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Protein and antigen profiles of *Leptospira interrogans* serovar Hardjo

Perfil proteico e antigênico da *Leptospira interrogans* sorovariedade Hardjo

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ABSTRACT

*The protein profile of the outer membrane of **Leptospira interrogans** serovar Hardjo subtype hardjoprajitno associated with the bovine natural immune response was investigated. The outer membrane proteins were extracted utilizing Triton X114 and precipitated with acetone. The protein sample was then resolved by SDS-PAGE and reacted in western blot against sera from a hyperimmune rabbit and from naturally infected bovines. In silver stained gels, 14 protein bands were observed, among which four proteins, with 22, 29, 47 and 63kDa, appeared as major constituents. Western blot tests with hyperimmune rabbit antiserum detected bands corresponding to proteins with 35; 27; 24; 21; 17 and 14kDa, while 32kDa and 45kDa proteins were the most immunoreactive with sera from naturally infected bovines.*

Key words: ***Leptospira interrogans****, outer membrane protein, hardjoprajitno, immunomodulatory proteins.*

RESUMO

*Estudou-se o perfil proteico da membrana externa da **Leptospira interrogans** sorovariedade Hardjo, amostra hardjoprajitno, associado à resposta imune natural de bovinos infectados. Foram utilizados Triton X114 para a extração das proteínas de membrana externa e acetona para precipitá-las. As proteínas extraídas foram analisadas por SDS-PAGE e western blot contra soro de coelhos hiperimunes e de bovinos naturalmente infectados. Em géis corados com nitrato de prata, 14 bandas proteicas foram identificadas, e quatro dessas bandas, com 22, 29, 47 e 63kDa, foram as mais proeminentes. Os western blots com soro hiperimune de coelho detectaram bandas correspondentes a proteínas com pesos moleculares de 35, 27, 24, 21, 17 e 14kDa, e bandas de 32 e 45kDa destacaram-se nos testes com soros de bovinos naturalmente infectados.*

Palavras-chave: ***Leptospira interrogans****, proteína de membrana externa, hardjoprajitno, proteínas imunomodulatórias.*

INTRODUCTION

Leptospirosis caused by ***Leptospira interrogans*** is a worldwide zoonosis that predominately afflicts bovine cattle, resulting in spontaneous abortions, embryonic death, infertility and mastitis (ARAÚJO et al., 2005). The most important serovar involved, Hardjo subtype hardjoprajitno, is responsible for significant economic losses in the milk and meat production.

According to serological classification, there are more than 230 ***Leptospira interrogans*** serovars (KOIZUMI & WATANABE, 2004). The strain diversity is due to the structure of lipopolyssacharides (LPS), the major components of the leptospiral outer membrane (OM) (CULLEN et al., 2005). Unlike LPS, most of proteins found in the cell surface are conserved among ***L. interrogans*** serovars, and these polypeptides can serve as indicators of pathogenicity between different ***L. interrogans*** serovars or virulence indicators in samples of the same ***L. interrogans*** serovar (FAINE et al., 1999). Due to the lack of LPS-based vaccines, identification and characterization of protein components of the leptospiral OM is critical for the development of alternative immunoprotection strategies (HAAKE et al., 2000).

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Studies suggest that the leptospiral OM has a relatively complex protein profile with a broad range of protein molecular weights (HAAKE et al., 1991; HAAKE et al., 2000; CULLEN et al., 2002). The first investigation of the ***L. interrogans*** OM (NUNES-EDUARDS et al., 1985) verified the OM protein composition of six ***L. interrogans*** serovars, identifying protein components with 63, 55, 51, 41, 38, 36, 35.5, 33 and 21kDa. According to BROWN et al. (1991), 25, 32, and 35kDa antigens were recognized in western blot of serovar Hardjo subtype hardjoprajitno whole-cell lysates probed with rabbit hyperimmune antisera. More recently, BISWAS et al. (2005) identified 67, 41, 35, 32, 28, and 22kDa proteins as major OM components for five different Leptospira serogroups. Western blots with hyperimmune rabbit antisera detected 67, 65, 60, 45, 43, 41, and 32kDa common proteins among them. The 32 and 45kDa were the most immunoreactive proteins using patient sera.

Despite the known relevance of serovar Hardjo subtype hardjoprajitno, only a few studies characterized the protein profile of this bacterium associated to antibody production. The aim of this study was to identify immunoreactive leptospiral proteins from hardjoprajitno that are expressed during the bovine immune response.

MATERIAL AND METHODS

Bacterial strain and cultivation: ***Leptospira interrogans*** serovar Hardjo subtype hardjoprajitno (WHO strain) was grown in three liters of EMJH liquid medium at 30ºC (ELLINGHAUSEN and MCCULLOUGH, 1965) for at least 1 week. The culture was then centrifuged at 13,000g for 10min at 4°C and the pellet was washed three times in PBS (pH 7.2) with 5mM $MgCl_2$. A final concentration of 1.5×10^{11} leptospires ml^{-1} were obtained, as determined by direct cell counting in a Petroff Hausser chamber.

Protein Extraction: The leptospiral OM was extracted according to the technique described by HAAKE et al. (1991) using Triton X114. The proteins extracted that were present in the aqueous and detergent phases were precipitated with 10vol. of acetone in an ice bath for 45min (CUNNINGHAM, 1988). The extracts were then solubilized in 460µL of the final sample buffer (125mM Tris-HCl (pH 6.8), 0.007% bromophenol blue, 10% glycerol, 200mM β-mercaptoethanol, and 2% SDS).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): After protein extraction, all extracts were tested for protein presence by SDS-PAGE. A 12% (w/v) poliacrylamide gel was used and 7-9µL of sample was applied in each lane. The electrophoresis was performed with 150V, amperes free at 4ºC in a Mini-PROTEAN II (Bio-Rad) for 60min. One gel was stained with silver nitrate and the other was reserved to western blot. The software Gel-Proanalyze 3.1 (MediaCybernetics®) was used to analyze the images and to estimate the molecular weight.

Sera: Serum was raised in rabbits against the serovar Hardjo subtype hardjoprajitno as recommended by the International Committee on Systematic Bacteriology, Sub-Committee on the Taxonomy of *Leptospira* cited by FAINE et al. (1999). Bovine sera were obtained from 13 animals and tested by MAT (RYU, 1970). Twelve sera were positive at dilution from 1:100 to 1:1,600.and one was negative and used as control.

Western Blot analysis: Western blot analysis was performed on proteins transferred to nitrocellulose membrane at 100V, amperes free for one hour in a Sigma Transblot (model: B2157), and blocked with 5% nonfat dry milk in Tris-buffered saline. Membranes were incubated with hyperimmune rabbit sera at a 1:100 dilution or bovine sera at a 1:50 dilution for one hour at room temperature. They were then washed three times with Tris-buffered saline for ten min. and were probed with a 1:1.000 dilution of rabbit IgG antiserum and bovine antiserum. Membranes were again washed three times with Tris-buffered saline and were exposed to the substrate for peroxidase, DAB, in order to visualize the antigen-antibody complexes. The nitrocellulose membranes were then photographed with a digital camera to estimate the molecular weight of the OM proteins (OMPs), as described above.

RESULTS AND DISCUSSION

The overall analysis of the leptospiral OMPs by SDS-PAGE demonstrated the presence of 14 bands, where the bands of 22, 29, 47, and 63kDa proteins were the major constituents (Figure 1, A). In contrast, in western blot with hyperimmune rabbit sera, six stained bands of leptospiral OMPs were determined, and the band sizes were analyzed (Figure 1, B). The stronger bands were r2 (35kDa), r3 (28kDa), r4 (24kDa), r5 (21kDa), r6 (18 kDa), and r8 (14kDa), located in L1 and L2 (Figure 1, B). When the western blot used bovine sera from 13 animals, 12 positive for serovar Hardjo subtype hardjoprajitno and one negative, 2 bands were strongly stained in the positive sera. The software Gel-Proanalyze 3.1 (MediaCybernetics®) analyzed the images of the positive sera and calculated the molecular weight values. Two bands, 47kDa and 29kDa, were observed.

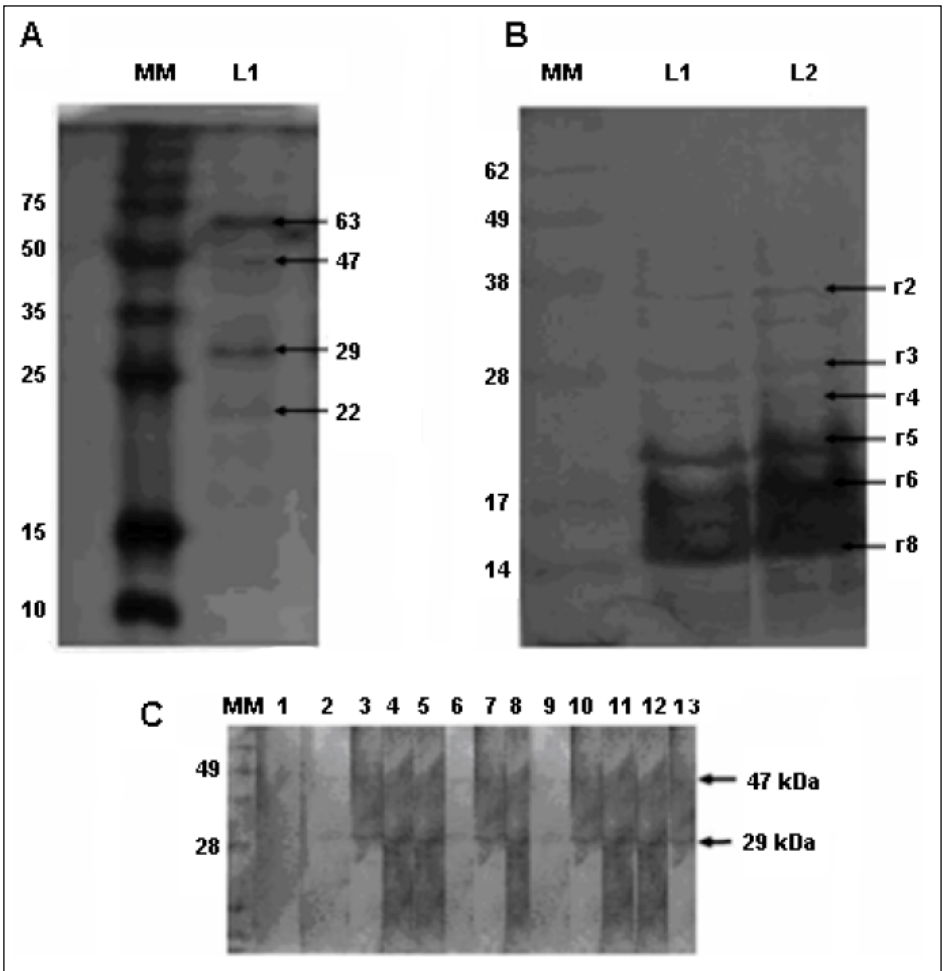

Figure 1 - SDS-PAGE and Western blot made with proteins of outer membrane of ***L. interrogans*** serovar Hardjo subtype hardjoprajitno. (A) SDS-PAGE; MM: molecular maker; L1: proteins extracted with triton x114 and stained with silver nitrate. (B) Western Blot; MM: molecular marker; L1 and L2: proteins reacted with hyperimmune rabbits serum produced with samples of the same serovar of Leptospira; r2 (35kDa), r3 (28kDa), r4 (24kDa), r5 (21kDa), r6 (18kDa), and r8 (14kDa) are codes for the proteins that reacted more strongly in the test. (C) Western Blot; MM: molecular marker; 1: negative control**;** 2 – 13: proteins reacted with sera of animals naturally infected by Leptospira serovar Hardjo.

The bacterial surface is the interface between pathogen and host and constitutes the site of interaction with host tissues during infection (CULLEN et al., 2005). The OMPs are the more complexes structure of the cellular surface and are the strongest immunoreactive structure, which makes the molecular detection of these polypeptides a key diagnostic approach. The use of bovine sera in the tests was useful to determine the potential of the OMPs to stimulate the bovine immune system. Through SDS-PAGE (Figure 1, A), the 22kDa protein appeared as a component of the OM for this ***L. interrogans*** serovar, like observed by KOIZUMI & WATANABE (2003). The polypeptide of 29kDa founded in serovar Hardjo subtype hardjoprajitno OM represents the main protein component, as observed via SDS-PAGE and in the western blot tested with bovine sera (Figure1, A and C). According to CULLEN et al. (2002), this 29kDa protein may be a degraded form of LipL32 (molecular weight 32kDa). Their immunogenicity was determined during antibody detection tests of naturally infected animals (Figure 1, C) and sera from patients detected a 32kDa polypeptide as the major immunoreactive protein (BISWAS et al., 2005).

A protein with an apparent molecular weight of 47kDa was observed via SDS-PAGE (Figure 1, A). It is included in a group of proteins that has not yet been described in the leptospire OMP family, and its function

is still undetermined (CULLEN et al. 2002). The protein found in greater amount, as showed via SDS-PAGE (Figure 1, A) has an apparent molecular mass of 63kDa and was previously described by NUNES-EDWARDS et al. (1985).

In the results of the western blots with rabbit sera (Figure 1, B), two bands (35kDa and 24kDa) yielded molecular weights that are very close to those found by BROWN et al. (1991). The slight variation between the molecular weights of these proteins could be explained by differences in result analysis, since those authors did not utilize a software package to calculate the molecular weights of the reactive proteins and only presented estimated values. Therefore, the 35 and 24kDa bands described in this study, most likely correspond to the 35 and 25kDa bands, respectively. These proteins appear to develop an important role in immunity of cattle affected by ***L. interrogans*** serovar Hardjo subtypes hardjobovis and hardjoprajitno. OMPL1 (LipL31), with an estimated molecular weight of 31kDa, undergoes a denaturation process during the extraction and its molecular weight is altered to almost 25kDa (CULLEN et al., 2004). In turn, one may consider that the 24kDa labeled protein corresponds to LipL31, a trans-membrane protein that is present in different ***L. interrogans*** serovars. This protein, in association with LipL41 (41kDa), is immunogenic when used during immunization processes in hamsters (HAAKE et al., 1999).

Regarding the minimal differences in molecular weights, the 35kDa band that was described by BROWN et al. (1991) may be the same 33kDa LPS found in ***L. interrogans*** serovars via SDS-PAGE by HAAKE et al. (1991). These molecular weight variations must be processed, through software utilization to determine the molecular weights of the proteins extracted, as well as for standardization of the processes for OMPs extraction.

A band that was lightly stained, which is located between bands r2 and r3 (Figure 1, B, L 2), did not have the calculated molecular weight. Even though calculations were not possible, estimation can be performed, by comparison with the bands of the molecular weight marker, which approximate the molecular weight at 32kDa. Most likely, the band is a non-degraded form of LipL32 (BROWN et al., 1991; CULLEN et al., 2002).

There was an extensive background seen in the membrane (Figure 1, B) between the bands r5, r6 and r8, like described by BROWN et al. (1991), It is considered a characteristic in western blot carried out with proteins extracted from ***L. interrogans*** serogroup Sejroe and reacted with sera from immunized animals with samples of the same serogroup. The background coloration may be a consequence of the large quantity of protein r5 (21kDa). Two distinct analyses of the results were probably performed: one considered the middle part of the bands (L 1), and other considered the top of the bands (L 2).

In the membrane (Figure 1, B L 2), r3, with a molecular weight of 36kDa, was intensely stained and correspond to LipL36. According to FAINE et al. (1999), there is a correlation between the expression of LipL36 and the virulence of the sample; This OMP is expressed only under in vitro conditions, after the sample had been cultivated in laboratory for a long time. In this case, the presence of LipL36 is fully consistent with the experimental conditions, since a standard sample was utilized throughout the study, without any contact with the host.

OMP r1, with a molecular weight of 51kDa, was detected only when the tests were made with great amount of antigen (Figure 1, B, L 2). However, HSIEH et al. (2005) described OMP52 with a molecular weight of 53kDa in ***L. interrogans***, which modulates the interaction between host cells and leptospires during the infection phase and, when the ***L. interrogans*** is grown in the laboratory, has its expression increased during the stationary phase of cultivation. Because of the similarity of molecular weights of proteins OMP52 and 51kDa found, none of the tests done here are able to tell us whether they are the same protein. However, one cannot ignore the hypothesis that this may be an isoform of the OMP52 described by HSIEH et al. (2005).

Although the results presented in figure 1, A and B are of great importance, the results presented in figure 1, C should be valued as that best represent the reality of ***L. interrogans*** samples kept from the environment. Based on these results, the proteins with molecular weights of 32 and 45kDa that were recognized by the bovine immune system are the most important antigens shown. The potential of these antigens must be further investigated during vaccines and diagnostic tests.

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