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Original article

Inmunoprotector potential of cellular vaccine formulations developed from *Leptospira interrogans* Ballum using *Mesocricetus auratus* as biomodel

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Background: In the last years, *Leptospira spp* Ballum has increased its representation in human clinical isolations in Cuba. Effective vaccines are needed to control this zoonotic disease.

Objective: With the objective of developing a new vaccine candidate able to generate an effective protection against this serovar, two monovalent formulations developed by two highly virulent strains were evaluated (FoBa and FoBb).

Methods: Clinical isolates of *Leptospira* serovar Ballum were subjected to serial passages in hamsters and monovalent vaccines were produced by modified methods developed for vax-SPIRAL®. The vaccine efficacy was tested in both experimental and control hamsters.

Results: The *Mesocricetus auratus* biomodel showed that both formulations generated a protection of 100% against the Ballum lethal infection together to high levels of IgG antibodies and were efficient in the elimination of homologous carrier state but not heterologous carrier states.

Conclusion: Both FoBa and FoBb vaccines were protective against leptospirosis with high IgG titers, absence of clinical signs and dead, and absence of leptospires in kidney of sacrificed animals.

Keywords: *Leptospira*, leptospirosis, *Mesocricetus auratus*, vaccine

Leptospirosis is one of the bacterial zoonoses more diffused and disregarded in the world [1]. The causal agent of this pathology belongs to the family Leptospiraceae, which are grouped into at least four saprophytic and 12 pathogenic species that include around 250 serovars [2]. The immunity against leptospirosis in most of the susceptible species is mainly of the humoral type and the immundominant antigen is the lipopolysaccharide (LPS) [3]. These microorganisms are resistant to the bactericidal activity of the normal serum and in absence of specific antibodies they are neither phagocytosed nor destroyed by the macrophages [4]. The immune response is implied in the pathogenesis of the leptospirosis through the immune complex formation, the cytokines liberation, and the generation of an autoimmune vasculitis. This way, the signs and symptoms of the lung, renal and hepatic involvement, appear in the immune phase when the specific antibodies begin to be detected [5]. Humans and animals become infected when they are directly exposed to pathogenic *Leptospira* from other infected animals or indirectly by contact with soil or water that has been contaminated with the urine of animals shedding the microorganism. Due to the morbidity and mortality in animals and man, as well as for their economic repercussion in the developed and developing countries, it constitutes an important and permanent concern for the human and veterinary medicine [4, 6, 7].

The currently available antileptospiral commercial vaccines are killed, whole-cell vaccines, with or without adjuvant that include in their formulations the serovars of common circulation in the region selected for their application [4, 8]. For a leptospirosis vaccine

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to be effective, in addition to being immunogenic and safe they must prevent both acute leptospirosis and the carrier state [9]. These biopharmaceutical products are available in some countries and they have some main disadvantages such as the generation of hypersensitivity reactions, a protection of short duration, and the lack of cross-protection against the serovars not included in the evaluated formulation [6, 10]. Killed vaccines are cheap and very simple to produce but many times may fail to prevent renal shedding in infected animals [11]. Other leptospirosis vaccines currently under development or in trials include recombinant protein vaccines, LPS vaccines, inactivated and attenuated vaccines [12].

In Cuba from the 90′s, it has been administered to the human population, a trivalent vaccine (vax-SPIRAL®) conformed by the serovars: Canicola, Copenhageni and Mozdok. The effectiveness and safety of this product to control and to diminish the lethality of the human leptospirosis have been confirmed thoroughly [13]. However, in the last years they have been changes in the immune epidemiological situation of leptospirosis in Cuba and the serovar Ballum has become one of common circulations and incidences together with Canicola, Mozdok, and Copenhageni [14]. Studies carried out in the Golden/Syrian hamsters (*Mesocricetus auratus*) biomodel [15, 16] have determined that the immunization with vax-SPIRAL® only protects in a range of 50-60% against the lethal challenge with Ballum virulent strains [17], which presupposes that with this vaccine the protection levels required to avoid the development of the leptospirosic illness are not reached. The objective of the current study was to develop killed vaccines from local isolates of *Leptospira interrogans* Ballum and determine their efûcacy (protection and immunogenicity) against Ballum and heterologous serovars included in vax-SPIRAL®.

Materials and methods

Bacteria strains

The selection of the vaccine strains was performed by starting from four clinical isolations of *Leptospira* serovar Ballum provided by the Provincial Centers of Hygiene and Epidemiology of Cuba. The strains belonging to serovar Canicola, Copenhageni and Mozdok used at challenge assays, were donated by Quality Laboratories of the Finlay Institute. Isolates had been sub-cultured a maximum of three times before storage at -70ºC.

Source and care of hamsters used in the study

Golden/Syrian hamsters (*Mesocricetus auratus*) were obtained from CENPALAB, Havana, Cuba. They were cared for and bred at the Animal House facilities of the Vice-presidency Research of Finlay Institute, Havana. Ethical approval for the study was obtained from the Ethics Committee of the Finlay Institute, Cuba.

Recovery of virulence through serial passage of leptospire isolates in hamsters

Isolates were passaged 4 times in golden/Syrian hamsters as previously described [16]. Hamsters weighing less than 55 g at approximately 4 weeks old were used, and two hamsters were used per isolate per passage. Brieûy, selected leptospires were grown in liquid Ellinghausen–McCullough/Johnson–Harris (EMJH) media (Becton Dickinson Microbiology Systems, Maryland, USA) to a density of 1×10^8 leptospires per ml. Each pair of hamsters was placed in separate cages and was inoculated intraperitoneally, using 1ml of culture by using a sterile tuberculin syringe with a 23 gauge 3/4 inch needle. Four days after inoculation, one hamster was sacriûced to recover the virulent leptospires while the other was maintained and the progression of disease monitored. Hamsters were euthanized in accordance with the guidelines published by the American Veterinary Medical Association (AVMA) [18]. Firstly, hamsters were placed in a chamber where they were exposed to carbon dioxide. When unconscious, (as evidenced by lateral recumbency and loss of pedal reûex), they were anaesthetized using a combination of 10% ketamine (Dutch Farm Veterinary Pharmaceutical Company, Barneveld, Holland) and 2% xylazine (Bromazine; Bomac Laboratories, Wiri Station Road, Manukau City, Auckland, New Zealand) administered intramuscularly at a dose of 100 mg/kg ketamine and 10 mg/kg xylazine in combination [18]. If the desired response was not achieved, more of the mixture was administered. The kidneys were harvested and macerated, and the resulting homogenate (containing leptospires) was mixed with 1ml of EMJH liquid medium, 100 µl of the neat suspension was then added to second tube of EMJH to produce a 1 in 10 dilution. One hundred microliters of each suspension was inoculated onto individual tubes of semi-solid EMJH culture media. A further 0.2 ml of the neat suspension was inoculated into another hamster for further passage.

LD_{50} determination

The two most virulent and immunogenic of the ten isolates subjected to serial passage in hamsters were chosen for LD_{50} determination, i.e. those isolates with the shortest time to death post-inoculation and the ability to elicit antibody response as determined by serum titres obtained in microscopic agglutination test (MAT). A total of 112 hamsters (9 weeks old) were used, 56 for each isolate, and were divided into 7 groups, each containing 8 hamsters which were given different concentrations of microorganism (control, 10^0,10^1,10^2,10^3,10^4,10^5 leptospires) intraperitoneally in 1ml quantities as described by Silva et al. [19]. Controls were administered the EMJH media used to grow the leptospires, while inoculated groups were given the leptospires suspended in their respective growth media. The animals were monitored twice daily for up to 21 days post-challenge but were euthanized whenever signs of terminal disease appeared (severe dehydration, anorexia and immobilization). From the results of the LD_{50} testing, the concentrations required for the vaccines were obtained using the Reed–Muench method [20].

Vaccine preparation

For the generation of monovalent vaccines of Ballum the production methodology developed for vax-SPIRAL® was used [21], with slight modifications. The selected isolates were propagated for 3 weeks in EMJH medium, until dense cultures were observed using dark-ûeld microscopy Olympus BX51 microscope with a dark-ûeld condenser. The leptospires were washed 3 times by centrifugation at 10,000×g for 30 minutes and resuspended in 40 ml of phosphate buffered saline (PBS). After the ûnal wash 0.5% neutral buffered formalin was added for 20 minutes, and the pellet was then similarly washed in tampon saline phosphate four times. The suspension was then diluted to a concentration of 6×10^8cells/ml. Aluminium hydroxide (1.0 mg/ml) was added as adjuvant and tiomersal (0.05 mg/ml) as preserve [22]. The vaccine produced was inoculated onto semi-solid EMJH medium to conûrm that there were no viable leptospires and a loopful onto blood agar and broth thioglycolate medium which was incubated at 37°C for 24 hours to conûrm the absence of aerobic and anaerobic microorganisms respectively. Microscopic agglutination test (MAT) was then used to verify that the strains were pure and of their original identities [23]. The vaccine preparations with satisfactory results in all the carried out controls were packed aseptically in volumes of 5.2 mL, using bulbs of glass of 10 mL with rubber cover and protective metallic and conserved 4°C until their use.

Experimental challenge of vaccinated, control animals and Post-mortem examinations

For the vaccine trial, three groups, each composed of 20 hamsters, aged four weeks, were used. A group 1 was inoculated with a formulation derived from strain FoBa, the group 2 with a formulation derived from strain FoBb and the third group was inoculated with PBS as a control. Animals were each administered (via intramuscular) a series of two inoculations, 6 weeks apart.

Blood was collected by venipuncture of the lateral saphenous vein [24] using microcapillary tubes before each inoculation, prior to challenge (2 weeks after inoculation) and then at death or 28 days post-inoculation at euthanasia

The challenged trial was conformed as it is described on **Table 1**. Animals were challenged (intraperitoneally) with 100 times LD_{50} of each isolates. These isolates were derived by culture of the tissues of the hamsters used in the fourth passage. Descriptions of the safety control groups of each formulation evaluated and placebos can be seen in **Table 2**. Before each inoculation, serum samples were taken from hamsters in the same vaccine group; these were pooled and tested for the presence of anti-leptospiral antibodies using ELISA [25].

Animals were monitored twice daily (for any sign of illness such as external hemorrhage, dehydration, rufûed hair coat, decreased activity and isolation from other hamsters in the cages) for up to 21 days post-challenge. The criteria used to determine the efûcacy of any of the vaccines were whether 80% of the immunized animals survived and 80% of the controls died [26].

Post-mortem examinations were performed and the liver and kidneys were harvested for in order to prove Koch's postulates [27]. To each animal were extracted both kidneys and the liver for their cultivation in EMJH.

Statistical issues and analyses

Data was analyzed at a 5% signiûcance level using STATISTICA version 6.1 (USA). Chi-square analyses were performed to determine if there were signiûcant differences in survival rates.

Results

Determination of virulence and LD_{50}

Only two isolates (i.e. FoBa and FoBb) were LipL32 positive (unpublished data) and tested as potential vaccine candidates due to high virulence (**Table 1**). These strains caused death within 11 to 14 days of inoculation. The non-vaccine candidate strains showed different survival behavior. Canicola tested caused death within 5 to 14 days of inoculation; on the other hand, Mozdok and Copenhageni caused death within 7 to 16 days. The LD_{50} value of FoBa and FoBb strains were 11 and 9 organisms, respectively. The statistical comparison between these results did not show significant differences among them.

Table 1. Phenotype classification and virulence of the study strains.

Strain	Phenotypic complex/ serovar	LD_{50} (CFU/mL)
FoBa	*Leptospira interrogans* Ballum	11
FoBb	*Leptospira interrogans* Ballum	9
FoBc	*Leptospira interrogans* Ballum	21
FoBd	*Leptospira interrogans* Ballum	19
CE1	*Leptospira interrogans* Canicola	4
CE2	*Leptospira interrogans* Mozdok	6
CE3	*Leptospira interrogans* Copenhageni	8

LD_{50} = Letal medium dose, CFU = colony former unit

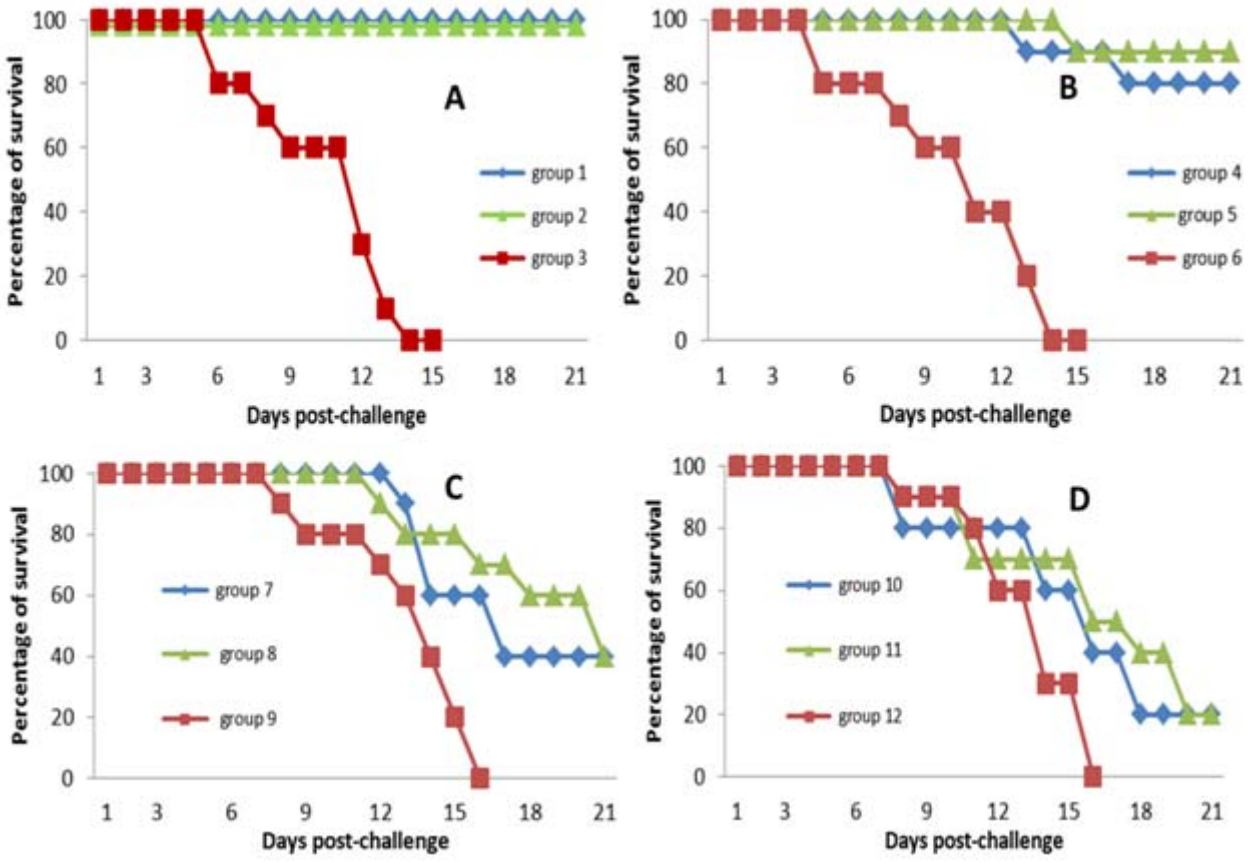

Figure 1. Percentage of hamsters surviving immunized with the formulations FoBa (blue line), FoBb (green line), and unimmunized (red line) 21 days post-challenge with **A**) Ballum, **B**) Canicola, **C**) Mozdok, and **D**) Copenhageni.

Experimental vaccination and assessment of carrier state

Table 2 summarizes the results of the vaccination, challenge and the titres obtained for each group from the time of the ûrst dose of vaccine until the end of the experiment are shown in **Figure 2**. Hamsters vaccinated with the experimental vaccines (FoBa and FoBb) belonging to groups 1 and 2, showed no clinical signs of leptospirosis and there was no post-challenge mortality. These animals were also culture-negative with no gross or histological pathological lesion. The IgG response against Ballum was revealed in 70% of the animals immunized with FoBa and in 80% of the group immunized with FoBb. After applying the second dose, the values of IgG generated by both monovalent vaccines were very significant regarding the values obtained after the first dose. Also 100% of the animals in the immunized groups were positive.

Mortalities in vaccinated groups challenged against heterologous strains, i.e., groups 3-12, occurred after the vaccination period. For the groups of hamsters inoculated with monovalent formulations and challenged against Mozdok and Copenhageni (groups 7, 8, 10, and 11) severe clinical signs were observed in 80 to 100% of individuals between 8 to 20 days post-challenge. For groups 4 and 5, the majority of deaths, 20 and 10% occurred between 12 and 20 days post-challenge respectively. In all cases, the titres remained near to zero similar at the unvaccinated control groups. In contrast, animals from groups 13-15 did not show mortality. The unvaccinated challenged hamsters displayed the greatest morbidity with 100% mortality.

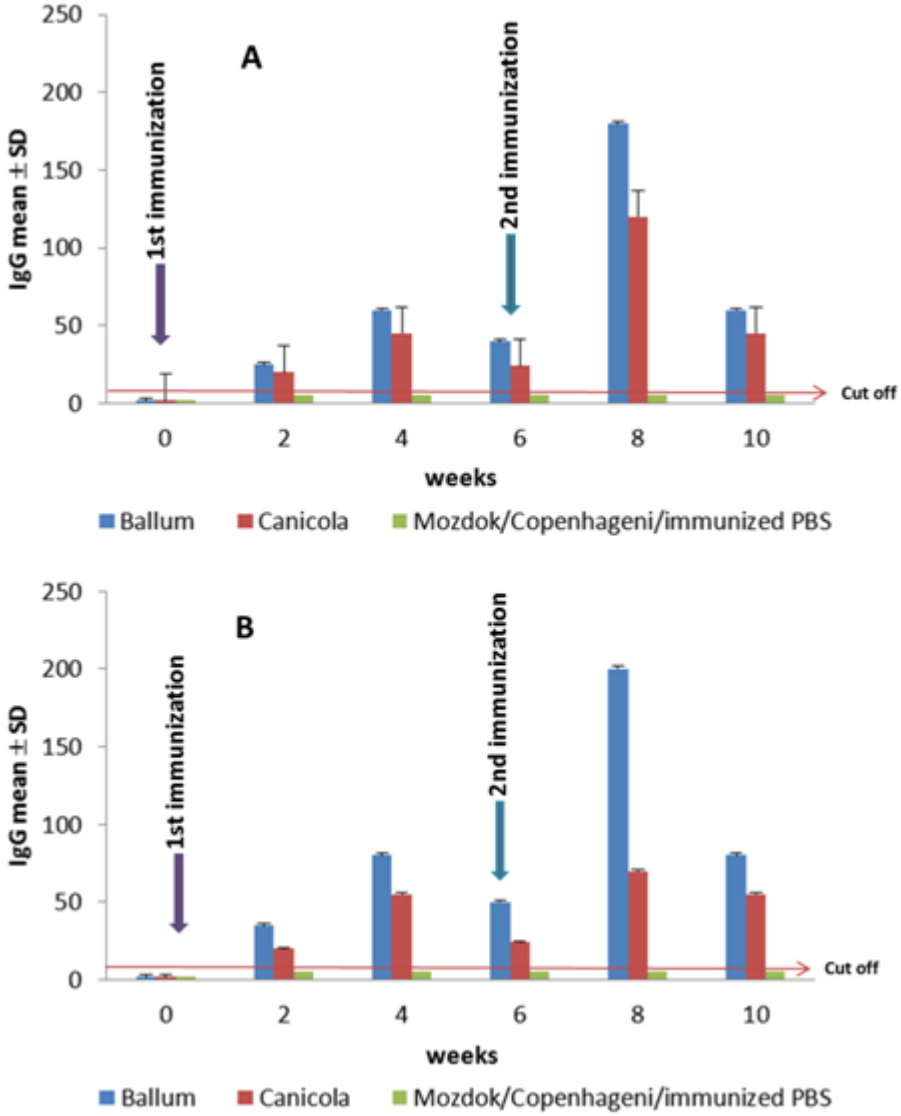

Figure 2. IgG response induced in hamsters against strains of different *Leptospira* serovars after the immunization with two doses of monovalent formulation of **A**) FoBa and **B**) FoBb.

Table 2. The groups of hamsters used in the experiment as well as the results of culture, mortality observed in each group

Group number	Treatment	Serovar of challenge	Number in group pre-challenge	Mortality (%)	# culture positive/ # livers evaluated	# culture positive/ # kidneys evaluated
1	FoBa	Ballum	10	0	0/10	0/20
2	FoBb		10	0	0/10	0/20
3	unimmunized group		10	100	10/10	20/20
4	FoBa	Canicola	10	40	0/10	0/20
5	FoBb		10	30	0/10	0/20
6	unimmunized group		10	100	10/10	18/20
7	FoBa	Mozdok	10	60	8/10	16/20
8	FoBb		10	60	8/10	12/20
9	unimmunized group		10	100	10/10	20/20
10	FoBa	Copenhageni	10	80	10/10	20/20
11	FoBb		10	80	8/10	16/20
12	unimmunized group		10	100	10/10	20/20
13	FoBa immunized	not challenged	10	0	0/10	0/20
14	FoBb immunized		10	0	0/10	0/20
15	unimmunized		10	0	0/10	0/20

Organ culture results from animals that died or were euthanized are summarized in **Table 2**. Animals from the groups 3, 6, 9, and 12 (non-vaccinated but lethally challenged animals) and hamsters immunized with FoBa or FoBb but challenged with Mozdok and Copenhageni displayed high levels of Leptospira-positive cultures of kidney and liver. By contrast the organ culture derived from the groups 1, 2, 13 to 15 were negative for the leptospira presence.

Discussions

One of the first steps for developing a whole cell vaccine is the correct selection and characterization of the vaccine candidate strains [8]. In this sense, the Commission of Experts of the WHO recommends that the vaccine strains should belong to the serovars toward which it is wanted to confer the protection and to be characterized by a high virulence [8, 28]. All four of the isolates tested as vaccine candidates were positive for the *lipL*32 gene that is found in pathogenic *Leptospira* [29]. However, only the FoBa and FoBb strains were highly virulent in the hamster model. The loss of the virulence of isolated leptospires of clinical samples is a phenomenon that is frequently observed due to the fact that the number of subcultures correlates negatively with the maintenance of virulence and growth. Some examples of virulence factors of *Leptospira* present only in pathogenic strains and that get lost with subcultures in microbiological medium are FlaA and FlaB proteins involved in the bacteria motility [30] and the proteins LigA and LigB, which bind to fibronectin [31]. LD_{50} experiments results showed that hamster death occurred between 7 to 15 days post-challenge. The timing of these deaths and clinical signs observed were consistent with those of other LD_{50} experiments in hamsters using these serovars [32].

When evaluating the protector capacity of the monovalent formulations FoBa and FoBb against the homologous lethal challenge, it was demonstrated that the same ones were very efficient when protecting 100% of the challenged animals. On the other hand, the cross-protection against the rest of the serovars was more limited, observing for both formulations a similar behavior. The statistical results demonstrated differences for Canicola with regard to Copenhageni and Mozdok, which allows us to affirm that under the present experimental conditions a cross-protection is appreciated between Ballum and Canicola strains. The studies guided to demonstrate the existence of cross-protection among different serovar from *Leptospira* have been very few. Until some years ago, it was established that the protection conferred after a natural infection or immunization with killed whole cell vaccine and LPS vaccine was serovar/serogroup-specific [33]. However, the concepts about the immunity against the leptospirosic infection have been modified in light of the current knowledge. Very recent works have reported experimental results that demonstrate the induction of a statistically significant cross-protection as a result of the immunization of protein fractions of strains of the serovar Autumnalis against of the challenge with Canicola in gerbils [34].

The results obtained revealed the absence of characteristic signs of the infection in the immunized animals challenged with Ballum serovar strains. Equally, the culture of several sections of organs of the leptospirosic illness demonstrated that in all the immunized animals challenged with Ballum and Canicola strains, there was absence of microbial growth after 60 days of incubation. In contrast, a high percentage of cultures of kidney tissue of animals immunized and challenged with Mozdok and Copenhageni were positive, suggesting that the vaccine formulations is only effective to prevent renal colonization against Ballum and Canicola serovars. The statistical comparison between both monovalent vaccine formulations relating to ability of eliminate the carrier state did not show differences. Although the results of the culture of organs in the EMJH medium obtained from the immunized animals showed negative results, it is possible that tissue colonization may be detected if more sensitive methods, such as PCR, were used [35]. In addition, histopathologic findings of target organs are important to support the efficacy of the vaccine to prevent morbidity.

Both of our in-house vaccines were efûcient in protecting against leptospirosis generated by strains of the serovar Ballum and Canicola, as no clinical signs were observed, no leptospires were detected in the kidney, and no deaths occurred. Future experiments would also include testing the effects of a combined vaccine based on serovars Canicola, Mozdok, Copenhageni and Ballum FoBa / FoBb or the effect of an immunization with one monovalent FoBa/ FoBb in animals immunized previously with vax-SPIRAL®.

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