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DIFFERENTIAL METHODS FOR ANALYZING THE OUTER MEMBRANE PROTEINS OF PATHOGENIC SEROGROUPS OF LEPTOSPIRA

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Abstract: The leptospiral outer membrane has a relatively complex protein profile Pathogenic mechanisms of Leptospira interrogans, the causal agent of leptospirosis, remain largely unknown. The outer membrane of pathogenic Leptospira species grown in culture media contains lipopolysaccharide (LPS), a porin (OMPL), and several lipoproteins, including LipL36 and LipL41. Loa22 was shown to be a protein having a C-terminal OmpA consensus domain. Loa22 was detected among pathogenic leptospires but not among non-pathogenic leptospires, suggesting the possible involvement of this protein in virulence. Loa22 is located in the outer membrane and a small portionis exposed on the cell surface. Thus, Loa22 may be a candidate for a novel vaccine against infection with pathogenic leptospires. The purpose of the study was to analyze the outer membrane proteins of pathogenic serogroups of leptospira by different methods whole cell solubilization, insoluble method and detergent method to identify the best method for extraction of proteins. Results of this study suggest that whole cell solubilization method was found promising for outer membrane protein extraction.

Keywords: Leptospira, OMP**,** Lipopolysaccharides, protein profiling.

Introduction

Leptospira is the etiologic agent of Leptospirosis, a bacterial zoonoses which is distributed worldwide and endemic in tropical environments (Katz *et al.*, 2002). Chronically infected domestic and wild animal species harbor leptospires in their renal tubules, which is shed into the environment upon urination. 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

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serve as indicators of pathogenicity between different *L. interrogans* serovars or virulence indicators in samples of the same *L. interrogans* serovar (Faine, 1999).

An important focus of current leptospiral research is identification of outer membrane proteins that are involved in the pathogenesis of leptospirosis (Bolin *et al.*, 1991). Leptospiral lipopolysaccharide (LPS) accounts for the antigenic diversity of pathogenic leptospires but the extensive serological diversity of leptospires has inspired a search for conserved outer membrane proteins (OMPs) that may stimulate heterologous immunity. Three classes of leptospiral OMPs have been identified. The most abundant class comprises the outer membrane lipoproteins and includes the major OMP and immunodominant protein antigen LipL32, the in-vivo down-regulated protein LipL36, LipL48 and the surface-exposed protein LipL41. Expression of major OMPs has been demonstrated both in culture and in host infections, and its surface exposure on the bacterial membrane has also been proven (Cullen P.A et al, loc.cit and Haake D.A 2002). Loa22 is a novel antigenic protein of pathogenic Leptospira, detected only among pathogenic leptospires, suggesting the possible involvement of this protein in virulence (Ristow, P. *et al.,* 2007). Immunofluroscence studies found that Loa22 is a surface exposed component of the leptospiral outer membrane and it was shown to be a lipoprotein having a C-terminal OmpA consensus domain. Thus, Loa22 may be a candidate for a novel vaccine against infection with pathogenic leptospires (Nobuo K, *et al*., 2003). In our work we have analyzed the outer membrane proteins of pathogenic serogroups of leptospira encompassing three genomospecies by different methods to identify the best method for extracting outer membrane protein.

Materials and Methods

Leptospiral strains and media

The spirochaete *L.interrogans* serogroups Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica and Pomona were utilised. The cultures were maintained at 28º-30º C in liquid EMJH (Ellinghausen McCullough Johnson Harrison) medium by routine subculture at 7 to 10 days intervals. The semisolid EMJH medium with agar (0.2 %) were used for maintenance of stock cultures.

Extraction of Outer membrane proteins

The leptospiral outer membrane has a relatively complex protein profile. The OMPs were extracted by three different methods.

i. Whole cell solublization method
ii. Insoluble / soluble membrane fractionation method.

iii. Detergent fractionation method.

Whole cell solublization method

Ten serogroups of leptospira (Table-1) were centrifuged at 12,000 g for 20 minutes. The pellets were washed twice in 5mM Magnesium chloride – phosphate buffered saline. Freeze thawed and resuspended in SDS-sample buffer, boiled for 10 min and subjected to polyacrylamide gel electrophoresis (PAGE).

Insoluble /Soluble membrane fractionation method

Leptospiral cultures (10 serogroups) were harvested and centrifuging at 12,000 g for 10 min and the pellets were washed twice in 5mM Mgcl2 –PBS and re suspended in lysis buffer (50mM Tris chloride l(pH 8),150mM Nacl,10mM EDTA, 1mg lysozyme /ml and 15% sucrose). The bacterial suspension was subjected to three cycles of freezing, thawing and tip sonication followed by centrifugation at 1, 00,000 g for 30 min to separate the soluble supernatant fraction (Periplasmic and cytoplasm) from the membrane pellet (cytoplasmic and outer membrane) fraction . The supernatant was precipitated with acetone, resuspended in SDS sample buffer and subjected to PAGE.

Detergent fractionation method

Cultured leptospira of 10 serogroups were pelleted and washed in 5mM Mgcl2-PBS and extracted in the presence of 1 percent Triton X-114, 10mM Tris (pH 8) , and 1mM EDTA at 4˚C. The insoluble material was removed by centrifuging at 17,000 g for 10 min. The Triton X-114 concentration in the supernatant was increased to 2 percent. Phase separation was performed by warming the supernatant to 37˚C and subjecting it to centrifugation for 10 min at 2,000 g. The detergent (outer membrane proteins (Omps) and aqueous (periplasmic) phases were separated and precipitated with acetone, followed by PAGE. .(Zuerner et al *loc.cit*).

SDS PAGE Gel Electrophoresis:

For one dimensional SDS –PAGE, samples were solubilized in SDS-PAGE sample buffer. The samples were heated to 100˚C for 10 min and electrophoresed in 12 % polyacrylamide gels. Molecular weight protein standards were used for analyzing the molecular weights of migrated proteins.

Results

We analyzed the outer memberane proteins in three complementary leptospiral fractionation procedures. The whole cell solubilization method separated the leptospiral proteins of the whole cell components (Fig-1). The insoluble/soluble technique separated into total membrane (cytoplasmic membrane and outer membrane) and soluble (cytoplasm and

periplasm) fractions (Fig–2). The detergent method demarcated the proteins into Triton X-114 soluble and insoluble fractions, followed by phase partitioning of the Triton X-114 soluble fraction into detergent (hydrophobic) and aqueous (hydrophilic) phases.(Fig-3). The presence of major outer membrane proteins was detected by SDS-PAGE in all method. The sizes of the proteins were correlated with protein molecular weight marker in all the three methods but sharp and neat bands could be seen only in whole cell solublisation method..Analysis of all the methodologies for OMP extraction conclude that whole cell solubilisation method is best suited for separation of outer memberane protein which can be further used for developing novel protein based diagnostics.

Discussion

In recent years, the identification of immunogenic outer membrane proteins of pathogenic Leptospira has become a major focus of Leptospira research (Nobuo. K.and Watanabe,H., *loc.cit*). Pathogenic *Leptospira* species possess a number of protein antigens that are expressed during infection of mammalian hosts and become targets for the host immune response (Guerreiro, H.,*et al* 2001). Studies suggest that the leptospiral OM has a relatively complex protein profile. The first investigation of the *L. interrogans* OM 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 weights (Haake D.A *et al., loc.cit*; Cullen P.A *et al., loc.cit*).

The goals of this study was to perform analyses of the protein extraction by three different techniques and to standardize one technique for OMP extraction in Leptospira. Several candidate virulence factors have been identified that may contribute to the pathogenesis of Leptospira infections. These include LPS, outer membrane proteins (OMPs) and other surface proteins, and adhesion molecules. To date, only a few leptospiral outer membrane proteins have been characterized in detail, Zuerner *et al., (1991)* have shown that major outer membrane protein (MOMP) could be solubilized by extraction of the outer membrane with the nonionic detergent. Among these, OMPs may be potential targets to induce and enhance immune responses against the disease. The protein Loa22 exhibits a bipartite structure, which includes an N-terminal domain that is unrelated to other eukaryotic or prokaryotic protein domains, followed by an OmpA domain. C-terminal amino acid sequence analysis of Loa22 revealed that other proteins of *L. interrogans* (LA4337, LA3685, LA0056, LA3615, and LB328) have sequence homology with members of the OmpA family.

These *L. interrogans* putative proteins, including Loa22, share between 46% and 59% sequence similarity in their C terminal domain, but they have significant amino acid sequence heterogeneity in their N-terminal domains (Ristow, P., *et al*. loc.cit) From this research we decided to give importance for Loa22 outer memberane protein extraction.

In our present study we used three techniques for protein extraction. Whole cell solublisation method, Insoluble/soluble membrane fraction method and detergent method. Previous leptospiral fractionation studies have demonstrated that the Triton X-114 insoluble material consists of the protoplasmic cylinder, including the cytoplasm, cytoplasmic membrane, and peptidoglycan cell wall, including penicillin-binding and flagellar proteins. The Triton X-114 detergent phase has been shown to contain outer membrane components, including leptospiral LPS, OmpL1 (an outermembrane porin), and several lipoproteins, including LipL32 (the major outer membrane protein), LipL36, and LipL41, while Triton X-114 aqueous phase would be expected to contain soluble periplasmic proteins (Nobuo, K and Watanabe, H *et al loc.cit*). Paul *et al* (2002) opined that the *L. interrogans* outer membrane consist of a minimum of 67 discrete protein units, including at least 12 that are the products of different genes. The present study concludes the whole cell solubulization method as the simple extraction method for studying the outer membrane proteins of leptospira.

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REFERENCES

[1] Nally, J. E. Chow, E. Fishbein, M. C. Blanco, D. R and Lovett, M. A. (2005) Infect. Immun. **73:**3251–3260.

[2] Zuerner, R.L., and Bolin, C.A. (1991) *Vet.Microbiol.* **24**: 355 366.

[3] Guerreiro, H., Croda, J., Flannery, B., Mazel,M., Matsunga,J., Galvao, R.M, Levett, P.N., Ko, A.I and Haake, D.A. (2001) *Infect Immun*. **69:** 4958-4968.

[4] Koizumi, N., and Watanabe, H. (2003) *FEMS Microbiol Lett* .**226:** 215–219.

[5] Haake, D.A., and Matsunaga, J. (2002) *InfectImmun***70:** 4936–4945.

[6] Cullen, P.A. Xu, X., Matsunga, J., Sanchez, Y., Ko, A.I., Haake, D.A and Alder, B. (2005) *Infect Immun*. **73:** 4853 – 4863.

[7] Ristow, P., Bourhy, P., Weykamp, D.A., Cruz McBride, F., Pereira Figueira, C. and Huerre. M, et al. (2007) *PLoS Pathog*. **3(7)**: e97.

[8] McBride AJ, Athanazio DA, Reis MG, Ko AI (2005) Leptospirosis. *Curr OpinInfect Dis* 18: 376–386.

[9] Bharti A.R, Nally J.E, Ricaldi JN, Matthias MA, Diaz MM, et al. (2003) *Lancet Infect Dis***3:** 757–771.

[10] Lerg. 2011. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group. Geneva, Switzerland: World Health Organization.

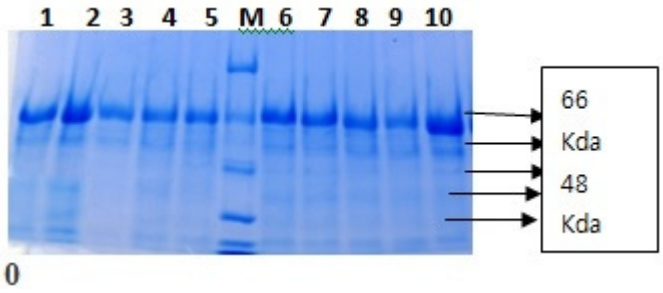
[11] Koizumi, N. & Watanabe, H. (2004) *Vaccine***,** 22: 1545-1552.

[12] Faine, S. (1999) 2.ed. Melbourne: *MediSci*, 272p.

[13] Bolin, C. A., Cassells, J. A. Zuerner, R. L. and G. Trueba**.** (1991) *Am. J. Vet.Res*. **52:**1639–1643.

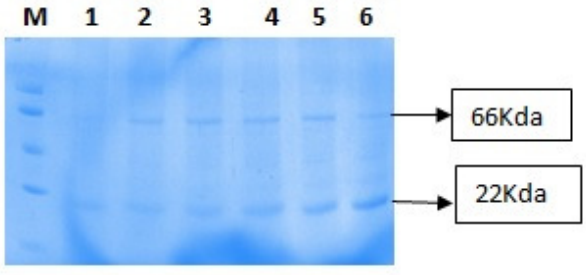
[14] Paul, A.C., Stuart, J.C., Dieter, M.B., Davis, A.H. and Ben, A. (2002) *Infect Immun**70*:** 2311-8.

Fig 1: Whole cell solubilisation method:

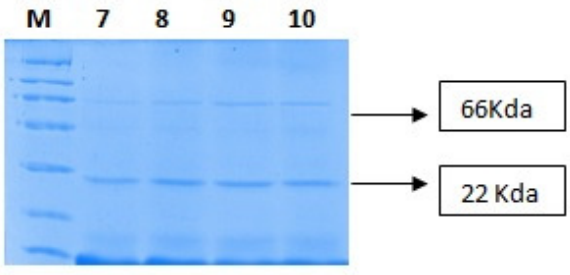


Legend: Lane1-10 leptospiral serogroups australis, autumnalis, ballum, canicola, grippotyphosa, hardjo, hebdomadis, icterrohaemorrhagiae, javanica, pomona

Fig 2: Insoluble Method

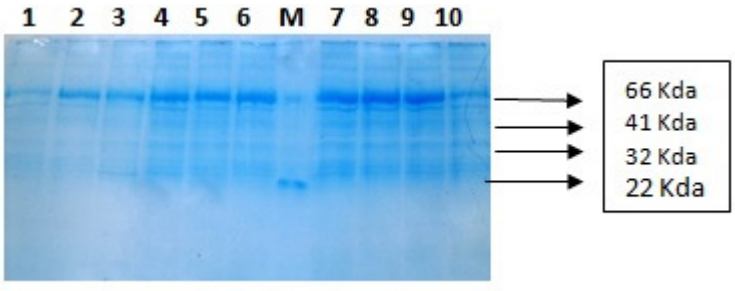


Legend: Lane1-6 leptospiral serogroups australis, autumnalis,ballum,canicola marker,grippotyphosa,hardjo



Legend: Lane 7-10 hebdomadis,icterrohaemorrhaegiae, javanica and Pomona..M-

Fig 3: Detergent method:



Legend: Lane1-10 leptospiral serogroups australis, autumnalis, ballum, canicola, grippotyphosa, hardjo,hebdomadis,icterrohaemorrhagiae,javanica, Pomona

Table 1: REFERENCE SEROGROUPS / SEROVARS / STRAINS OF LEPTOSPIRA

S.No	CODE	SEROGROUP	SEROVAR	STRAIN
1	AUS	Australis	australis	Ballico
2	AUT	Autumnalis	bangkinang	Bangkinang I
3	BAL	Ballum	ballum	Mus127
4	CAN	Canicola	canicola	HondUterecht IV
5.	GRI	Grippotyphosa	grippotyphosa	Moskva V
6.	SEG	Sejroe	hardjo	Hardjopraj
7.	HEB	Hebdomadis	hebdomadis	Hebdomadis
8.	ICT	Icterohaemmorrhagiae	icterohaemorrhagiae	RGA
9.	JAV	Javanica	poi	Poi
10.	POM	Pomona	pomona	Pomona