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Production of Leptospiral LipL32 Antigen in *Pichia pastoris* and Its Use in an Enzyme-Linked Immunosorbent Assay

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ABSTRACT

The production of recombinant LipL32 protein using Escherichia coli *has been used extensively for the development of vaccines and diagnostic tests for leptospirosis. However,* E. coli *has demonstrated limitations, including low yield and lack of post-translational modifications. In this study, rLipL32 was produced in eukaryotic expression system* (Pichia pastoris) *and evaluated the antigen by enzyme-linked immunosorbent assay (ELISA). The yield obtained from the culture supernatant reached 270 mg/L and ELISA showed an accuracy of 95.34%. In summary, the production of rLipL32 using* P. pastoris *did not impair the antigenic characteristics of this antigen and ensured its use for detecting the leptospiral antibodies in swine sera.*

Key words: *Pichia pastoris*, LipL32, ELISA, Leptospirosis

INTRODUCTION

Leptospirosis is the most spread infectious disease worldwide that affects the humans and a wide variety of animals, caused by the spirochetes of the genus *Leptospira*. In pigs, signs of leptospirosis include reproductive failure, abortion, stillbirths, fetal mummification, weak piglets and agalactia (Adler and Moctezuma 2010). The definitive serological investigation for leptospirosis is the microscopic agglutination test (MAT), which measures the antibody levels in sera from the suspected animals (Faine et al. 1999). Although this technique has been performed in the clinical laboratories throughout the world for several years, it presents drawbacks such as periodic verification of each serovar in the culture and a high number of cross-reactions among serovars (Adler and Moctezuma 2010).

Several virulence factors have been shown potential for the development of diagnostic assays for leptospirosis, including proteins OmpL1 (Haake et al. 1993), Lig (Leptospiral immunoglobulin-like) (Matsunaga et al. 2003), LenA/LenD (Stevenson et al. 2007), Loa22 (Ristow et al. 2007) and subsurface lipoprotein 32 (LipL32) (Haake et al. 2000; Pinne and Haake 2013). Among these proteins, LipL32 is the most abundant antigen found in the leptospiral total protein profile and highly conserved among the

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pathogenic *Leptospira* species but has no orthologs in the saprophyte *Leptospira* (Haake et al. 2000). The production of recombinant LipL32 protein (rLipL32) in *Escherichia coli* has been extensively used for the development of vaccines (Adler and Moctezuma, 2010) and diagnostic tests for human (Flannery et al. 2001), cattle (Bomfim et al. 2005), dog (Dey et al. 2004) and swine (Hartleben et al. 2012). However, this system has demonstrated potential limitations, including low yield (Hartwig et al. 2010), improper folding and lack of post-translational modifications (Cos et al. 2006).

Pichia pastoris has emerged as an important alternative expression system, because this yeast has the ability of growing in minimal medium at very high cell densities, secreting the heterologous protein simplifying its recovery (Cos et al. 2006), and perform many of the post-translational modifications such as processing of signal sequences, folding, disulfide bridge formation, certain types of lipid addition, and O and N-linked glycosylation (Cereghino et al. 2000; Cregg et al. 2000; Hohenblum et al. 2004; Cos et al. 2006). In this study, rLipL32 was produced in *P. pastoris* and the antigen was evaluated by enzyme-linked immunosorbent assay for detecting the leptospiral antibodies in swine sera.

MATERIALS AND METHODS

Production of rLipL32

The expression of rLipL32 MutS secretory phenotype was performed using the eukaryotic system based in *P. pastoris*, as described previously (Hartwig et al. 2010). Briefly, a recombinant clone was grown in a baffled flask containing BMGY broth (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.00004% biotin, 1 % glycerol, 100 mM potassium phosphate and 2 % agar, pH 6.0). The expression was induced during 96 h by the addition of methanol to a final concentration of 0.5%. The Microcon YM-30 Amicon Bioseparations (Millipore) were used to concentrate the recombinant proteins expressed in the supernatant, following the manufacturer's protocol. The recombinant protein was analyzed by the SDS-PAGE (12%) and visualized by staining with Coomassie Blue and Western blotting (WB). The rLipL32 concentration was determined by the BCA™ Protein Assay Kit (PIERCE) method using bovine serum albumin (BSA) as a standard.

Microscopic Agglutination Test (MAT)

Swine serum samples were obtained from the sera bank of the Laboratory of Bacterial Zoonosis, Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo, and subsequently tested by the MAT according to (Faine et al. 1999). A total of 86 samples were collected from the animals at a pig farm with reproductive disorders such as abortions, premature births and stillbirths. Reciprocal agglutination titers of higher than or equal to 100 were considered positive reactions (Faine et al. 1999).

ELISA

Polystyrene ELISA microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, NY, USA) were sensitized with rLipL32 (100 ng/well). Samples were diluted 1:100 in phosphate-buffered saline Tween 20 and added to the plate. Then, rabbit anti-pig IgG peroxidase conjugate (Sigma-Aldrich, USA) was added and the reaction was revealed by adding a solution containing OPD and hydrogen peroxide. The colorimetric reaction was stopped by adding 25 µL of 2 M H_2SO_4 per well and the OD was measured at 492 nm using the VICTOR™ X5 Multilabel Plate Reader (Perkin Elmer, USA). The cut-off value was determined as the mean OD + two standard deviation of the swine serum pool (n = 47) diluted 1:100 among the serum samples from those animals, which were negative in the MAT (Bomfim et al. 2005). The evaluation of ELISA was carried out regarding sensitivity (capacity of ELISA to identify the positive serum samples, which were also positive on the MAT) and specificity (capacity of ELISA to identify the negative serum samples, which were also negative on the MAT) (Mariya et al. 2006). These experiments were repeated three times. Control serum samples from the healthy swine (n = 20) and from the swine with other bacterial diseases, except leptospirosis (n = 30) were used. These samples were composed of sera from the animals with positive serology for enzootic pneumonia (n = 20) and brucellosis (n = 10). The concordance between the MAT and ELISA assays was determined using the Kappa coefficient (Epi Info ™ 7 version).

RESULTS AND DISCUSSION

Several immunochemical approaches to detect the antibodies in human (Flannery et al. 2001) and animal sera (Dey et al. 2004; Bomfim et al. 2005; Mariya et al. 2006) have been proposed using rLipL32 from *E. coli*, including the pigs (Hartleben et al. 2012). In this study, out of 86 serum samples, 39 (46.98%) showed agglutination for one, or more *Leptospira* serovars with titers ranging from 100 to 6,400. In addition, these 39 samples MAT positive also reacted with rLipL32 in the ELISA assay, suggesting that the recombinant protein produced in *P. pastoris* retained the antigenic characteristics (Table 1). These findings further supported the hypothesis that during the infection of the mammals by the pathogenic *Leptospira*, LipL32 was an antigen recognized in the host humoral immune response (Haake et al. 2000).

The ELISA showed an accuracy of 95.34% relative to the MAT. The sensitivity (100 %) was the same reported for swine (Hartleben et al. 2012), cattle (Bomfim et al. 2005), and higher than reported for canine (Dey et al. 2004) and convalescent human sera (Flannery et al. 2001) using rLipL32 produced in *E. coli.* Furthermore, the specificity (91.48%) was higher for the same species previously reported (Hartleben et al. 2012). Four MAT negative samples reacted with rLipL32 in the ELISA. These samples were tested by the immunoblotting assay using LipL32 antigen. Out of four samples tested, two identified a band of 32 kDa (data not shown), suggesting false negative results in the MAT and two false positive results in the ELISA. No positive reaction was observed when sera from the animals with other bacterial diseases were tested. In comparison to "gold standard" serodiagnostic method of leptospirosis, MAT, the ELISA had an excellent agreement (kappa = 0.906977). These results demonstrated that ELISA developed here could be used as a screening test before confirmation by the MAT.

Table 1 - Microscopic agglutination test and ELISA results for swine leptospirosis diagnosis.

MAT		**ELISA Positive**		**ELISA Negative**	
Nº of samples	Titer[a] (≥100)	Nº of samples	OD ± SD[b]	Nº of samples	OD ± SD[b]
47	-	4	≤ 0.192 (0.031)	43	≤ 0.121 (0.023)
10	100	10	≤ 0.291 (0.053)	-	-
8	200	8	≤ 0.439 (0.033)	-	-
2	400	2	≤ 0.543 (0.052)	-	-
4	800	4	≤ 0.592 (0.051)	-	-
6	1600	6	≤ 0.775 (0.071)	-	-
6	3200	6	≤ 0.881 (0.079)	-	-
3	6400	3	≤ 0.897 (0.086)	-	-

[a] Antibody titers for MAT are expressed as the reciprocal of the highest serum dilution that resulted in 50 % agglutination of leptospires. [b] Mean scores of the ODs obtained of three experimental replication and standard deviation (SD).

Table 2 - Anti-leptospiral antibody detection in swine sera by ELISA compared to MAT.

ELISA	**MAT**		**Total**
	Positive	Negative	
Positive	39	4	43
Negative	0	43	43
Total	39	47	86

Sensitivity = 100 % (95 % confidence interval between 88.8 % and 100 %); Specificity = 91.48 % (95 % confidence interval between 78.7 % and 97.2 %); Kappa = 0.906977.

The large-scale production of proteins with potential diagnostic value is important for the pharmaceutical, biomedical and biotechnological applications (Cos et al. 2006; Hartwig et al. 2010). An efficient secretion system is of great interest since these proteins may be easily recovered from the culture supernatant and maintain the conformational structure (Hartwig et al. 2010). Therefore, in recent years *P. pastoris* has been used for the production of recombinant proteins (Cos et al. 2006). In order to evaluate the potential of rLipL32 produced in *P. pastoris* as a diagnostic tool, LipL32 coding sequence was cloned in pPICZαB allowing the secretion in this expression system. A band compatible with 32 kDa was observed (data not shown) and the yield obtained

from the culture supernatant (270 mg/L) was higher than rLipL32 produced in *E. coli* system (40 mg/L) (Seixas et al. 2007; Hartleben et al. 2012). Therefore, *P. pastoris* provided a cost-effective expression system for the production of LipL32 protein.

CONCLUSIONS

In conclusion, compared to *E. coli*, *P. pastoris* could express rLipL32 in larger quantities, which minimized the variations during the standardization of serological tests. In addition, the production of rLipL32 using this yeast did not impair the antigenic characteristics and ensured the use of antigen for detecting the leptospiral antibodies in swine sera.

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