Full Length Article

FATTY ACID METHYL ESTER PROFILE OF THE LIPOPOLYSACCHARIDES FROM SELECTED SEROVARS OF PATHOGENIC *LEPTOSPIRA*

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ABSTRACT

Leptospirosis is one of the most important zoonotic disease caused by the pathogenic leptospires *Leptospira interrogans*. In this study, four pathogenic *Leptospira* serovars were selected based on the seroprevalence data, their LPS extracted, assessed for its purity and seroreactivity and processed for the determining the fatty acid profiles. The average concentration of the LPS extracted from the cultures across the serovars was 742.75±19.4 µg/ml with the polysaccharides contributing to 77.2 % of the LPS (573.6+18.01 µg/ml). The common fatty acids that were present in all the four serovars included butyric acid (C4:0), capric acid (C10:0), palmitoleic acid (C16:0), eicosapentaenoic acid (C20:5n3) and docosapentaenoic acid (C22:6n3). The heptadecanoic acid (C17:1) was present in serovars australis and autumnalis; pentadecanoic acid (C15:0) in canicola and hardjo serovars and caproic acid (C6:0) in the serovars autumnalis and canicola. The fatty acid eicosatrinoic acid (C20:3n3) was unique to the serovar autumnalis and the myristic (C13:0) and docosadienoic acid (C22:2) being unique to the serovar hardjo. Future studies to understand the fatty acid profile of the other common pathogenic circulating *Leptospira* LPS will add scientific evidence to the minor cross-reactivity observed across the serovars in MAT.

Keywords*: MAT, sero-reactivity, cross-serovar reactivity, LPS*

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INTRODUCTION

Leptospirosis, one of the most significant zoonoses, is brought on by the pathogenic *Leptospira interrogans*. *Leptospira* are obligate aerobic spirochetes that exhibit both the characteristics of gram-positive and gram-negative bacteria (Haake and Zückert, 2015). Leptospires are classified into more than 200 serovars (SVs) and 24 serogroups based on the structural diversity of its major extracellular component the lipopolysaccharide (LPS) and studies in mice, humans, and hamsters reveal its role in eliciting the production of agglutinating, opsonic, and protective antibodies. The LPS component of a gram-negative bacterium is made of the lipid A (the hydrophobic component), the core oligosaccharide (hydrophobic) and the Oantigen or Opolysaccharide (repeated hydrophilic distal oligosaccharide) (Caroff and Novikov, 2020). Despite the structural, biochemical, and immunological similarity of leptospiral LPS to gram-negative bacterial LPS, they are known to be at least 10-fold less toxic for animals or cells (Bulach *et al.,* 2000). It is well known from reports that the LPS in *Leptospira* contributes to serogroup as well as serovar-specific diagnosis and the use of a whole-cell vaccine elicits a broader immune response. This is justified by the fact that there is no cross-protection with widely cross-agglutinating saprophytic *L. biflexa* and the cross-reactivity observed in MAT, LPS epitopes are unable to share the cross-protection (Gitton *et al.,* 1994). The degree of acylation pattern in the fatty acyl chains, the length of the fatty acids, methyl and phosphate group of the lipid a component of the LPS contribute to the biological and pathological properties (Rietschel *et al.,* 1996). In this context, it would be interesting to profile the fatty acids from selected reference serovars and assess their uniqueness and shared profile. Hence, in this study, we extracted the LPS from the serovars australis, autumnalis, canicola and hardjo, assessed for their purity and seroreactivity and planned to determine the fatty acid profile by fatty acid methyl ester analysis (FAME).

MATERIALS AND METHODS

Culture and extraction of LPS from selected *Leptospira* reference serovars: The study included the reference pathogenic *Leptospira interrogans* serovars australis and autumnalis and clinical isolate from canine (identified as *L. interrogans* serovar canicola) and bovine (identified as *L. interrogans* serovar hardjo) maintained in Ellinghausen McCullough Johnson Harris (EMJH) (Difco) liquid media supplemented with 10 % enrichment supplement (Difco) were grown for a week at 29°C. Around 200 mg wet weight of the *Leptospira* cell pellet was used for the LPS extraction using the hot-phenol method (Westphal and Jann, 1965).

Purity and concentration of the extracted LPS from the *Leptospira* serovars: The thiobarbituric acid assay was performed to determine the concentration of LPS in the extracted samples (Skozha and Mohos, 1976) and the LPS from *E.coli* served as a standard. As the LPS is made of the core and the O-polysaccharide, the extracted LPS was

subjected to phenol-sulphuric acid assay using glucose as standard (Du Bois *et al.,* 1956) to determine the polysaccharide content. The presence of other residual proteins in the LPS preparation was determined by the bicinchoninic acid (BCA) assay. The purity of the extracted LPS was determined by electrophoresing 10 µg/well in a 15 % SDS PAGE gel followed by silver staining (Kittelberger and Hilbink, 1993) and the results were documented.

Sero-reactivity of the extracted *Leptospira* LPS: A panel of dog serum samples that tested positive and negative by the standard microscopic agglutination assay (MAT) was used to determine the seroreactivity of the extracted LPS by dot immunoblot and indirect ELISA. For the dot immunoblot, the *Leptospira* LPS (2 µl at a concentration of 5 µg/µl) was dotted onto an activated PVDF membrane, air dried and the positive sera resulted in a brown dot. For the indirect ELISA, the freeze dried LPS (200 ng/well) was coated on a PVDF membrane, air dried and was assessed using a known positive and negative at different dilutions of the serum. After the standard ELISA protocol the absorbance was measured at 490 nm in an ELISA reader (Biorad, Hercules, CA, USA). Following the choice of the optimum dilution of the serum, the sera panel classified by MAT were used to determine reactivity of the extracted LPS.

Fatty acid methyl ester (FAME) analysis of the *Leptospira* LPS by GC-FID:

4 ml of methanol was added to the freeze dried *Leptospira* LPS (100 mg) and incubated at 80°C for 30-60 minutes for methanolysis and was performed the analysis was performed in Netel Michrograph 9100 GC with a Flame ionization detector (FID). The peaks obtained for the extracted *Leptospira* LPS from the selected serovars samples peaks were compared with the Supleco 37 component FAME standard mix (Sigma-Aldrich) to determine the fatty acid profile.

RESULTS AND DISCUSSION

Leptospirosis is an infection caused by the pathogenic spirochaete *Leptospira* species with ubiquitous distribution. It is a known fact that *Leptospira* LPS plays a major role in immunity and hence is used as a potential antigen in a variety of diagnostic formats (Wang *et al.,* 2010; Widiyanti *et al.,* 2013). In this study, we have generated data on the profile of fatty acids that are unique and common across the LPS extracted from selected reference pathogenic *Leptospira* serovars.

Purity and concentration of the extracted LPS from the *Leptospira* serovars

The four pathogenic serovars of *Leptospira interrogans*: australis, autumnalis, canicola (canine isolate) and hardjo (bovine isolate) were chosen based on seroprevalence studies by MAT in humans and animals over the last decade (Senthil Kumar *et al.,* data to be published) and the hot-phenol method was followed for the extraction of the LPS in this study as they have been reported to be effective in *Leptospira* (Bonhomme *et al.,* 2020; Vanithamani *et al.,* 2021) and also

from other gram-negative bacteria (Davis and Goldberg, 2012).

The 3-Deoxy-D–manno-oct-2-ulosonic acid (Kdo), an 8-carbon sugar that is ubiquitous in gram-negative LPS have been targeted for detection methods and used for the indirect quantitative analysis of the extracted LPS (Osborn, 1963) by the thiobarbituric acid assay. The concentration of the LPS based on the KDO sugar for the selected pathogenic serovars australis, autumnalis, canicola and hardjo were 747 µg/ml, 760 µg/ml, 749 µg/ml and 715 µg/ml respectively (742.75±19.4 µg/ml across the serovars). The concentration of the LPS and the low levels of total protein indicated the quality and purity of the extracted LPS with the hot-phenol method. The polysaccharide content of the LPS component determined by the phenol sulphuric acid method which revealed a concentration of 582.5 µg/ml, 547.5 µg/ml, 588 µg/ml and 576.1 µg/ml for the serovars australis, autumnalis, canicola and hardjo respectively (573.6±18.01 µg/ml across the serovars) and contributes to 77.2 % of the LPS component. The SDS-PAGE was performed to visually the purity of the extracted LPS and we observed 14 to 34 kDa molecular weight bands following silver staining (Figure 1). The seroreactivity profile observed in the dot-immunoblotting and indirect ELISA(1:400 serum dilution) correlated with the MAT results indicating that the extracted LPS is antigenically intact and capable of differentiating sera samples into positive and negative (Figure 2).

Fatty acid methyl ester profile of the LPS of the selected *Leptospira* pathogenic serovars

Lipopolysaccharide (LPS) is a highly immunogenic compound and is responsible for serovar specificity (Kelson *et al.,* 1988; de la Pena-Moctezuma *et al.,* 1999). Gas chromatography (GC) coupled with the flame ionization detector (FID) had been applied for determining the fatty acid profile in few of the studies (Yi and Hackett 2000). To determine the profile, the chromatograms were generated with the fatty acid standards and the same was matched with the test sample for the analyses. In our approach, the Sulpeco 37 mix (containing 37 different fatty acids) was used as a standard, and the chromatograms and retention time are shown in Figure 3.

The fatty acids that were present in all the serovars were butyric acid (C4:0), capric acid (C10:0), palmitoleic acid (C16:0), eicosapentaenoic acid (C20:5n3) and docosapentaenoic acid (C22:6n3). Tridecanoic acid (C13:0) was present in the *L. interrogans* serovars australis, autumnalis, canicola whereas myristic acid (C14:0) in the serovar hardjo. (Table1). Eicosatrienoic acid (C20:3n3) was a unique fatty acid in the serovar autumnalis. Pentadecanoic acid (C15:0) was found in serovars canicola and hardjo whereas, the heptadecanoic acid (C17:0) was found in the australis and autumnalis serovars. Eicosapentaenoic acid (C20:5n3) and docosapentaenoic acid (C22:6n3) was unique in hardjo serovar (Table 1).

In a previous study, the fatty acids palmitic acid (C16:0), hydroxyl lauric acid (3-

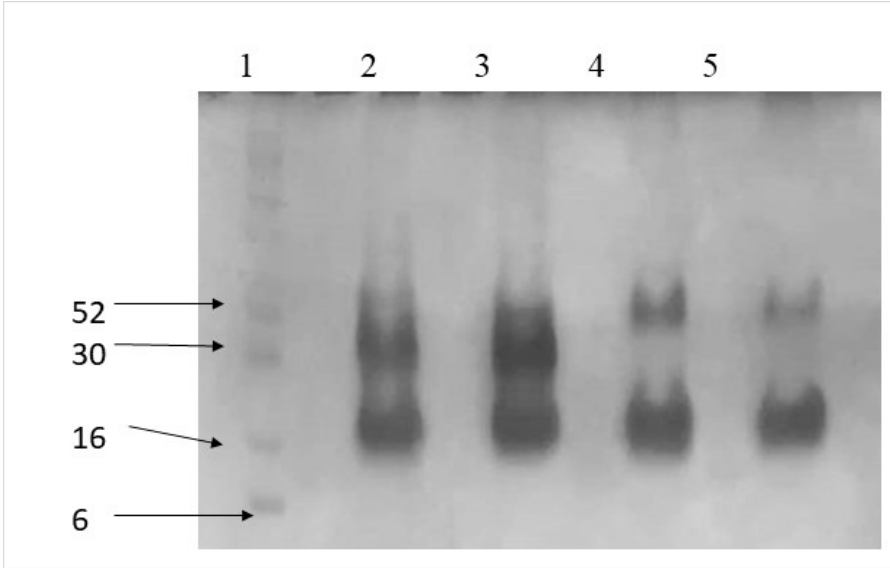

Figure 1. The SDS-PAGE analysis of the LPS extracted from the reference pathogenic serovars to determine their purity

The PAGE profile revealed 14 to 34 kDa molecular weight bands in all the samples. Lane 1- Protein ladder, Lane 2 australis LPS, Lane 3 autumnalis LPS, Lane 4- canicola LPS and Lane 5- hardjo LPS.

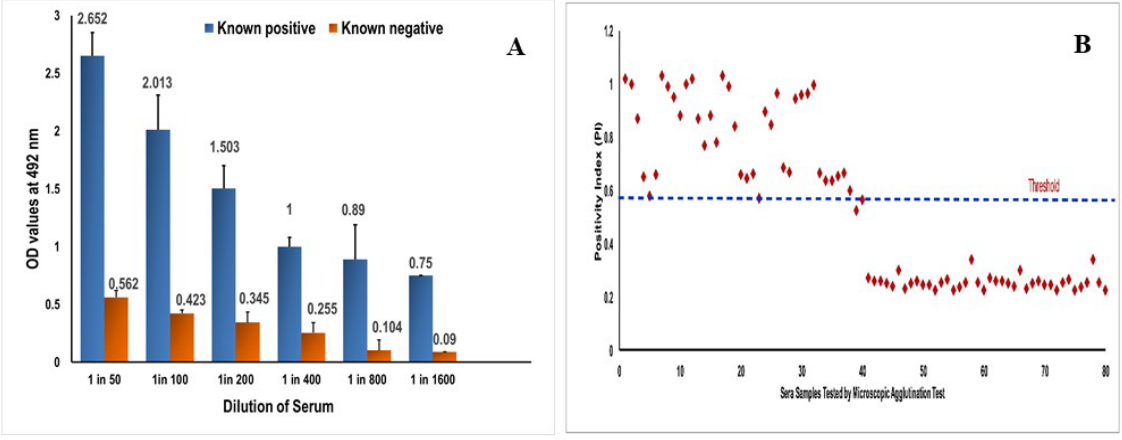

Figure 2. A. Setting the optimum serum dilution for the indirect ELISA using a known MAT positive and negative serum B. Preliminary screening of the MAT positive and negative serum using the LPS extracted from pathogenic serovars

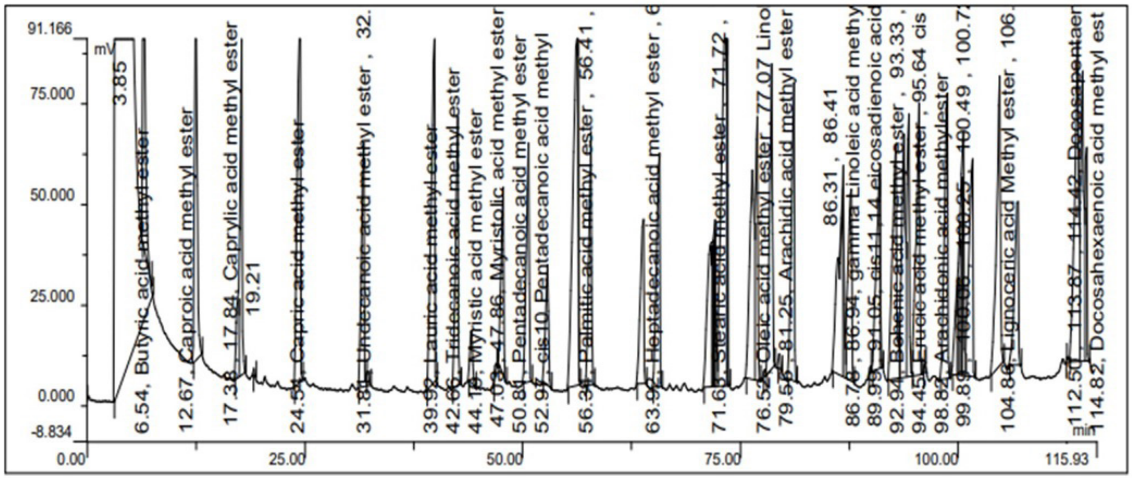

Figure 3. Fatty acid methyl ester analysis of the Supleco 37 component standard mix

OH C12:0), oleic acid (C18:0) and 3-hydroxy palmitate (3-OH C16:0) were reported to be the major components of the fatty acid chain of LPS in both pathogenic, non-pathogenic leptospires and intermediate virulent strain leptospires (Patra *et al.,* 2015; Vanithamani *et al.,* 2021). Other fatty acids such as stearic acid (C18:0); myristic acid (C14:0) and hydroxyl-myristic acid (C14:1) were also reported in the leptospires. The myristoleic acid (C14:1) and palmitoleic acid (C14:1) were reported to be the minor fatty acids in the lipid component of the LPS (Patra *et al.,* 2015). The LPS samples of hardjo included decanoic (C 10), tridecanoic (C 13), and branched fatty acids (a,z-C15) (Vinh *et al.,* 1989). Similar fatty acids, such as 3-hydroxy-decanoic acid (3-OH-C10), were reported to be present in the LPS of the strains L171 and LI87, however the strain LI3 (hardjoprajitno) LPS included various fatty acids, such as 2-hydroxydecanoic (2-OH-C10), 2-hydroxydo-decanoic (2-OH-C12), palmitic, and stearic acids. This study in addition to confirming the fatty acid profile of australis, autumnalis and hardjo has also reported the profile in canicola that has not been reported so far. The fatty acids C16-C18 (Up to C24 in certain bacteria) have been shown to contribute to the virulence of pathogenic bacteria (Johnson *et al.,* 1970; Cacciapuoti *et al.,*1991). The list of fatty acids which that are present in the four selected pathogenic serovars that have been analyzed in this study (Table 1) provides explanation to the limited cross serovar reactivity in MAT. Future studies need to be targeted to determine the profile from the common circulating pathogenic serovars and this might provide an understanding to their pathogenicity. Understanding the fatty acid profile of LPS in *Leptospira* and in other gram negative bacteria is much more important to understand the low

Table 1. The fatty acid methyl ester profile of the LPS extracted from pathogenic Leptospiral serovars australis, autumnalis, canicola and hardjo.

S.#	australis LPS		autumnalis LPS		canicola LPS		hardjo LPS	
	Fatty acid	Con.	Fatty acid	Con.	Fatty acid	Con.	Fatty acid	Con.
1.	*Butyric acid (C4:0)*	*0.96*	*Butyric acid (C4:0)*	*0.69*	*Butyric acid (C4:0)*	*0.65*	*Butyric acid (C4:0)*	*0.93*
2.	-	-	*Caproic acid (C6:0)*	*0.13*	*Caproic acid (C6:0)*	*0.12*	-	-
3.	**Capric acid (C10:0)**	**75.1**	**Capric acid (C10:0)**	**76.5**	**Capric acid (C10:0)**	**73.6**	**Capric acid (C10:0)**	**75.77**
4.	**Tridecanoic acid (C13:0)**	**30.07**	**Tridecanoic acid (C13:0)**	**20.9**	**Tridecanoic acid (C13:0)**	**21.8**	**Myristic acid (C13:0)**	**22.13**
5.	-	-	-	-	*Pentadecanoic acid (C15:0)*	*0.12*	*Pentadecanoic acid (C15:0)*	*0.12*
6.	*Palmitoleic acid (C16:0)*	*0.32*	*Palmitoleic acid (C16:0)*	*0.17*	*Palmitoleic acid (C16:0)*	*0.3*	*Palmitoleic acid (C16:0)*	*0.29*
7.	*Heptadecanoic acid (C17:1)*	*0.06*	*Heptadecanoic acid (C17:1)*	*0.24*	-	-	-	-
7.	-	-	*Eicosatrienoic acid (C20:3n3)*	0.37	-	-	-	-
8.	*Eicosapentaenoic acid (C20:5n3)*	*0.52*	*Eicosapentaenoic acid(C20:5n3)*	*0.07*	*Eicosapentaenoic acid (C20:5n3)*	*0.4*	*Eicosapentaenoic acid (C20:5n3)*	*0.12*
9.	-	-	-	-	-		*Docosadienoicacid (C22:2)*	*0.51*
10.	*Docosapentaenoic acid (C22:6n3)*	*0.58*	*Docosapentaenoic acid (C22:6n3)*	*0.55*	*Docosapentaenoic acid (C22:6n3)*	*0.4*	*Docosapentaenoic acid (C22:6n3)*	*0.9*

The concentration of the fatty acids determined to be a constituent of the LPS from the four reference pathogenic serovars is indicated as percentage (%) The *ash-coloured* highlighted cells indicate the fatty acids present in all the serovars tested;*Orange cells* are the common fatty acid across autumnalis and canicola serovar; *Green cells* are the common fatty acids across serovars australis, autumnalis and canicola; *Pink cells* are the fatty acid common across canicola and hardjo; *Yellow cells* are the common fatty acid across australis and autumnalis serovar; ***Clear cells are the unique fatty acid present.*** Bold font indicates the fatty acids that are present in high concentration; Italics and underlined indicate the fatty acids that are present in low concentration.

immunogenicity as reported for the leptospiral LPS. The study of the pathogenic and non-pathogenic Leptospires would also be more valuable in understanding the same.

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