manufactured with reference strains of Icterohaemorragiae serovar, have been sufficient to protect animals against clinical disease, this protection was not capable to protect against kidney carrier state for Copenhageni serovar.

Protection conferred by two imported canine anti-leptospirosis bacterins and manufactured with reference Canicola serovar strains were sufficient to protect animals against

disease, infection and kidney carrier state in a challenge performed with indigenous strain of Canicola serovar.

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