RESEARCH

Preliminary characterization of the antigens recognized by sera from vax-SPIRAL® vaccinees in outer membrane preparations of Leptospira pomona *serovar* mozdok

✍ Mariela Naranjo, Maylen Machado, Marta González, Roger Medina, Erick Abreu, Jennifer Abreu, Niurka Batista, Vismar Torres, Andrés González, Julio Oramas

Centro de Investigación-Producción de Vacunas. Ave. 27 # 19805 e/ 198 y 202, La Coronela, La Lisa, Ciudad de La Habana, AP 16017, CP 11600
Fax: 53(7) 2086075; E-mail: marielanar@finlay.edu.cu

ABSTRACT

The preliminary identification of antigens recognized by sera from individuals vaccinated with the antileptospirosis vaccine vax-SPIRAL® in outer membrane protein preparations from *Leptospira pomoma* serovar *mozdok* was made. In this paper we start with the solubilization of the outer membrane components of *Leptospira interrogans* serovar *mozdok*, using the Nunes-Edward method. In addition, we characterized the obtained fractions; the molecular weight of the protein present in this fraction and its immunogenic capacity by western and dot blot were determined. Our results show that this is an appropriate method to identify antigens for vaccine purposes. Humoral response was addressed to antigens absents in the outer membrane preparations with the aforementioned method. Coomassie stain showed a profile characterised by 16 bands; 6 main bands, 49.4, 45.8, 36.7, 28.3 and 21.0 kDa and 10 minor bands such as 66.4, 57.2, 34.1 y 32.8 kDa. The silver stain showed the presence of lipopolysaccharide. Dot and western blot techniques showed the recognition of these proteins by the sera from vaccinated individuals. The humoral response was addressed mainly to the bands of 66.1, 58.3, 47.2, 45.3, 34.5, 30.0, 29.8 and 17.2 kDa.

Key words: Leptospira, outer membrane proteins, OMP, SDS, extraction, purification

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RESUMEN

Caracterización preliminar de antígeno reconocidos por sueros de individuos vacunados con vax-SPIRAL® en preparaciones de membrana externa de *Leptospira pomona* serovar *mozdok*. Se realizó la caracterización preliminar de los antígenos reconocidos en preparaciones de membrana externa de Leptospira pomona serovar mozdok, por el suero de individuos inmunizados con la vacuna trivalente antileptospirósica vax-SPIRAL®, a partir de la solubilización de los componentes de la membrana externa de Leptospira interrogans serovar mozdok por el método de Nunes-Edwars. Se realizó, además, la caracterización de las fracciones obtenidas, y se determinó el peso molecular de las proteínas presentes en ellas, así como su capacidad inmunogénica, mediante las técnicas de western y dot blot. Los resultados demostraron que el método empleado no es adecuado en la identificación de antígenos con fines vacunales. Se obtuvo respuesta humoral a antígenos no presentes en las preparaciones de membranas externas. La tinción con azul de Coomassie mostró un perfil caracterizado por 16 bandas: 6 bandas mayoritarias, entre ellas, 49.4, 45.8, 36.7, 28.3 y 21.0 kDa, y 10 bandas minoritarias, como 66.4, 57.2, 34.1 y 32.8 kDa. La tinción con plata reflejó la presencia de lipopolisacáridos. Con las técnicas de dot y western blot se apreció el reconocimiento de esas proteínas por el suero de individuos vacunados. La respuesta humoral estuvo dirigida principalmente a las bandas 66.1, 58.3, 47.2, 45.3, 34.5, 30.0, 29.8 y 17.2 kDa.

Palabras claves: Leptospira, proteínas de membrana externa, OMP, SDS, extracción, purificación

*I*ntroduction

Leptospirosis is a disease with a worldwide distribution [1]. Most available vaccines for human or veterinary use are based on the use of whole cells inactivated by physical (heat) or chemical (formaldehyde or phenol) methods [2-6]. Most of these vaccines offer only very limited protection, since they are typically not effective against the asymptomatic development of renal infections and the subsequent leptospiuria in animals, which later become asymptomatic carriers that perpetuate the animal-to-animal transmission of the disease and may potentially endanger the lives of human beings upon close contact [7].

For an immunogen to be effective in the control of leptospirosis, it must be safe, able to protect against the clinical manifestations of the disease and against asymptomatic renal infections, and induce relatively long-term immunity [7].

Urinary transmission is the only means known so far by which *Leptospira* is spread from animals to man. Therefore, the ability of an immunogen to prevent renal infections is of major relevance in the control of leptospirosis, and vaccine research in this field should focus on the development of a new generation of more effective vaccines, based on preparations of outer membrane components from *Leptospira interrogans*. It is known that the components of the outer membrane are not only immunogenic and capable of inducing a protective response in immunized animals comparable to that achieved by whole-cell immunization [4, 8, 9], but also, and perhaps most importantly, it can prevent renal infections [6].

There are several reports indicating that the outer membrane from *Leptospira* contains antigens involved in its infection, transmission, virulence, survival and

✍ Corresponding author

1. Vinet JM. Leptospirosis. Curr. Opin. Infect. Dis 2001;14:527-38.

2. González M, Naranjo M, Rodríguez Y, Oliva R, Batista N, González I, Izquierdo L, Sierra G. Vacuna antileptospirósica adsorbida para uso humano. Primer ensayo evaluativo de reactogenicidad e inmunogenicidad en un grupo de voluntarios adultos. VacciMonitor 1997;6: 12:2-10.

3. Masuzawa T ***et al.*** Comparison of protective effects with tetravalent glicolipid antigens and whole cell-inactivated vaccine in experimental infecction of ***Leptospira***. Zlb. Bak 1991;35:199-208.

4. Russell FB, Nancy E, Auran, Russell CJ. Immunogenicity of whole cell and outer envelope leptospiral vaccine in hamsters. Infect Immun 1974;10(5):1051-6.

adaptation to the environment, which are, additionally, excellent candidates for the development of diagnostic tools and DNA vaccines [10-15].

One of the components identified at the outer membrane of this pathogen is the lipopolysaccharide (LPS) or lipopolysaccharide-like substance (LLS), a biomolecule which can induce a protective immune response [16-18]. Furthermore, it has been reported that some outer membrane proteins can enhance the response against LPS or LLS, resulting in higher levels of protection in hamsters [19].

A large number of publications describe different purification methods for outer membrane proteins (OMP) [10-20]. They differ mainly in the extraction step, which is the most important stage of the process due to its influence not only in the final yield, but in the protective capacity upon immunization, the number of contaminants which must later be eliminated, and the conditions for downstream processing [21]. The nature of the detergent used in this process is also very important; some of the detergents most commonly used are: sodium dodecyl sulphate (SDS), Triton-X-100 (TX-100), Triton-X-114 (TX-114), sodium deoxycholate (DOC) and N-lauryl sarcosinate (Sarkosyl).

The standard method for the purification of the outer membrane of *Leptospira* consists of treating the cells with a hypertonic saline solution, which shrinks the cells and detaches the outer membrane from the protoplasmatic cylinder (PC). The outer membrane is then released with the ionic detergent SDS [22, 23].

A trivalent vaccine has previously been obtained in Cuba, consisting of whole *Leptospira* cells from serovars *canicola*, *copanhageni* and *mozdok*, inactivated and adsorbed to aluminum hydroxide gel (vax-SPIRAL®). It is known from passive immunization experiments in hamsters that this vaccine induces a humoral immune response in humans. Therefore, it is necessary to characterize this response to know the antigens it targets in addition to LPS or LLS, in order to characterize other biomolecules, such as proteins, which may be relevant in establishing an immune status.

The main objective of this work is the preliminary identification of the antigens recognized in outer membrane preparations from *Leptospira pomona* serovar *mozdok* by sera from individuals vaccinated with the antileptospiral trivalent vaccine vax-SPIRAL®. Furthermore, this study compares the method for the solubilization of outer membrane components proposed by Auran *et al.* [22] with the modification to this method published by Nunes-Edwards [23], to determine which one yields the highest possible number of antigens that can be used in the characterization of anti-vax-SPIRAL® sera.

Materials and methods

Cell culture and production of biomass

A strain of *Leptospira interrogans*, serogroup *Pomona*, serovar *mozdok*, was cultured in protein-free media [24] using a 35-L Chemap fermentor, starting from a 10% (v/v) inoculum grown in a Tween-albumin medium [25] on an orbital shaker at 130 rpm and , at 28 °C, for 7 days. The fed-batch fermentation started at a culture volume of 20 L, which was supplemented with 10 L of fresh protein-free medium at 18 to 22 hours after inoculation. Samples were taken at inoculation (sample I), before supplementation (sample II) and at the end of the process (sample III). These samples were controlled for growth by measuring the optical density at 400 nm on an spectrophotometer, and for viability by direct observation under a dark field optical microscope, Gram staining, and virulence [26]. Other parameters under control were pH, dissolved oxygen percentage, air flow and temperature.

The culture was centrifuged at 3260 x g for 30 minutes at 4 °C after 48 hours of growth. The yield of wet biomass was measured by weighing the cell pellet, which was then washed three times in phosphate-buffered saline (PBS), pH 7.2 before further processing.

Outer Membrane Proteins(OMP) extraction

The OMP were extracted using the SDS solubilization technique described by Auran *et al.* (1972), and modified by Nunes-Edwards (1985). The wet biomass obtained from every 5 L of culture was resuspended in 10 mL of distilled water, mixed then with 200 mL of a 1 M NaCl solution, and kept for 2 hours at 25 °C. It was then treated with 200 mL of 0.04% SDS for 30 minutes and subjected to four centrifugations, the first at 4 260 g and the others at 23 200 g. Finally, the mixture was centrifuged at 40 000 g. The pellet obtained from each centrifugation step was resuspended in 10 mL of PBS, and its protein concentration was measured by the Lowry method [27].

SDS-polyacrylamide gel electrophoresis

The proteins were electrophoresed using the discontinuous system of Laemmli [28]. Twenty micrograms of protein were loaded per well, and after completing the electrophoretic run (30 mA, 100 and 120 V for 1 hour), the gel was stained with Coomassie blue [26]. Some gels were stained with silver for the detection of LPS [29]; in this case only 5 µg of protein were loaded per well. A mixture of proteins of 94, 57, 43, 30, 20.1 and 14.4 kDa were used as molecular weight markers (Pharmacia Biotech).

Immunotransfer

Western blot

This technique was carried out as described by Burnnette [30]. After electrophoresis, the samples were transferred to 0.45 µm nitrocellulose membranes, using a buffer composed of 20 mM Tris and 20% (v/v) methanol. The transfer was performed at 0.2 A for 2 hours, using a high-voltage power source. The nitrocellulose strips were incubated first for 1 hour at 37 °C in a blocking solution containing 5% (w/v) skimmed milk (Merck) in PBS, and then for 14 hours at 4 °C with the sera under study diluted 1/3 in PBS-1% (w/v) bovine serum albumin (BSA)-0.05% (v/v) Tween 20. After five washes in PBS-0.01% (v/v) NP40, the strips were incubated with a Protein A-peroxidase conjugate (Sigma) diluted 1/6000 in the same buffer for 1 hour. Finally, the membranes were washed and the signals were developed using 4-chloro-1-naphtol and hydrogen peroxide as substrates for peroxidase (5 mg

5. WHO. Report of the Discission of the WHO working group on leptospiral vaccine development and vaccinology. Nagoya, Japan; 1993.

6. Schreiber P, Martin V, Najbar W, Sanquer A, Gueguen S, Lebreux B. Prevention of renal infection and urinary shedding in dogs by leptospira vaccination. Vet Microbiol 2005;108(1-2):113-8.

7. Glosser JW, Johnson RC, Sulzer CR, Auran NE. Immunogenic properties of a leptospiral outer envelope bacterin in hamster and foxes. Am J Vet Res 1974; 35(5):681-4.

8. Russel FB, Russell CJ. Immuno-genicity and Humoral and Cell-mediated imnune responses to leptospiral whole cell outer envelope and protoplasmic cilinder vaccines in hamsters and dogs. Am J Vet Res 1982;43(5):835-40.

9. Yan Y, Chen Y, Liou W, Ding J, Chen J, Zhang J *et al*. An evaluation of the serological and epidemiological effects of the envelope vaccine to *Leptospira*. Chin Med Assoc 2003; 66(4):224-30.

10. Nally JE, Whitelegge JP, Aguilera R, Pereira MN, Blanco DR, Lovett MA. Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of *Leptospira interrogans* serovar *copenhageni*. Proteomics 2005; 5(1):144-52.

11. Okuda M, Sakai Y, Matsuuchi M, Oikawa T, Watanabe M, Itamoto K *et al*. Enzyme-linked inmunoabsorbent assay for the detection of canine Leptospira antibodies using recombinant OmpL1 protein. Vet Med Sci 2005;67(3):249-54.

12. Hsie WJ, Chang YF, Chen CS, Pan MJ. Omp52 is a growth-phase-regulated outer membrane protein of *Leptospira santarosai* serovar *shermani*. FEMS Microbiol Lett 2005;243(2):339-45.

13. Koizumi N, Watanabe H. Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. FEMS Microbiol Lett 2003;226(2):215-9.

14. Gamberini M, Gomez RM, Atzingen MV, martins EA, Vasconsellos SA, Romero EC *et al*. Whole-genome analysis of *Leptospira interrogans* to identify potencial vaccine candidates against leptospirosis. FEMS Microbiol Lett 2005;244(2):305-13.

15. Cullen PA, XU X, Matzunaga J, Sanches Y, Ko AI, Haake DA, *et al*. Surfaceome of *Leptospira ssp*. Infect Immun 2005;73(8): 4853-63.

16. Masuzawa T, Nakamura R, Shimitzu T, Iwamoto Y, Monte T, Yanahihara Y. Imnunological characteristics of glicolipid antigen of *Leptospira interrogans* serovar *lai*. Infect Immun 1989; 57:2502-06.

17. Masuzawa T. Protective activity of glycolipid antigen against infection by *Leptospira interrogans* serovar *canicola*. J Gen Microbiol 1990;136(2):227-30.

18. Nally JE, Chow E, Fishbein MC, Blanco DR, Lovett MA. Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect Immun 2005;73(6): 3251-60.

19. Jost BH, Adler B, Faine S. Experimental immunization of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J Med Microbiol 1989;29: 115-20.

of 4-chloro-1-naphtol, 1 mL of methanol, 24 mL of TBS and 15 µL of 30% (v/v) H_2O_2).

Dot blot

Ten microliters of the samples were directly dotted under vacuum on a nitrocellulose membrane. The strips were incubated for 1 hour at 37 °C in a blocking solution, and were processed afterwards as described above for the *western blot*.

Immune sera

The immune sera used were obtained from individuals vaccinated with one and two doses of vax-SPIRAL®,6 weeks apart [2]. Sera from non-vaccinated individuals were used as negative controls.

Molecular weight determination

A regression curve was obtained from the logarithm of the molecular weight of the MWM and the observed electrophoretic mobilities. The molecular weights of the different bands visualized in the electrophoresis or after *western blot* were estimated by the interpolation of this curve.

Determination of the LD50

The LD50 was estimated using the methodology described by Reed and Muench [31].

Results and discussion

The vax-SPIRAL® vaccine, manufactured by the Finlay Institute, is composed of whole *Leptospira interrogans* cells from serovars *canicola, copenhageni* and *mozdok*, and it protects against leptospirosis. Clinical trials carried out in our country before registering this vaccine showed an efficacy of 93%, prompting the establishment of routine vaccination for all high-risk personnel since 1996.

This study, which aims to identify the outer membrane antigens of serovar *mozdok* of *Leptospira interrogans* serogroup *pomona* involved in eliciting an immune response in individuals vaccinated with vax-SPIRAL®, started with a fermentation to obtain the necessary biomass for the extraction of the OMP components.

The culture conditions determine or lead to the expression of both structural and soluble outer membrane components in any microorganism [32]. In the specific case of *Leptospira interrogans*, it is very important to control its biological activity, since this is closely related to its ability to induce a protective response in immunized individuals [2]. This is why virulence is the main parameter under control in the cultures intended for the purification of structural or soluble OM components. The results from the 30 L fermentative processes of *Leptospira mozdok* are shown in table 1. The wet biomass yield was 26.62 g, corresponding to 0.88 g/L of culture.

Once the biomass was obtained, the OMP were extracted. Since the culture volumes were higher than those used in the literature, the treatment time using the NaCl and SDS solutions were adjusted to 2 hours and 30 minutes respectively, on the basis of dark field microscopy observations.

Table 1. Results of the culture of Leptospira interrogans serovar mozdok to obtain biomass for the solubilization of outer membrane components

Controlled parameters	Samples I	II	III
Temperature (°C)	28.4	29.6	28.2
Air flow (L/min)	2	2	2
Dissolved oxygen percentage (%)	100	98	60
OD (400 nm)	0.031	0.526	0.661
Gram staining	S	S	S
Viability	S	S	S
Virulence	S	S	S

I = Sample taken at inoculation

II = Sample taken at supplementation

III = Sample taken at the end of fermentation

S = Satisfactory result (Number of LD_{50} within the established range)

One of the goals of this study is the comparison of two OMP purification methods described by Auran *et al.* [22] and Nunes-Edwards *et al.* [23]. According to the latter, the two additional centrifugation steps introduced, performed at 23 200 g/ 30 minutes before the ultracentrifugación, are for eliminating the protoplasmatic cylinders. Another additional centrifugation step was introduced here since, in our study, the methodology described in the literature did not guarantee the complete elimination of these components. The pellets obtained in each centrifugation step were however not discarded, but resuspended in 10 mL of PBS and, after determinating their protein concentration by Lowry (table 2), they were analyzed by electrophoresis and Coomassie blue staining to determine if these fractions contained antigenic components that might be related to the induction of an immune response (figure 1).

The fractions had a complex electrophoretic profile, with protein bands having apparent molecular weights that ranged from 14 kDa to more than 80 kDa, similar to that reported by other authors [33-35].

The electrophoretic profile of these fractions was characterized by approximately 6 major and 10 minor bands shared among all samples. There were no qualitative differences among the fractions (FI-IV) regarding their electrophoretic profiles, although there were quantitative differences related to the intensity of the bands in FIV compared to the others, as well as the protein concentration for each fraction (table 2).

After using linear regression (described in the Materials and Methods section), it was determined that the major bands had apparent molecular weights

Table 2. Yields during the process for the isolation of outer membrane components solubilized with SDS

Sample in each centrifugation	Protein concentration by Lowry (mg/mL)	Total volume of the sample (mL)
C2 (FI)	6.210	10
C3(FII)	1.139	10
C4(FIII)	0.614	10
UC(FIV)	4.560	10

C2: Pellet from the second centrifugation

C3: Pellet from the third centrifugation

C4: Pellet from the fourth centrifugation

UC: Pellet from ultracentrifugation

20.Haake Da, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infect Immun 2002; 70(9):4936-45.

21.Janson C, Uppsala LAS. Protein purification. Principle, high resolution methods and aplication. American Press USA; 1991.

22.Auran NE, Johnson RC, Ritzi DM. Isolation of the outer enveloped of Leptospira and its inmunogenic properties in hamsters. Infect Immun 1972;65:968-75.

23.Nunes-Edwards PL, Thierman AB, Bassford PJ, Straman LV. Identification and characterization of the protein antigens of *Letospira interrogans* serovar *hardjo*. Infect Immun 1985;48:492-7.

24.Estévez L. Evaluación de un medio libre de proteínas químicamente definido para *Leptospira interrogans*. VacciMonitor 1999;6:11:2-10.

25.Ellinghausen HC, Mc Cullough WC. Nutrition of *Leptospira pomona* and growth of 13 others serotype fraction of oleic albumin complex and medium of albumin and polysorbate 80. Am J Vet Res 1995;26:45-51.

26.Suplikp V. Metodología para la evaluación de la virulencia de *L. interrogans*. J Vet 1963;7(3):9-13.

27.Lowry OH. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-8.

28.Laemmli NK. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. Nature 1970;227: 680-5.

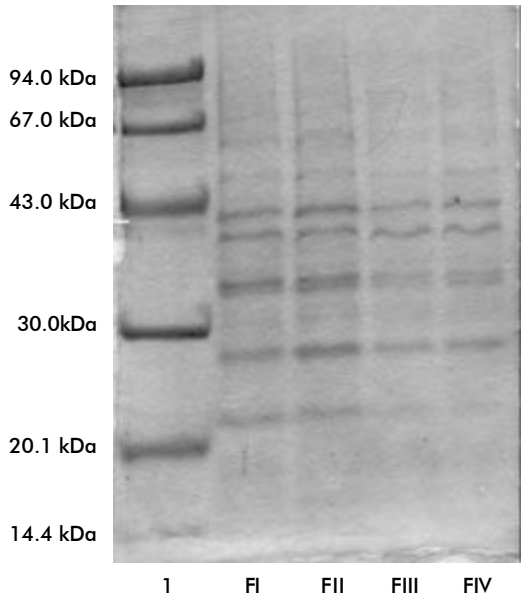

Figure 1. SDS-Polyacrylamide gel electrophoresis (12.5%). Twenty micrograms were loaded per well from each of the fractions obtained after the second (FI), third (FII) and fourth (FIII) centrifugations, and after ultracentrifugation (FIV) during the OMP extraction process.

of 49.4, 45.8, 36.7, 35.3, 28.3 and 21.0 kDa, with the minor bands corresponding to 66.4, 57.2, 34.1 and 32.8 kDa.

These results are similar to those obtained by Nicholson and Prescott [36], which used sarkosyl as the detergent and the sonication for OMP extraction in *Leptospira alstoni* serovar *grippotyphosa*, *L. borgpetersenii* serovar *hardjo* and *L. interrogans* serovar *autumnales*, *bratislava*, *canicola*, *icterohaemorrhagiae* and *pomona*. They identified 7 protein bands shared between the 3 pathogenic species under analysis, with molecular weights of 77.0, 66.0, 59.5, 42.0, 35.5, 24.0 and 18.0 kDa. The 44.0 kDa stained poorly with Coomassie blue, which might be explained if it was a lipoprotein, a glycoprotein, or just because it was present in small amounts.

In both extraction methods (sarkosyl- or SDS-based) the fractions are usually contaminated with endoflagelar components, represented by a double band with approximate weights of 34.5 and 36 kDa [23-33]. This study identified this double band with species appearing at 35.3 and 36.7 kDa, since their intensity matches previous reports in the literature [30-33]. The insoluble fraction obtained using either method can have proteins associated both to the peptidoglycan layer or the outer membrane.

According to Haake *et al.* [32], who used 0.1% Triton-X-114, the 66 kDa protein (observed in this work) together with other two proteins of 35 and 39 kDa are unique to virulent strains. In contrast, the 31 kDa (OmpL1) and 38 kDa proteins are found only in attenuated strains, which explains their absence in the electrophoretic profiles of our samples. Taking into account that vax-SPIRAL® is a whole-cell inactivated vaccine prepared from a non-attenuated strain, it is logical to expect that the immune response will be directed against the main protective antigens of virulent strains, instead of those conserved in attenuated strains. Both virulent and attenuated strains contain similar amounts of the major detergent-soluble proteins, with molecular weights of 41 y 44 kDa. The latter may correspond to the band observed in our study at an apparent molecular weight of 45.8 kDa, since its mobility might have been altered due to the presence of LPS (data not shown). A similar explanation could be used for the protein reported by Haake *et al.* [32] at 59.5 kDa, which in our case appeared at 75.2 kDa. LPS or LLS in *Leptospira* samples subjected to SDS-PAGE which is detected by a characteristic band pattern upon silver staining and *western blot* [32]. Its presence in our preparations, however, is of prime importance, taking into account the high number of findings that point to LPS as the main antigen responsible for immunity against leptospirosis [8, 17, 19, 34].

The 32.8 kDa band seems to correspond to the major outer membrane protein (MOMP), reported by other authors at 32 kDa. The protein observed at 49.4 kDa may correspond to that identified by Brown *et al.* [33] at around 50 kDa. These results are similar to those reported by Chapman *et al.* [37], who evaluated the immune response in persons who were given a bivalent *hardjo-pomona* vaccine, in which the main protein bands were those of 32 kDa and the 34.5/35 kDa doublet, besides other components.

The protein found at 21 kDa (figure 1) was described by Haake [38] as one of the proteins isolated when using sucrose for OMP purification, and was identified by other authors as Lip21, one of the most abundant proteins of the leptospiral surface [15].

Since there were no qualitative differences between the electrophoretic profiles of fractions I to III (shown in figure 1), FI and FIV were selected for the evaluation of their immunogenic capacity, given the possibility of finding differences in their antigenic composition arising from the centrifugation conditions used for their isolation that can not be detected by electrophoresis.

Using these two fractions, variants A = FI and B = FIV were prepared, adjusted to the same concentration and adsorbed to aluminum hydroxide gel. No free protein was detected after absorption, evidencing the high efficiency of this step.

Figure 2 shows the bands recognized in each fraction by the sera of vax-SPIRAL® vaccinees. It can be seen that the intensity of the signal is lower after only one immunization, as compared to the sera from humans receiving two doses, whose response has been boosted. No antigens were recognized by the negative control serum. Besides, it is evident that the LPS in the sample is being recognized by the immune sera, indicating that the immune response induced by vax-SPIRAL® is also directed against LPS as one of the major components of the outer membrane. It also confirms what is argued by other authors who consider that the LPS plays an important role in immunity against *Leptospira*, conferring even higher protection levels when combined with OMP that enhances this response [16-19].

Figure 3 shows the results of *western blot* evaluating the detection of outer membrane components (FI and FIV) by the sera of individuals vaccinated with one and two doses of vax-SPIRAL®, as well as unvaccinated

29.Hitchcock PJ, Brown TM. Morphological herogenicity among Salmonella lipopolysacharide chemotypes in silver polyacrilamide gels. J Bact 1993;154: 269-277.

30.Burnnette WN. Western Blotting electrophoretic transfer of protein from sodium dodecyl suphate polyacrylamide gel to unmodified nitrocelulose and radiographic detection with antobodies and radioiodinated protein A. Anal. Biochem 1980;112:192-200.

31.Reed LJ, Muench H. Asimple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493-7.

32.Haake DA, Walker EM, Blanco DR, Bolin CA, Miller JN, Lovett MA. Changes in the surface of *Leptospira interrogans*serovar *griphotiphosa* during *in vitro* cultivation. Infect Immun 1991;59(3):1131-40.

33.Brown JA, Lefebver RB, Pan MJ. Protein and antigens profile of prevalent serovar *Leptospira interrogans*. Infect Immun 1991;59(5):1772-7.

34.Gitton X, Fontaine GA, Francois A, Geniere JP. Immunoblotting study of the antogenic relationship among eigth serogroups of *Leptospira*. Vet Microbiol 1992;32:293-303.

35.Luo YH, Yan J, Mao YF, Li SP. Determination of the genus-specific antigens in outer membrane proteins from the strains of *Leptospira interrogans* and *Leptospira biflexa* with different virulence. Infect Immun 2004;66(7):3210-7.

36.Nicholson VM, Prescott JF. Outer Membrana Proteins of the three pathogenic *Leptospira species*. Vet Microb 1993;36:123-38.

37.Chapman AJ, Everard COR, Fine S, Adler B. Antigens recognized by the human immune response to severe leptospirosis in Barbados. Epidemiol Infect 1991;107: 143-55.

38.Haake DA. Inventor. The regents of the university of California. Asignee. *Leptospira* outer membrana proteins. US patent; 26274, 1997;(July):24.

Figure 2. Dot blot results, showing the recognition of 1) Whole cells from L. mozdok, 2) FI (Pellet from the second centrifugation), 3) FIV (Pellet from ultracentrifugation), and 4) L. mozdok LPS, by the sera from individuals vaccinated with one or two doses of the vax-SPIRAL® vaccine. Each well was loaded with 10 μL of the samples. Sera from non-vaccinated individuals was used as a negative control.

individuals. In terms of the number and intensity of the bands, there is a higher recognition of the OMP by the sera from individuals vaccinated with two doses of the vaccine, evidencing once again, the identified booster effect of the second dose. Eight bands are recognized by all the sera from individuals vaccinated with 2 doses in the immunotransfers of fractions FI and FIV, with quantitative differences between these bands. Furthermore, two additional proteins are recognized in the last fraction (FIV). These might represent proteins that precipitated during ultracentrifugation, which would explain their absence in FI. Using linear regression, it is found that the bands detected in both fractions have molecular weights of 66.1, 58.3, 47.2, 45.3, 34.5, 30.0, 29.8 and 17.2 kDa, whereas the proteins unique for FIV migrate at 26.2 and 24.1 kDa.

The proteins recognized in FI and FIV by the sera from individuals immunized with one or two doses of the antileptospirosis vaccine have apparent molecular weights of 66.1, 58.3, 45.3, 34.5 and 29.8 kDa. These seem to correspond to the 66.4, 57.2, 45.8, 34.1 and 28 kDa bands observed in the electrophoretic pattern, respectively.

These results, are also very similar to those published by Nicholson y Prescott [36], who found, in immunoblots from different *Leptospira* strains, six antigens which were recognized by all their sera, with molecular weights of 66, 59.5, 44.0, 42.0, 35.5 and 18.0 kDa.

Although the electrophoretic profile (figure 1) does not have bands below 21 kDa, *western blot* shows a 17.2 kDa band that may correspond to the 18 kDa species described by Nicholson and Prescott [36]. Its absence in Coomassie-stained gels may imply that although it is present in quantities below the detection limit of this staining technique, it is immunogenic enough to be recognized by the sera of vax-SPIRAL® vaccinees, even in those who have received only one dose.

The same reasoning can be applied to explain the presence of other three additional bands which are detected by *western blot* but not by electrophoresis alone. They correspond to 30.0 kDa, present in both fractions (FI and FIV), and 24.1 and 26.2 kDa, present only in FIV. The 24.1 kDa band seems to be that reported by Nicholson and Prescott [36] in electrophoresis, and the one with 30.0 kDa, although not mentioned often in the literature, was first described by Haake in 1997 [36], using a French press for its isolation. The 26.0 kDa band has not been previously reported. This is a very important finding, since this is the first description of an outer membrane protein from *Leptospira interrogans* with that molecular weight involved in immunity. This protein could be used, after verification studies, for the design of new generation vaccines against human leptospirosis based on defined, highly purified components; or for enhancing the current vaccine. It will be necessary to find out whether this protein is involved in the induction of a protective response and what is even more important, in the prevention of renal infections.

Upon evaluation of the method of Nunes-Edwards [16], which is just a modification of the protocol described by Auran *et al.* [15] claimed by its authors to eliminate the protoplasmic cylinder by using two centrifugations before ultracentrifugation, it was seen that these fractions had immunogenic components, which is undesirable for the objectives of this study.

Since one of the goals of our research is the characterization of the immune response in persons vaccinated with vax-SPIRAL® through the recognition of OMP preparations by their sera, it is imperative to have at our disposal a preparation from which no OMP has been depleted. Therefore, the use of the methodology proposed by Auran *et al.* (1972), without

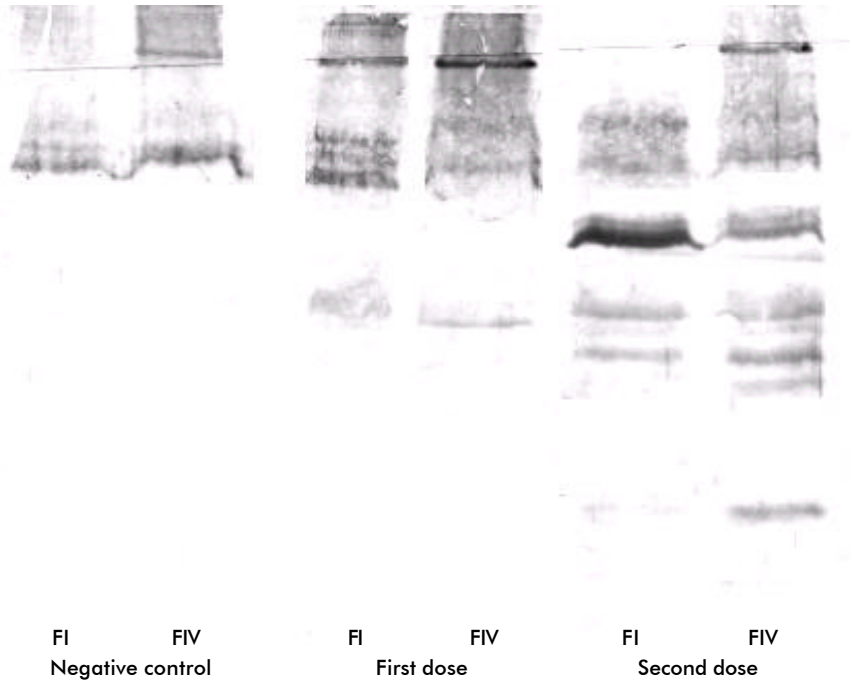

Figure 3. Recognition by western blot of the fractions FI (Pellet from the second centrifugation) and FIV (Pellet from ultracentrifugation) by sera from persons immunized with one or two doses of the vax-SPIRAL® vaccine or from non-vaccinated individuals.

partial centrifugations before ultracentrifugation, is proposed for future studies.

Our results have allowed the identification of proteins which are important in the immunogenicity of the vax-SPIRAL® (*L. mozdok*) vaccine, as evaluated by the induced response to its antigens. The findings reported here will be essential in future studies for characterizing the vaccine components that are not only immunogenic, but relevant for the induction of a protective response, and will guide research focused on the purification of these antigens for improving the current vaccine, or for the development of second generation vaccines against human leptospirosis.

It was shown by dot and western blot that the OMP are recognized by sera from vax-SPIRAL® vaccinees. In western blot, bands of 66.1, 58.3, 47.2, 45.3, 34.5, 30.0, 29.8 and 17.2 kDa were recognized in both fractions, and of 26.2 and 24.1 kDa in FIV.

A 26.0 kDa protein detected by the sera of human vaccinees was identified for the first time. This protein is involved in the immunity to *Leptospira* and might be used as an antigen in new generation vaccines.

The use of the methodology for OMP extraction described by Nunes-Edwards depletes outer membrane preparations of some antigens which may be important in the induction of an immune response against *Leptospira*.

The use of the methodology described by Auran *et al*. is recommended if a representative preparation, containing all the antigens involved in the induction of an immune response, is needed.

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