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Efficacy of Leptospiral vaccine (vax-SPIRAL®) against challenge with strains isolated from leptospirosis epidemic in Nicaragua using the hamster as biomodel

Luis Alfredo Rosario Fernández[1]*, Niurka Batista Santiesteban[2], Daniel Francisco Arencibia Arrebola[2], Bárbara Yolanda Valdés Abreú[2], Willian Jirón Toruño[3], Chistyane Duttman[3]

1. Institute of Pharmacy and Food Science (IFAL, UH), 222 street between 25 and 27 avenue. Lisa Municipality, Havana, Cuba. 2. Research Vicepresident (Finlay Institute), 17 avenue between 198 and 200 street. P.A.16017, P.C. 11600, Playa Municipality, Havana, Cuba. 3. Veterinary School, Autonomous National University of Nicaragua (UNAN), León, Nicaragua.
*Corresponding author email: darencibia@finlay.edu.cu, nbatista@finlay.edu.cu
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Abstract

A hamster model was used to determine the protector capacity of vax-SPIRAL® against the lethal infection after challenge with 10,000 LD_{50} of four strains isolated of a leptospirosis outbreaks in Nicaragua. These strains were selected from a primary group of 16 belonging to the serogroups strains Ballum, Icterohemorragiae, Pomona, Serjoe and Pyrogenes. Most of these, except Pyrogenes serogroup, reproduced the infection in animal model and the most virulent representatives of each serogroup were selected for the challenge. The results of this study suggest that vax-SPIRAL® conferred high percents of heterologous protection which aimed to consider it as a prophylactic measure useful in situations of floods and natural disasters in Nicaragua.
Keywords: *Leptospira*, Heterologous protection, Natural Disaster, Biomodel, Endemic disease, Serogroup.

Introduction

Leptospirosis is a zoonosis caused by a large group of pathogenic *Leptospira* comprises about 250 serovars grouped into 20 genomic species (1). It is a disease of worldwide distribution where the man is an accidental host (2). Contamination of drinking water with sewage waste is the major risk factor in cases of floods and natural disasters. That is why vaccination is recommended for people exposed to this kind of risk especially in those countries where natural events occur that lead to the development of outbreaks in certain seasons (3). However, despite its growing incidence in humans, this practice has not been widespread and only a very small number of countries that apply it, mainly due to the low availability of vaccines for humans and the little information concerning to them (3, 4).

At present, studies on leptospirosis vaccines have focused on developing vaccine candidates based on lipopolysaccharides (5,6) lipoproteins (7,8), outer membrane proteins (9,10) and potential virulence factors (11,12). The aim of these has been to eliminate the disadvantages of the use of inactivated/killed whole cell vaccines.

However, any vaccine based on these principles will be available in a short term, although much progress has been made in addressing new strategies for developing leptospiral vaccine safely and effective. The inactivated whole cell vaccines have been widely used in countries such as China, Japan, Israel, Russia and Cuba and are currently the only permissible alternative to combat the scourge of the disease in spite of the main drawbacks reported: reactogenicity and limited protection to serovars present in the formolution.

In Cuba was designed, developed and evaluated a trivalent leptospiral vaccine adsorbed on aluminum hydroxide gel, consisting of inactivated whole cells of serovars indigenous strains: Canicola, Copenhageni and Mozdok. Product registration was achieved in 1998 under the trade name of vax-SPIRAL® (13), with

satisfactory results of safety, immunogenicity and protective homologous capacity (14-16). Its use in the population has shown an overall efficiency in the field of more than 60% which has contributed significantly to reduce morbidity and mortality caused by this disease in Cuba (17).

In October 2007, after hurricane Felix whipped Nicaragua was reported a total of 6334 suspected cases of leptospirosis. With the aim of combating the epidemic was requested immunization of the affected population with vax-SPIRAL® but due to the intrinsic limitations of this vaccine was necessary to conduct an experimental study in the biomodel Syrian hamster (*Mesocricetus aureatus*) that would evaluate the protective capacity of this vaccine against strains that was circulating in that country. These results were necessary to generate an approach to the commercial house about recommending or not the formulation to Nicaraguans sanitary authorities to combat the outbreak.

Materials and Methods

Bacterial strains and serological classification: Sixteen strains of clinical isolates recollected during the period October-November 2007 from the departments of Leon and Chinandega (after hurricane Felix whipped Nicaragua) was used in this study. These strains were classified to serogroup by the microscopic agglutination test (MAT) (18, 19), using reference polyclonal antisera produced in the International Reference Laboratory WHO (Tropical Medicine Institute, Netherlands). All strains were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) (Difco Labratories) liquid media at 28 ° C and were subsequently stored at -70°C in liquid nitrogen and Fletcher's semisolid media (20,21).

The strains belonging to serogroups Pomona and Icterohaemorrhagiae were faced also by MAT to the reference monoclonal antibodies F70C7, F70C24 (*L. interrogans* serovar Copenhageni) and F46C5, F46C9, F58C2 (*L. interrogans* serovar Mozdok) respectively obtained from Leptospirosis Reference Laboratory WHO (Netherlands).

Animals: Female Syrian hamsters (CENPALAB, Havana) 3–4 weeks old were used in the study. The animals had *ad libitum* access to a commercial pelleted ration and drinking water. The experiments were conducted according to the protocol approved by IACUC (Institutional Animal Care and Use Committee) at Finlay Institute to comply with biosecurity principles. All work was conducted in compliance with regulations, policies, and principles of the Animal Welfare Act; the Public Health Service for Policy on Humane Care and Use of Laboratory animals used in Testing, Research, and Training; the NIH Guide for the Care and Use of Laboratory Animals.

Evaluation of virulence: The virulence of all isolates was estimated qualitatively in the Syrian hamster model, according to the methodology described by González and colleagues (22). Groups of five animals of 45-50 g, were injected intraperitoneally with doses of 0.8, 0.4, 0.1 and 0.05 mL of a bacterial suspension adjusted to 7.5 x 10^7 cells / mL (direct counting in Petroff-Hausser chamber) (Fisher Scientific) (23). This bacterial suspension came from a static culture of 5-6 days in EMJH media. The inoculated animals were observed over a period of 14 days post infection to evaluate the occurrence of clinical signs, including the presence of nasal bleeding, dehydration, pilo erection and prostration. Animals that showed symptoms and signs of leptospirosis were sacrificed following the code of ethics of animal protection (24) and kidneys were cultured in EMJH liquid media at 28°C. The strains were considered highly virulent if the desease occurred at least in all the animals inoculated with doses of bacterial suspension 0.1 mL. If the desease of the inoculated animals took place only at dose 0.4 mL the strain was considered of moderate virulence and for lower doses levels the strains were considered of low virulence or avirulent. Only highly virulent strains were used in the study of median lethal dose (LD_{50}).

LD_{50} determination in hamster: The median lethal dose was evaluated according to the methodology proposed by Reed and Muench (25) in three different tests with different batches of animals. The animals were observed until 15 days

after infection. As negative control were used animals inoculated with EMJH liquid media.

Evaluation of the vax-SPIRAL® protection against the challenge with the virulent strains: For the evaluation of heterologous protection (or crossprotection) of the vaccine, 50 female hamsters were immunized (n = 10 per group) with vax-SPIRAL®. The animals received two doses of 0.5 ml, according to route, dose and schedule proposed by Naranjo and colleagues (26). Fourteen days after completing the scheme, they were challenged intraperitoneally with 10,000 LD_{50} (0.5 ml of inoculum) of each of the highly virulent strains. Fifty un-immunized animals were used as control testing and were inoculated with a dose of challenge isolates as described above. The animals were kept under appropriate conditions and were observed daily for a period of 14 days after which the survivors of each group were euthanised.

The prevalence of the microorganism in the main target organs of *Leptospira* infection were determined by a microbiological culture of liver and kidney in EMJH media (27). The organ cultures were observed periodically under the dark field microscope (Standard Option 25) and it was considered as a negative result the absence of growth after 60 days of incubation at 30°C under static conditions.

Statistical analysis: Data was analyzed at a 5% significance level using STATISTIC version 6.0 (Statsoft®, Texas, US). Chi-square ($\chi 2$) analyses were performed to generate a contingency table with the purpose of determine whether animal survival was due to vaccination and also to compare the proportions between the different experimental groups.

Results

Serological classification of clinical isolates: The sixteen clinical isolates were confirmed as *Leptospira spp*. These strains were then classified to serogroup (Table-1) by MAT with polyclonal serum reference. The strains belonging to serogroups Pomona and Icterohaemorrhagiae were faced with specific monoclonal antibodies to serovars *Mozdok* and *Cophenageni* and results obtained allowed affirm that none of the isolates coincided serologically with the strains present in the vax-SPIRAL®.

Virulence and LD_{50} determination: All the strains belonging to serogroups Icterohaemorrhagiae, Pomona, Serjoe and Ballum reproduced the infection in hamsters, with differences between them respect to the levels of lethality (Table-1). The most virulent strains of each of these serogroups were listed with the codes 3507, 6307, 8807 and 4207. In contrast none of the strains belonging to serogroup Pyrogenes caused infection in animals, not even after three passes in animals.

LD_{50} average values of the strains 6307 and 8807 required inocula between 30 and 35 leptospira cells to produce infection and

Table-1. Serologic classification and virulence characteristic of the strains used in the study.

No.	Serogroup	Code strain	Origin	Cualitative virulence classification
1	Icterohaemorrhagiae	3507	León	High virulence
2	Icterohaemorrhagiae	3504	Chinandega	Low virulence
3	Icterohaemorrhagiae	3506	León	Moderate virulence
4	Pomona	6307	Chinandega	High virulence
5	Pomona	6308	León	Low virulence
6	Pomona	6310	Chinandega	Low virulence
7	Pomona	6309	León	Moderate virulence
8	Ballum	8807	Chinandega	High virulence
9	Ballum	8806	León	Moderate virulence
10	Ballum	8808	Chinandega	Low virulence
11	Serjoe	4206	León	Low virulence
12	Serjoe	4207	Chinandega	High virulence
13	Serjoe	7407	León	High virulence
14	Pyrogenes	8707	Chinandega	Avirulent
15	Pyrogenes	3907	León	Avirulent
16	Pyrogenes	5007	Chinandega	Avirulent

subsequent death of the infected animals, while strains 4207 and 3507 needed an inocula below the seven leptospira. These values showed, in general, little variability between trials for the same strain. In all cases, clinical signs could be observed from the third until the eighth day post-inoculation.

Evaluation of the vax-SPIRAL® protection against the challenge with virulent strains: The results of the active protection of hamsters immunized with vax-SPIRAL® and challenged with strains of high virulence showed high percentages of protection. Figure- 1 shows a 100% mortality in animals unimmunized and challenged.

The statistical comparison demonstrated that they do not exist significant differences among the values of the survival percentages of animals immunized with vax-SPIRAL® and subsequently challenged with Pomona, Icterohaemorrhagiae and Ballum. On the other hand were observed differences among these three groups regard to Serjoe.

In groups where some animals died may be noted that these deaths happened from day 10. Macroscopic analysis of the organs of the immunized animals that survived the challenge, in all cases revealed the absence of characteristic signs of infection (inflammation, hardening, bleeding or jaundice). Besides the organs cultures of in EMJH media showed the absence of microbial growth after 60 days of incubation. Contrary, the unimmunized animals where when examining the organs we showed all the hallmarks of this entity and the cultures of sections of organs such as liver and kidney showed the growth of *Leptospira* (Table II).

Discussion

Traditionally it has been suggested that leptospirosis vaccines obtained by whole-cell inactivation provide specific protection to antigenically related serovars that are part of that vaccine formulation (28-30). The lack of cross protection among some serovars, the need for annual or biannual revaccination and the high frequency of side effects are serious drawbacks that have limited the availability of these vaccines for human use. However, studies with vax-SPIRAL® in Cuba show that this is safe, low reactogenic and point out cross-protection against serovars not included in it (12, 13, 16). The introduction of a vaccine of this type in a region must be preceded by a small study that allows predicting the effectiveness of the product to solve the health problem. This is necessary to have strains isolated from the affected region. These strains must be characterized by a high virulence and be representative of the serotypes that are in circulation (11, 29, 30).

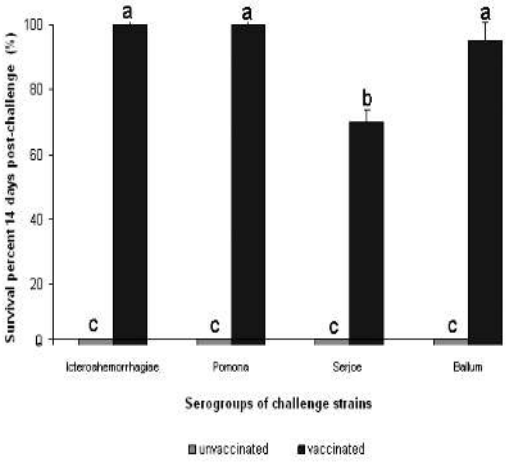

Figure-1. Protection against lethal infection conferred by the vaccine vax-SPIRAL® in hamsters after challenge with 10,000 LD_{50} of strains 3507 (*L. Icteroahemorrhagiae*), 6307 (*L. Pomona*), 4207 (*L. Serjoe*) and 8807 (*L. Ballum*) at 14 days post-inoculation. The letters on the bars represent the statistical comparison results obtained from the application of the Chi-square test with significance level $p < 0.05$.

In the period from October to November the

Table-2. Prevalence of Leptospira in the main infection target organs

Challenge Strains	Animals unvaccinated		Animals vaccinated with vax-SPIRAL®(survivors)	
	Liver	Kidneys	Liver	Kidneys
L. Icteroahemorrhagiae	10/10	10/10	0/10	0/10
L. Pomona	10/10	10/10	0/10	0/10
L. Serjoe	10/10	10/10	0/10	0/10
L. Ballum	10/10	0/10	0/10	0/10

local health authorities in the departments of Leon and Chinandega obtained a group of sixteen clinical isolates confirmed as *Leptospira spp*. The classification of these strains demonstrated that all were patogens and they belonged to the serogroups Pomona (4), Icterohaemorrhagiae (3), Ballum (3), Serjoe (3) and Pyrogenes (3). Likewise the strains belonging to the first two serogrupos mentioned previously didn't coincide in the classification at serovar level with the strains present in vax-SPIRAL®.

Often the virulence of pathogens is directly quantified in animal models by LD_{50} determination, in our case were used primarily qualitative methods because although they are less accurate, are equally reproducible and require fewer animals. These methods are being more appropriate to assess the virulence of a high number of strains simultaneously (24). In this study only 13 of the 16 strains reproduced the infection in the animal model and of these only five were classified as highly virulent. Which generally led to an early death of animals between the 5^{th} and 8^{th} day post inoculation. Coincidentally the three strains that did not reproduce the infection belonged to serogroup Pyrogenes. Although there were several passes to them in the animal model was impossible to recover. Consequently, the strains classified as high virulence were ruled to be evaluated in further studies. Although this trial was excluded strains of epidemiological studies, ensures a reduction of animal suffering and costs. Also manages the negative controls immuno-protection test work properly, ie values are obtained close to 100% lethality in this group that assigns greater accuracy to statistical comparisons.

The loss or attenuation of virulence has been demonstrated by different investigators but its causes remain a highly contested phenomenon. In leptospira numerous studies show substantial differences in the expression of important antigens in the pathogenesis between virulent and avirulent isogenic strains (28). Extracellular proteins, outer membrane lipoprotein and adhesins are expressed mostly by strains virulent than their avirulent variants (29). Apparently this unequal antigenic architecture is reversed in uneven invasive capacity of these different strains, and as a result generates an immune response in biomodel evaluated, which may be more or less successful.

The comparison of LD_{50} values for selected strains show differences between them. The lowest values were for strains belonging to serogroups Icterohemorriageae and Serjoe, which showed differences with the rest of the scores level, but all were below the dose known to microorganisms belonging to these serogroups and classified as highly virulent (31). The behavior of the survival curves of these animals was also consistent with those reported by other authors (32), despite the differences in the LD_{50} the range of deaths was only five days for an average period of seven days of death.

Vax-SPIRAL® was able to protect against the Leptospira strains isolated from the epidemic in Nicaragua. The best results were obtained in groups challenged with Icterohemorriageae and Pomona that which is expected if we consider that these serogroups are included in the vaccine, although they do not belong to the same serovars. For the rest of the serogroups a value of survival bigger than 60% was observed. These results were consistent with the conclusions of an effectiveness clinical trials carried out in the counties Holguin and Villa Clara (Cuba) where a protection of 60,4% was detected against serogroups not included in the vaccine (13). It was also recently published a study that displayed the protection of vax-SPIRAL® against most common strain of the outbreak in Honduras after hurricane Mitch (16). All these results demonstrate explicitly heterologous cross-protection of the vaccine, in the Syrian hamster model.

Few studies have been intended to demonstrate the existence of cross-protection among different serogroups of Leptospira. Until a few years ago was well established that the protection conferred after natural infection or immunization was serovar / serogroup specific (33), but the concepts of immunity against this infection have been modified in the light of current knowledge. Very recent works have reported experimental results that demonstrate the induction of a statistically significant cross protection as a result of immunizing leptospira protein fractions (12, 34).

At the present time the theory more accepted regarding the leptospira cross protection it is that outlines that cross protection exists, for those related serovares that sharing a high similarity in the LPS polisacaridic nucleus and for the non related serovares that present key proteins demonstrated as highly conserved factors of virulence (12).

Following the recommendations of the WHO was demonstrated that the immunization conferred a total protection against carrier status in this animal model (table II). These results endorse the effectiveness of the vaccine because even it is known that the carrier state in man is of short duration after natural infection and usually is not a major source of leptospira spread, the human vaccines should eliminate the carrier state in preclinical studies. Among the possible elements that it contributes to the elimination of carrier state it has been related with the presence of adyuvants in these vaccines (35). The aluminum hydroxide stimulates the immune system inducing a strong immune response characterized by a patron TH-2 type (36, 37). In this sense it is well established that the development of a successful humoral immune response against leptospirosis characterized for the production of agglutinating and opsonizing antibodies against antigenic determinants present in the outer membrane of the microorganism, such as LPS and lipoprotein is important in resistance to infection (12, 38-40).

The results of this study reveal that vax-SPIRAL® shows a percentage of protection between 60 and 100 % against strains belonging to serogroups Icterohemorragiae, Pomona, Ballum and Serjoe isolated from Nicaragua in 2007 when is tested in a model recommended by WHO as optimal for the evaluation of leptospirosis vaccines; the Syrian hamster. Based on experimental evidence and the correlation between the biomodel and humans protection it could be argued that the vaccine would help to reduce the magnitude of outbreaks with these characteristics, although the tests are not conclusive regarding the protection of the vaccine on the Pyrogenes serogroup because it was not possible to obtain virulent strains able to reproduce the disease in hamsters. It is important to add that the eradication of this health problem will not be possible only with the immunization of the affected population, this also requires the creation of a set of measures that include the vaccination of animals, decline rates of infestation by rodents in areas near human settlements and control of animal and human wastes; together with the training of public health and direction of medical personnel in order to improve epidemiological studies.

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Conflict of interest

Authors declare that they have no conflict of interest.

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