Characterization and Taxonomic Significance of Lipopolysaccharides of *Leptospira interrogans* Serovar *hardjo*

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Lipopolysaccharides (LPSs) from *Leptospira interrogans* serovar *hardjo* (reference strain hardjoprajitno and strain hardjobovis) were prepared by the hot phenol–water procedure. High yields of LPSs were found in the phenol phase. Gel electrophoresis of the phenol phase LPSs showed similar patterns for all strains in contrast to the different patterns found in the water phase LPSs. Sugar composition was also similar among all strains with rhamnose as the predominant sugar. Mannosamine was detected by high performance thin layer and gas-liquid chromatography. 2-Keto-3-deoxyoctonic acid (KDO) was comparable with authentic KDO by paper chromatography. Periodate oxidation at near neutral pH with or without prior hydrolysis showed that most of the KDO was substituted. The fatty acid composition of strain hardjobovis LPS was slightly different from that of the reference strain hardjoprajitno. Myristic and 3-hydroxymyristic acid were not detected in any of the LPS preparations. In conjunction with genetic and other data, the two strains are sufficiently different to be regarded as members of two separate species sharing common antigens. There is sufficient evidence to rename the hardjoprajitno strain type *L. interrogans hardjo-p*, and the hardjobovis strain type *L. borgpeterseni hardjo-b*.

INTRODUCTION

Leptospira interrogans serovar *hardjo* is a common causative agent of leptospirosis in Australia and New Zealand (Adler & Faine, 1980; Blackmore & Schollum, 1982) as well as in other parts of the world (White *et al.*, 1981; Thiermann, 1984).

Leptospiral lipopolysaccharide (LPS) has been shown to be an important antigen capable of eliciting the production of protective antibody during infection (Adler & Faine, 1978*a, b*), thus playing an important role in leptospiral immunity (Jost *et al.*, 1986; Chapman *et al.*, 1988). Ultrastructurally, leptospiral LPS, consisting of ribbon-like structures, resembles the LPS of other Gram-negative bacteria (Vinh *et al.*, 1986*a*). LPSs extracted from virulent strains of serovar *copenhageni* (Vinh *et al.*, 1986*a*; Isogai *et al.*, 1986) or serovar *canicola* Moulton strain (Shimizu *et al.*, 1987*a*) lack hydroxytetradecanoic acid and 2-keto-3-deoxyoctonic acid (KDO), characteristic components of Gram-negative, especially enterobacterial, LPS. However, a KDO-like component, giving a positive reaction with thiobarbituric acid, was found in these preparations (Vinh *et al.*, 1986*a*; Shimizu *et al.*, 1987*a*). Furthermore, these LPSs did not possess the endotoxic activity that is well-documented in the LPS of other Gram-negative bacteria (Finco & Low, 1967; Vinh *et al.*, 1986*b*; Isogai *et al.*, 1986; Shimizu *et al.*, 1987*b*).

Leptospire are classified by serology. The antigens involved are LPS components. At present a single species of pathogenic leptospire, *L. interrogans*, is recognized officially, based on agglutination–absorption tests (Kmety & Dikken, 1988), but new species based on genetic differences have been proposed (Yasuda *et al.*, 1987). The recognized type strain of serovar *hardjo* is hardjoprajitno (Wolff, 1953), but the prevalent infecting strain isolated from cattle and

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Abbreviation: KDO, 2-keto-3-deoxyoctonic acid (3-deoxy-D-manno-octulosonic acid).

humans is hardjobovis (Robinson *et al.*, 1982; Thiermann & Ellis, 1986) which differs genetically (Lefebvre *et al.*, 1987) but not serologically from hardjoprajitno. In this paper we have characterized and compared the LPS from hardjoprajitno and hardjobovis strains; the taxonomic significance of the findings for the two strains of serovar *hardjo* is also discussed.

METHODS

Leptospire. *Leptospira interrogans* serovar *hardjo* (reference strain hardjoprajitno, L13, and strain hardjobovis, L171 and L187) were used. Strain L171 was a New Zealand bovine isolate obtained from Dr R. Marshall, Massey University, New Zealand, typed by him as hardjobovis. Strain L187 was isolated from a human placenta in Australia (Faine *et al.*, 1984); its characteristics resembled L171. The spirochaetes were grown and maintained as described previously (Vinh *et al.*, 1984).

LPS extraction. Leptospiral LPSs were extracted by the hot phenol-water procedure of Westphal & Jann (1965) as described previously (Vinh *et al.*, 1986a). Because of the low yield of LPS in the water phase (<1% total cell dry wt), LPS was extracted from the phenol phase as follows. The phenol phase was separated and dialysed extensively against tap-water at room temperature to completely remove the phenol, and was then dialysed against distilled water. The dialysed solution was then concentrated by ultrafiltration. An equal volume of absolute ethanol was added and the mixture was stirred overnight at 4 °C in the presence of 0.01% (w/v) sodium acetate. The precipitate formed was discarded and the supernate was brought to 90% (v/v) ethanol. The mixture was again stirred overnight at 4 °C in the presence of 0.01% (w/v) sodium acetate. The precipitate was collected and purified by ultracentrifugation as described previously (Vinh *et al.*, 1986a). Samples of LPS in double-glass-distilled water (100 µg ml⁻¹) were examined by UV absorption spectrophotometry to detect the presence of nucleic acids and proteins.

Escherichia coli O127:B8 LPS was purchased from Difco and *Salmonella minnesota* LPS from Sigma.

SDS-PAGE. Samples of LPS preparations (10 µg) were electrophoresed (Laemmli, 1970) using a 1 mm thick slab gel in a Bio-Rad Protean II gel apparatus. A 15% (w/v, acrylamide) separating gel and a 5% stacking gel without SDS (Hitchcock & Brown, 1983) were used and electrophoresis was done as described previously (Jost *et al.*, 1988). Proteinase-digested whole cell lysates were prepared as described by Hitchcock & Brown (1983). LPS bands were detected by the silver staining technique (Tsai & Frasch, 1982) as modified by Hitchcock & Brown (1983) with a minor modification in the developer solution whereby a concentration of 0.25% (w/v) of citric acid was used instead of 0.05% (w/v). The increase in citric acid concentration was found to circumvent the overdevelopment of the bands, thus giving better control of colour development.

Production of monoclonal antibodies. The production of monoclonal antibodies against *Leptospira interrogans* serovar *hardjo* LPS was described previously (Farrelly *et al.*, 1987).

Immunogold labelling. For immune electron microscopy cultures (5 ml) of leptospire in late exponential phase were used. After centrifugation for 10 min at 8000 g at room temperature, the leptospiral pellet was washed with tissue culture medium 199 (M199) containing 15 mM-HEPES without foetal calf serum, and resuspended in the same medium (0.5 ml). A drop of leptospiral suspension was deposited onto a carbon-Formvar-coated, 400-mesh copper grid for 20 min at room temperature. Excess fluid was removed with filter paper. The grids were then inverted onto a drop of a 1% (w/v) solution of bovine serum albumin (BSA) in M199 at room temperature for 20 min to prevent non-specific binding. After removal of excess fluid the grids were inverted onto a drop of either monoclonal antibody (MUM/F1-4/*hardjo*) dilution or Dulbecco's Modified Medium (control) for 1 h at 30 °C. Excess fluid was removed and the grids were washed with M199 containing 1% (w/v) BSA. The grids were then incubated with goat anti-mouse immunoglobulin colloidal gold conjugate (Auroprobe EM GAMIgG + IgM G5; Jannsen) for 30 min at room temperature. The grids were washed with M199 containing 1% (w/v) BSA, then with distilled water and stained with an aqueous solution of 2% (w/v) ammonium molybdate. After air drying, the grids were examined with a JEOL 100 electron microscope at 60 kV.

Chemical analyses. Protein was measured by the micromethod of Bradford (1976) using Bio-Rad protein assay reagents. Total carbohydrate was determined by the phenol/sulphuric acid method (Dubois *et al.*, 1956) using glucose as standard. Heptose and hexosamines were determined according to methods described by Nowotny (1979). Phosphorus was determined according to Chen *et al.* (1956). 2-Keto-3-deoxyoctonic acid (KDO) was estimated by the thiobarbituric acid reaction described by Karkhanis *et al.* (1978) after hydrolysis of LPSs either with 0.1 M-H₂SO₄ at 100 °C for 20 min or 4 M-HCl at 100 °C for 1 h. Commercial KDO (Sigma) was used as a standard.

Determination of KDO without prior hydrolysis (Droge *et al.*, 1970). To 50 µg LPS in 50 µl water were added 20 µl 25 mM-sodium periodate and the tube kept in the dark at 4 °C overnight. Sodium arsenite (20 µl; 0.2 M) in 0.5 M-HCl was added to stop the oxidation. After complete reduction of periodate 410 µl water was added and the tubes were heated in a boiling water-bath for 15 min and cooled in iced water. The reaction was then continued as described above.

Gas-liquid chromatography (GLC). To prepare samples for GLC analysis of fatty acids and monosaccharides, 1 mg of each LPS was hydrolysed with 4 M-HCl for 1 h at 100 °C. The fatty acids were extracted with

chloroform/methanol (1 : 1, v/v), dried with a stream of dry nitrogen and methylated as described previously (Vinh *et al.*, 1986a). The solution containing monosaccharides was dried *in vacuo* in the presence of KOH.

Fatty acid methyl esters were dissolved in 50 µl high purity grade hexane (Hipersolv; BDH) and 1 µl was injected into a Varian 3700 gas-liquid chromatograph equipped with a 25QC2/BP10 0.25 mm capillary column (SGE) and a flame ionization detector. The injector was operated with a 1 : 60 split ratio. Nitrogen was used as carrier gas at a rate of 30 ml min⁻¹. The injector and detector temperatures were 230 °C and 300 °C respectively. The temperature was programmed from 120 to 270 °C at a rate of 8 °C min⁻¹. The fatty acid profile was recorded with a Spectra Physics model SP4270 integrator and fatty acids were identified by comparison with known standards (Bacterial fatty acid methyl esters: Supelco).

Trimethylsilyl derivatives of monosaccharides were prepared by adding 100 µl TriSil (Pierce Chemicals). The mixture was incubated at 60 °C for 30 min and then samples (1 µl) were injected into a gas-liquid chromatograph as described above except that the injector was operated at a 1 : 35 split ratio and the temperature program started at 160 °C for 4 min, increasing to a maximum temperature of 260 °C at a rate of 5 °C min⁻¹. The sugars were identified by comparison with known standard sugars. To detect amino sugars, the alditol acetate derivatization method of Blakeney *et al.* (1983) was used.

Paper chromatography. KDO was identified by ascending paper chromatography using Whatman no. 1 filter paper with ethyl acetate/pyridine/water (2:1:2, by vol.) as solvent. The spots were detected with the thiobarbiturate reagent described by Nowotny (1979).

Detection of mannosamine by high-performance thin-layer chromatography (HPTLC). Monosaccharide hydrolysate (0.5 µl, prepared as above) was applied to 5 × 7 cm aluminium-backed Merck Kieselgel 60 F254 HPTLC plates 1 cm from the bottom edge. The separation was done as described by Rebers *et al.* (1986) using acetonitrile/acetic acid/ethanol/water (13:1:2:4, by vol.) as the solvent system. D-Glucosamine, D-galactosamine and D-mannosamine (Sigma) were used as reference compounds. After separation, the plates were dipped in 0.2% (w/v) ninhydrin in ethanol followed by heating at 100 °C to locate amino sugars. Solutions of silver nitrate in acetone and sodium hydroxide in ethanol (Stahl, 1969) were also used to detect reducing sugars.

RESULTS

LPS extraction

Leptospiral LPSs of serovar *hardjo* were found in both water and phenol phases when extracted by the phenol-water procedure of Westphal & Jann (1965). The yield of LPS in the water phase was very low (<1% cell dry wt). In contrast, the yield of LPS in the phenol phase accounted for 5–7% cell dry wt for all three strains. All LPS preparations were checked by UV absorption. The main leptospiral LPS peak was located at 192 nm as described previously (Vinh *et al.*, 1986a). No absorption peaks corresponding to nucleic acids or proteins were detected at 260 nm and 280 nm respectively. Serovar *hardjo* LPS from either phase had a ribbon-like ultrastructure observed by electron microscopy (data not shown) as described previously for serovar *copenhageni* (Vinh *et al.*, 1986a).

SDS-PAGE

The silver-stained gel profiles of all LPS preparations are shown in Fig. 1. Unlike the ladder-like profile of smooth-type enterobacterial LPSs (lanes 1 and 2), LPSs extracted from *L. interrogans* serovar *hardjo* showed different patterns on polyacrylamide gels. LPSs obtained from the phenol phase had similar patterns for all three strains (lanes 6, 7 and 8), whereas those obtained from the water phase showed different profiles between strain *hardjoprajitno* (lane 5) and the other two *hardjobovis* strains (lanes 3 and 4), both of which were similar. The phenol phase LPSs of all three strains and the water phase LPS of strain *hardjoprajitno* possessed three main characteristic and diffuse bands located between 18 and 30 kDa, whereas one main band at 28 kDa was found in the water phase LPSs of the two *hardjobovis* strains. A lower molecular mass band located in the region of the 'core-lipid A' of smooth Gram-negative bacterial LPSs (lanes 1 and 2, Fig. 1) was present in all LPSs; however it was much less intense in the water phase LPSs. The silver-stained profile of purified LPS was similar to that of the proteinase-K-digested whole cell lysate (data not shown). When transblotted and immunostained with a monoclonal antibody MUM/F1-4/*hardjo*, the three main bands described above were found to be reactive as described previously (Farrelly *et al.*, 1987) for all LPS preparations (data not shown). However, the monoclonal antibody did not react with the low molecular mass 'core-lipid A' band.

Fig. 1. Silver-stained SDS-PAGE profile of aqueous and phenol phase LPS preparations. Lane 1, *E. coli* O127:B8 LPS; lane 2, *Salmonella minnesota* LPS; lanes 3, 4 and 5, aqueous phase LPSs of serovar *hardjo*, hardjobovis strains L171 (lane 3), L187 (lane 4) and hardjoprajitno strain L13 (lane 5); lanes 6, 7 and 8, phenol phase LPSs of hardjobovis strains L171 (lane 6), L187 (lane 7) and hardjoprajitno strain L13 (lane 8). The small arrowhead (◄) indicates the 28 kDa band and the big arrowhead (►) indicates the band corresponding to the 'core-lipid A' region of Gram-negative LPS.

Table 1. *Composition of LPSs of L. interrogans serovar hardjo*

Values are means of two determinations $\pm$ SE. ND, Not determined.

Component	Amount present [μ g (mg LPS) $^{-1}$]			
	hardjoprajitno L13	hardjobovis		<i>E. coli</i> O127:B8
		L171	L187	
Protein	58 $\pm$ 5.0	36 $\pm$ 1.0	35 $\pm$ 1.0	58 $\pm$ 5.0
Lipid	350 $\pm$ 10.0	420 $\pm$ 8.0	370 $\pm$ 15.0	290 $\pm$ 10.0
Total carbohydrate	290 $\pm$ 10.0	292 $\pm$ 10.0	300 $\pm$ 15.0	175 $\pm$ 5.0
Amino sugars	60 $\pm$ 5.0	54 $\pm$ 5.0	54 $\pm$ 5.0	ND
Heptose	7 $\pm$ 0.1	8.5 $\pm$ 0.3	8 $\pm$ 0.1	ND
KDO	8 $\pm$ 0.5	12 $\pm$ 1.0	12.5 $\pm$ 1.0	14 $\pm$ 1.0
Phosphorus	8 $\pm$ 0.5	10 $\pm$ 1.0	9 $\pm$ 0.5	ND

Immunogold labelling. Immunogold staining of serovar *hardjo* (strain hardjoprajitno) with anti-LPS monoclonal antibody MUM/F1-4/*hardjo* showed that gold particles attached both to the surface of the leptospire as well as to LPS which had apparently detached from the leptospiral cell (Fig. 2).

Chemical analyses. Because of the low yield of water phase LPSs, all chemical analyses were done on phenol phase LPSs which were shown to have similar profiles on silver-stained SDS-PAGE gels.

Fig. 2. (a) Immunogold labelling of serovar *hardjo*, strain hardjoprajitno L13, with MUM/F1-4/*hardjo* anti-LPS monoclonal antibody. (b) Control. Bars, 1 µm.

The general composition of serovar *hardjo* LPS is shown in Table 1, and the fatty acid composition is shown in Table 2. Branched fatty acids (*a,i*-C15), decanoic (C10) and tridecanoic (C13) acids were found in all the LPS samples. LPS of strains L171 and L187 contained similar fatty acids including 3-hydroxy-decanoic acid (3-OH-C10), whereas strain L13 (hardjoprajitno) LPS contained different fatty acids including 2-hydroxydecanoic (2-OH-C10), 2-hydroxydecanoic (2-OH-C12), palmitic and stearic acids. Myristic and hydroxymyristic acids were not detected in any of the LPS samples.

Table 2. *GLC analysis of the fatty acid composition of LPSs of L. interrogans serovar hardjo*Values are means of two determinations $\pm$ SE. —, Not detected.

Fatty acid	Percentage of total lipid fraction in:		
	hardjoprajitno L13	hardjobovis	
		L171	L187
Decanoic (C10)	29.4 $\pm$ 0.1	21.5 $\pm$ 0.1	48.5 $\pm$ 0.5
2-Hydroxydecanoic (2-OH-C10)	12.8 $\pm$ 1.0	—	—
3-Hydroxydecanoic (3-OH-C10)	—	9.5 $\pm$ 0.1	19.5 $\pm$ 0.1
2-Hydroxydodecanoic (2-OH-C12)	18.4 $\pm$ 1.2	—	—
Tridecanoic (C13)	7.3 $\pm$ 0.1	23.7 $\pm$ 0.5	21.4 $\pm$ 0.5
13-Methyltetradecanoic (<i>i</i> -C15)	10.2 $\pm$ 0.2	9.4 $\pm$ 0.1	3.7 $\pm$ 0.1
12-Methyltetradecanoic (<i>a</i> -C15)	—	6.7 $\pm$ 0.1	4.2 $\pm$ 0.1
Pentadecanoic (C15)	14.7 $\pm$ 0.5	—	—
Palmitic (C16)	3.2 $\pm$ 0.1	—	—
Stearic (C18)	Trace	—	—

Table 3. *GLC analysis of the sugar composition of LPSs of L. interrogans serovar hardjo*Values are means of two determinations $\pm$ SE.

Sugar	Percentage of total carbohydrate fraction in:		
	hardjoprajitno L13	hardjobovis	
		L171	L187
Unknown	4.5 $\pm$ 0.5	5.0 $\pm$ 0.2	4.8 $\pm$ 0.2
Arabinose	10.5 $\pm$ 0.5	13.7 $\pm$ 1.0	11.0 $\pm$ 1.0
Rhamnose	22.0 $\pm$ 1.0	25.0 $\pm$ 0.8	24.8 $\pm$ 0.6
Ribose	15.0 $\pm$ 0.5	7.4 $\pm$ 0.9	10.8 $\pm$ 0.4
Fucose	2.4 $\pm$ 0.1	2.5 $\pm$ 0.1	1.2 $\pm$ 0.1
Xylose	15.6 $\pm$ 0.1	17.7 $\pm$ 0.5	19.5 $\pm$ 1.2
Mannose	8.1 $\pm$ 0.1	10.5 $\pm$ 0.3	9.3 $\pm$ 0.1
Galactose	1.2 $\pm$ 0.0	6.7 $\pm$ 0.1	3.5 $\pm$ 0.1
Glucose	3.1 $\pm$ 0.1	3.0 $\pm$ 0.5	3.5 $\pm$ 0.6
Glucosamine	1.6 $\pm$ 0.0	1.5 $\pm$ 0.1	2.4 $\pm$ 0.0
KDO	1.1 $\pm$ 0.3	1.5 $\pm$ 0.1	0.9 $\pm$ 0.1

Table 4. *KDO content of LPSs of L. interrogans serovar hardjo as determined by the thiobarbituric acid reaction with or without prior hydrolysis*For details of the hydrolysis conditions see Methods. Values are means of two determinations $\pm$ SE.

Source of LPS	Amount of KDO detected [$\mu\text{g (mg LPS)}^{-1}$]		
	Without prior hydrolysis	With prior hydrolysis	
		Mild	Strong
hardjoprajitno L13	10.0 $\pm$ 1.0	9.0 $\pm$ 0.5	55.0 $\pm$ 5.0
hardjobovis L171	12.0 $\pm$ 1.0	12.5 $\pm$ 1.0	40.0 $\pm$ 5.0
hardjobovis L187	12.5 $\pm$ 1.0	13.0 $\pm$ 1.0	65.0 $\pm$ 6.0

The sugar composition is shown in Table 3. All LPSs contained a similar number of monosaccharides. The major component was rhamnose, representing 20% (w/v) of the total monosaccharides. By using HPTLC for amino sugar analysis a spot having the same R_F as that of the mannosamine standard was detected. This component was identified as an amino sugar

by reaction with ninhydrin as well as with silver nitrate. This was confirmed by GLC of alditol acetate derivatives, when a peak having the same retention time as D-mannosaminitol was detected.

As shown in Table 4 the amount of KDO detected in all the LPSs increased considerably when the LPS samples were subjected to strong acid hydrolysis (4 M-HCl at 100 °C for 1 h), indicating the presence of substituted KDO. To confirm this observation, the procedure of Drogue *et al.* (1970), using periodate oxidation at pH 5–6 with or without prior hydrolysis, was followed. The results (Table 4) showed that with periodate oxidation of LPSs prior to hydrolysis only 6–12 µg (unsubstituted) KDO (mg LPS)⁻¹ were detected, whereas the amount of KDO detected increased up to 40–65 µg (mg LPS)⁻¹ after hydrolysis, indicating that most of the KDO in serovar *hardjo* LPS was substituted at position C4 and/or C5.

Paper chromatography of KDO from LPSs of serovar *hardjo* revealed that this compound had the same mobility as that of authentic KDO (R_F 0.30).

DISCUSSION

The present study describes the composition and gel profiles of LPSs extracted from *Leptospira interrogans* serovar *hardjo*, strain *hardjoprajitno*, which is the standard reference laboratory strain, and two local field isolate *hardjobovis* strains by the hot phenol–water procedure of Westphal & Jann (1965). LPSs of serovar *hardjo* were found in both aqueous and phenol phases. However, very little LPS could be obtained from the aqueous phase. One possible reason for this may be that the content of lipid in serovar *hardjo* LPS is higher ($\geq 30\%$, Table 1) than that of water-soluble *E. coli* LPS (Table 1), thus rendering the leptospiral LPS less soluble in water; hence it partitions preferentially into the phenol phase. It has been suggested that the solubility of the LPS molecule is dependent on the relative proportion of the hydrophilic polysaccharide and the hydrophobic lipid A (Galanos & Luderitz, 1984). The LPS of certain other bacteria has also been found in both phases (Raff & Wheat, 1968; Kreutzer *et al.*, 1979; Jessop *et al.*, 1988). LPS of other leptospiral serovars (*copenhageni*, *pomona*) is also found in the water phase. Clearly *hardjo* is different. On the other hand the original LPS-like TM antigen of serovar *kremastos* (Shinagawa & Yanagawa, 1972) was prepared from the phenol phase. The phase partitioning properties may vary between serovars and not be related to serovar specificity.

Structurally, the LPS preparations showed ribbon-like forms similar to that described previously for serovar *copenhageni* (Vinh *et al.*, 1986*a*). When examined by immunogold staining with a specific monoclonal antibody, LPS appeared to be distributed homogeneously on the outer membrane of the leptospire as shown by the binding of gold particles.

The different silver-stained gel profiles of the aqueous phase LPSs of the *hardjobovis* strains and the reference strain *hardjoprajitno* could be useful in distinguishing these two subtypes. In addition, our results indicate that it is inappropriate to rely solely upon standard water phase LPS in comparing profiles (Cinco *et al.*, 1986).

The general chemical composition was similar in all three strains of serovar *hardjo* LPSs which have a lipid content (35–40%) higher than that of serovar *copenhageni* LPS (33%) (Vinh *et al.*, 1986*a*) and *E. coli* O127:B8 LPS (29%).

The fatty acid composition of the reference *hardjoprajitno* and *hardjobovis* strains was somewhat different in that palmitic acid (C16:0) and stearic acid (C18:0) were not present in the *hardjobovis* strains. On the other hand, all LPSs possessed a fatty acid identified as isobranched pentadecanoic acid (*i*-C15:0). Branched chain fatty acids have been found generally in Gram-positive bacteria and in some Gram-negative bacteria (Jantzen & Bryn, 1985; Wollenweber *et al.*, 1980). Isobranched fatty acids were shown to be present in the LPSs of *Pseudomonas* species (Moss *et al.*, 1973; Wilkinson *et al.*, 1973). Whether or not the presence of this branched chain fatty acid in serovar *hardjo* LPSs has any taxonomic significance cannot yet be established.

The sugar composition of LPSs from serovar *hardjo* was similar to that previously reported in the LPS extracted from serovar *copenhageni* (Vinh *et al.*, 1986*a*) with a high content of rhamnose. The presence of mannosamine detected by HPTLC and by GLC as alditol acetate derivatives is the first reported for leptospiral LPS. Mannosamine could not be detected as

a trimethylsilyl derivative because it was insoluble in the trimethylsilyl derivatizing reagent thus masking its detection by GLC. Although we did not quantify the amount of mannosamine in LPS it appears that mannosamine was a major amino sugar in leptospiral LPS. Mannosamine has been found in the LPS of *Salmonella* groups J and T, *E. coli* O31 and Arizona 15 (Luderitz *et al.*, 1968), in the polysaccharide of pneumococcus type XIX (Shabarova *et al.*, 1962), and recently of group E *Streptococcus* strain K131 (Rebers *et al.*, 1986). The presence of mannosamine and other amino sugars in the LPS of other leptospiral strains is currently under investigation.

Unlike the KDO-like sugar detected in the LPS of serovar *copenhageni*, KDO in the LPSs of serovar *hardjo* was found to have the same mobility as that of the authentic KDO on ascending paper chromatography. The amount of KDO estimated by the thiobarbituric acid reaction after mild acid hydrolysis was 6–12 µg (mg LPS)⁻¹. However, when the strength of acid for hydrolysis of LPSs was increased to 4 M-HCl the amount of KDO detectable increased considerably [40–65 µg (mg LPS)⁻¹]. The small amount of KDO detected after mild acid hydrolysis may be due to the substitution at positions C4 and/or C5, thus requiring strong acid hydrolysis conditions to break the linkages of these substituents (Brade *et al.*, 1983; Zamze & Moxon, 1987). This was confirmed by periodate oxidation at near neutral pH with or without prior hydrolysis of LPS (Drogue *et al.*, 1970). The results showed that only a small amount of KDO which was not substituted at positions C4 and C5 was detected without prior hydrolysis, suggesting that most of the KDO was substituted at these positions. Substituted KDO has been shown to react weakly or not at all with thiobarbituric acid (Drogue *et al.*, 1970; Charon & Szabo, 1972; Chaby & Szabo, 1975). The apparent absence of authentic KDO in LPS from some leptospiral strains (Vinh *et al.*, 1986a; Isogai *et al.*, 1986; Shimizu *et al.*, 1987a) has prompted some workers to suggest that leptospires do not possess typical Gram-negative LPS (Shimizu *et al.*, 1987a). Recently, Cinco *et al.* (1988) found only traces of fatty acids in the phenol–water extract from *L. interrogans* serovar *copenhageni* (strain Teramo), and concluded that LPS was not present in *L. interrogans*. However, our present results with serovar *hardjo* indicate that leptospiral LPS is structurally and biochemically very similar to classical Gram-negative LPS.

Although there were physicochemical differences between the LPSs of hardjoprajitno and bovis, they are antigenically very similar (Farrelly *et al.*, 1987) and only minor epitope differences have been detected using monoclonal antibodies. The two strains are indistinguishable on the basis of agglutinating absorption tests (Kemety & Dikken, 1988) which measure mainly, if not exclusively, LPS antigens. This is consistent with the almost identical monosaccharide composition of the three strains, and with the carbohydrate nature of the agglutinating epitopes in *Leptospira* (Jost *et al.*, 1986; Terpstra *et al.*, 1987; Farrelly *et al.*, 1987). Both hardjoprajitno and hardjobovis share major epitopes involved in diagnosis, classification and possibly immunity, and both have a high lipid content which may be responsible for their LPS partitioning mainly in the phenol phase. However, the two strains show significant differences on PAGE of their aqueous phase LPS, and are genetically different. It has been shown that these two strains can be identified separately by restriction endonuclease DNA analysis (Robinson *et al.*, 1982; Thiermann *et al.*, 1986) and DNA hybridization and G+C content (Lefebvre & Thiermann, 1986; Lefebvre *et al.*, 1987). The hardjoprajitno strain has a G+C content of 34.9 mol% (Lefebvre *et al.*, 1987; Yasuda *et al.*, 1987) which would place it in the proposed new *interrogans* species, while hardjobovis has a G+C content of 39.9 mol%, consistent with the proposed species *borgpeterseni* (Yasuda *et al.*, 1987). These results give good reason to divide the current *L. interrogans* serovar *hardjo* into two different species, new species *L. interrogans* serovar *hardjo*, which is the hardjoprajitno strain and new species *L. borgpeterseni* serovar *hardjo* which is the hardjobovis strain.

The use of the infrasubspecific nomenclature for the strain types 'hardjoprajitno' and 'hardjobovis' is cumbersome and impractical, especially since serology by agglutination or ELISA with whole organisms or fragments or LPS cannot distinguish between them. DNA probes or gel profiles of aqueous phase LPS can, but are not readily available. For practical purposes of identification and nomenclature, the use for the time being of the trinomials *L. interrogans hardjo-p* for the strains of the hardjoprajitno type, and *L. borgpeterseni hardjo-b* for

the hardjobovis type is recommended. They cannot be referred to legitimately as different serovars because they are not serologically different according to the rules of leptospiral taxonomy (Faine, 1982).

The presence of substituted KDO requires the use of strong acid hydrolysis to obtain an accurate measurement of KDO, and mannosamine is a constituent of leptospiral LPS.

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