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Analysis of lipopolysaccharides of *Pasteurella multocida* and several Gram-negative bacteria by gas chromatography on a capillary column

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Gas chromatography의 capillary column을 이용한 *Pasteurella multocida* 및 기타 그람음성 세균의 lipopolysaccharide 분석

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Abstract : Lipopolysaccharides (LPS) of *Pasteurella multocida (P multocida)* and several Gram-negative bacterial pathogens were analyzed by methanolysis, trifluoroacetylation and gas chromatography (GC) on a fused-silica capillary column. The GC analysis indicated that LPS prepared from a strain of *P multocida* by phenol-water (PW) or trichloroacetic acid (TCA) extraction were quite different in chemical composition. However, LPS prepared from *Salmonella enteritidis* by the two extraction methods were very similar. PW-LPS extracts from different *Pasteurella* strains of a serotype had essentially identical GC patterns. Endotoxic LPS extracted from 16 different serotypes of *P multocida* by PW or by phenol-chloroform-petroleum ether procedures yielded chromatograms indicating similar composition of the fatty acid moieties but minor differences in carbohydrate content. When the chemical composition of endotoxic LPS extracted from several Gram-negative bacteria (*P multocida, Pasteurella haemolytica, Haemophilus somnus, Actinobacillus ligniersii, Brucella abortus, Treponema hyodysenteriae, Escherichia coli, Bacteriodes fragilis, Salmonella abortus equi* and *Salmonella enteritidis*) were examined, each bacteria showed a unique GC pattern. The carbohydrate constituents in LPS of various Gram-negative bacteria were quite variable not only in the O-specific polysaccharides but also in the core polysaccharides. The LPS of closely related bacteria shared more fatty acid constituents with each other than with unrelated bacteria.

Key words : lipopolysaccharides, *Pasteurella multocida* , gas chromatography.

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Introduction

Pasteurella multocida (P multocida) is a microorganism capable of infecting most animal species including man and is responsible for economically important animal diseases such as fowl cholera and pneumonic *Pasteurellosis* in cattle[3,4]. The scientific and economic importance of *P multocida* has lead to the use of various bacterial fractions in the development of immunizing agents and for the study of bacterial pathogenicity. Studies on subcellular fraction of *P multocida* have indicated that many fractions with demonstrated immunogenicity contained lipopolysaccharide (LPS) as an important constitutent[7-9,19,20-23].

The *P multocida* LPS extracted by conventional methods (phenol-water, phenol-chloroform-petroleum ether or trichloroacetic acid) has been observed to evoke a variety of endotoxic activities in a host including pyrogenicity, depression, diarrhea and death in mice, rabbit and chickens and lethality for chicken embryos[1,4,8]. Since these endotoxic activities have also been demonstrated in LPS of many other Gram-negative bacteria, it has been generally believed that *P multocida* LPS is structurally similar to other Gram negative bacterial LPS[1,21]. However, the chemical relathionship between LPS of *P multocida* and other Gram-negative bacteria have not been studied extensively. In addition, the chemical relationships between the endotoxic LPS of *P multocida* prepared by different methods have not been studied.

Methods previously utilized for the chemical characterization of *P multocida* LPS were based on detection of fatty acids and colorimetric assays for 2-keto-3-deoxy-octonate (KDO), glucucosamine, L-glycero-D-mannoheptose (LD-heptose) and neutral sugars that are well-known components of Gram-negative bacterial LPS (especially *Salmonella* species). However, this chemical analysis requires several different assay systems that are time consuming. Also, the fatty acid content of bacterial LPS should be determined as well for it may be the carbohydrate content. This is indicated by the studies on synthetic *Salmonella* LPS analogues which indicated that variant composition of fatty acids exhibited differences in biological activities such as antigenicity, lethality, pyrogenicity, mitogenicity and complement activity[12]. Recently, Bryn and Jantzen[3] demonstrated that analysis of LPS by methanolysis, trifluoroacetylation and gas chromatography on a capillary column was a highly useful method for determining the chemical composition of bacterial LPS. The objection of this study were 1) to analyze the chemical composition of purified *P multocida* for future analysis of LPS-containing immunogens of the organism and 2) to examine chemical relationships between the endotoxic LPS of *P multocida* prepared by different methods and from different serotypes, and 3) to compare LPS from *P multocida* with those from other Gram-negative bacterial pathogens.

Materials and Methods

Cultivation of bacteria : *P multocida* and *Pasteurella haemolytica* (*P haemolytica*) were grown in Roux bottles containing dextrose starch agar (Difco Laboratories, Detroit, MI). *Salmonella abortus equi* (*S abortus equi*) was grown in Roux bottle containing trypticase soy agar (Difco). *Actinobacillus ligniersii* (*A ligniersii*) was grown in brain-heart infusion broth (BHI; Difco) with aeration. *Haemophilus somnus* (*H somnus*) was grown in Roux bottle containing BHI, 1.5% agar (Difco) and 10% sterile bovine serum with 5% CO_2 aeration. After incubation at 37℃ for 18h, the Roux bottle-grown bacteria were suspended in 0.15M sterile phosphate buffered saline solution (PBS, pH 7.2). These bacteria and the broth-grown bacteria were harvested by centrifugaton. Cells were washed three times with PBS by centrifugation and resuspended in distilled water.

LPS preparation by phenol-water extraction : Phenol-water extracted LPS (PW-LPS) of *P multocida* (strain P-2383 and P-1062; capsular type A, somatic type 3), *P haemolytica* D-80 (biotype A, serotype 1), *H somnus* 8025, *A ligniersii* and *S abortus equi* were prepared according to the method of Westphal and Jann[26] with slight modifications. Briefly, the bacterial suspension in distilled water heated to 65℃ and an equal volume of prewarmed (65℃) 90% phenol was added. This mixture was stirred vigorously, incubated for 15 min at 65℃ and centrifuged at 3,000×g at 4℃. The water phase was aspirated off, the original volume of distilled wat-

er was added to the remaining phenol phase, and the extraction was repeated. The first and second water phases were combined and dialyzed against daily changes of distilled water for 4 days. The dialyzed water phase was treated with ribonuclease and deoxyribonuclease (Sigma Chemical Co., St. Louis, MO) for 24h at 37℃ and centrifuged at 105,000xg for 3h at 4℃. The pellet was washed once by centrifugation, resuspended in distilled water and lyophilized.

LPS preparation by trichloroacetic acid(TCA) extraction : TCA extracted LPS (TCA-LPS) of *P multocida* (P-2383) was prepared according to the method of Staub[24] Briefly, the washed bacteria were suspended in cold (4℃) distilled water and an equal volume of 0.5N TCA was added. After incubation for 3h at 4℃, the mixture was centrifuged at 3,000x g for 30 min. The supernatant was removed, warmed to room temperature, neutralized to pH 6.5 with 3N NaOH, cooled to 0℃ in an ice bath, and added to 2 volumes of 100% ethanol (-20℃). The precipitate, which formed following overnight incubation at -4℃, was sedimented by centrifugation. The supernatant fluid was discarded and the precipitate was dissolved in distilled water to 0.1 volume of the original bacterial suspension. Microbial debris was removed by centrifugation at 27,00x g and the supernatant fluid was lyophilized.

Other bacterial LPS : LPS from 16 somatic types of *P multocida* by either the PW procedure (26) or the phenol-chloroform-petroleum ether (PCP) procedure[6] were kindly provided by Dr. R.B. Rimler, National Animal Disease Center (NADC), U.S. Department of Agriculture (USDA), Ames, Iowa. A PW-LPS preparation from *P haemolytica* P-101 (biotype A, serotype 1) was obtained from Dr. G.H. Frank, NADC, USDA. A *Brucella abortus* (*B abortus*) LPS f5 was prepared by PW procedure with extraction from the phenol phase rather than water phase as previously described[14] and was supplied by Dr. B.L. Deyoe, NADC, USDA. PW-LPS of *Treponema hyodysenteriae* (*T hyodysenteriae*), *Escherichia coli* (*E coli*) K235 and *Bacteriodes fragilis* (B fragilis 9F were obtained from Dr. M.J. Wannemuehler, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa. TCA-LPS and PW-LPS of *Salmonella enteritidis* (*S enteritidis* were purchased from Sigma.

Standards : Various individual standards were used for determination of the retention times as well as internal standards in GC analysis. Rhamnose, fucose, ribose, galactose, mannose, glucosamine and KDO were obtained from Sigma. Dr. P.A. Rivers, NADC, USDA provided ID-heptose and D-glycero-D-mannoheptose (DD-heptose). Unhydroxylated fatty acids from carbon chains 12 to 17 were obtained from Applied Science (State College, PA) and 3-hydroxy-dodecanoate (3-OH-12 : O), 2-hydroxy-tradecanoate (2-OH-14 : O) and 3-hydroxy-tetradecanoate (3-OH-14 : O) were purchased from Foxboro/Analabs (North Haven, Conn). A bacterial fatty acid mixture (Cat. No 4-7080) was purchased from Supelco (Bellefonte, PA).

Preparation of samples : Derivatization of the LPS was performed by the method of Bryn and Janzen[3] with slight modification. Briefly, 2 to 5mg of bacterial LPS were suspended in one ml of 2M HCl in methanol (Supelco) in a Tefion-lined screw-capped vial (Supelco) and held at 85℃ for 18h. Methanolysates were concentrated to dryness at room temperature with nitrogen gas and trifluoroacetic acid (TFA, gold label ; Aldrich Chemical Inc., Milwaukee, WI) in acetonitrile (HPLC grade ; Fisher Scientific, Fair Lawn, NJ) and heating in a boiling water bath for 2 min. After cooling to room temperature, the reaction mixture was diluted with acetnitrile to a final TFA concentration of 10% and injected into the column.

Gas chromatograpy (GC) and peak identificantion : The GC analysis was carried out on Hewlett-Packard 402 gas chromatography system equipped with a flame-ionizing detector that had been modified for the use of a capillary column. Fused-silica capillary column (GB-1, 25m x 0.25mm ID, Foxboro/Analabs, or SPB-1, 30m x 0.25 ID, Supelco) were operated in split mode and with helium carrier gas. The column temperature was held for 5 min at 95℃ and then programmed to increase at 4℃ per min up to 230℃. Peaks of individual standards and bacterial LPS were recorded. The retention times were calculated by the distance of the individual peak moved from the injection line on the chromatogram.

Results

Determination of retention times of standard compounds : As indicated by Bryn and Janzen[3], most monosaccharide

derivatives were resolved into defined peaks by a capillary column. The specific GC patterns of standard compounds in this experimentation were very similar to those reported by Bryn and Janzen[3]. However, it was necessary to separate the components of LPS with splitter open in contrast to the procedure of Bryn and Janzen, which was conducted in splitless mode. Also, they observed the third galactose peak between the second glucose peak and the second mannose peak, but in our study, it appeared at the same position as the first glucose peak. Retention times of the individual standard compounds on the GB-1 column are listed in Table 1

Table 1. Gas chromatographic distribution of some commonly occuring LPS monosaccharides as trifluoroacetylated, methyl-glycosides on a fused silica capillary column (GB-1)

Component	Retention times (min)			
Rhamnose	6.1	7.35		
Fucose	6.35	6.75	7.8	
Ribose	4.9	6.15	6.4	9.1
Galactose	10.3	11.15	11.85	
Mannose	11.6	12.45		
Glucose	11.85	12.0		
DD-heptose	15.0			
LD-heptose	15.8			
Glucosamine	17.35			
KDO	19.85	20.65		

and 2. The SPB-1 column gave slightly different retention times, but the chromatographic sequence was the same as the GB-1 column.

Chemical composition of *P multocida* LPS : Since LPS extracted from enterobacteria is a classical example of bacterial endotoxin, LPS prepared from *S enteritidis* was utilized as a standard for comparisons of *P multodica* and other bacteria. In contrast to LPS preparations from *S enteritidis* (Fig 1), LPS prepared from a *P multocida* strain by TCA of PW extraction was quite different in chemical composition (Fig 2). The PW-LPS of *P multocida* contained mamy constituents characteristic of other Gram-negative bacterial LPS including LD-heptose, glucosamine, KDO, tetradecanoate and 3-hydroxy-tetradecanoate. However, TAC-LPS of the organism appeared to contain more glucose and certain longer chain fatty acids that were absent in the PW-LPS. LPS extracted from 15 different serotypes exhibited similar chemical composition with identical fatty acid constituents regardless of the extraction methods. The differences were found that LPS extracted from encapsulated *P multocida* (serotype 3, 9, and 13) by the PW procedure contained polysaccharides than the LPS extracted from non-capsulated serotypes by the PCP procedure. Also, minor vairation was found in individual carbohydrate constituents. LPS of all serotypes contained glucose, galactose, LD-heptose, glucosamine and KDO. However, some serotype contained an additional constituent. For example, LPS of serotype 2 and 5 contained DD-heptose, but this compound was not detectable in LPS of other serotype. In this respect, LPS from

Table 2. Retention times of common LPS fatty acids as O-trifluoroacetylated methyl esters on a fused-silica capillary column (GB-1)

Chain length	Retention times		
	Unhydroxylated	2-hydroxylated	3-hydroxylated
C-12	19.25	-	21.95
C-13	22.45	-	-
C-14	25.35	27.05	27.45
C-15	28.2	-	-
C-16	30.8	-	-
C-17	33.4	-	-

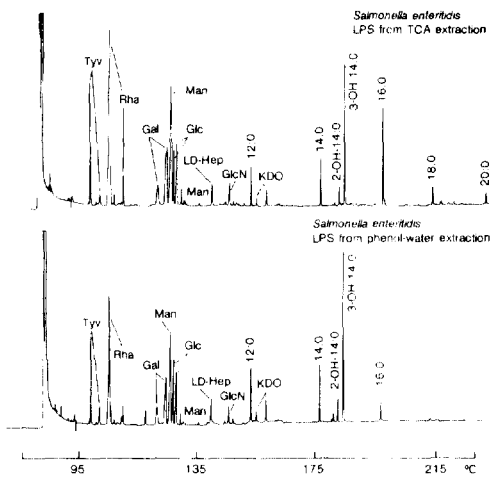

Fig 1. Gas chromatograms from a fused-silica capillary column (SPB-1) of two *S enteritidis* LPS prepared by TCA and PW extractions after methanolysis and trifluoroacetylation. Conditions of sample preparation and chromatography are given in the text. Abbreviations: Tyv, tyverose; Abq, abequose; Rha, rhamnose; Gal, galactose; Man, mannose; Glc, glucose; DD-heptose, D-glycero-D-mannoheptose; LD-heptos, L-glycero-D-mannoheptose; GlcN, glucosamine; 2-keo-3-deoxy-octonate, KDO; 12:0, dodecanoate; 3-OH-12:0, 3-hydroxy- dodecanoate; 14:0, tetradecanoate; 2-OH-14:0, 2-hydroxy- tetradecanoate; 3-OH-14:0, 3-hydroxy- tetradecanoate; 16:0, hexadecanoate; i-16:0, 14-methyl-pentadecanoate; 3-OH-16:0, 3-hydroxy hexadecanoate; 17:0, heptadecanoate; 18:0, octadecanoate; 18:1^{9}, cis-9-octadecanoate.

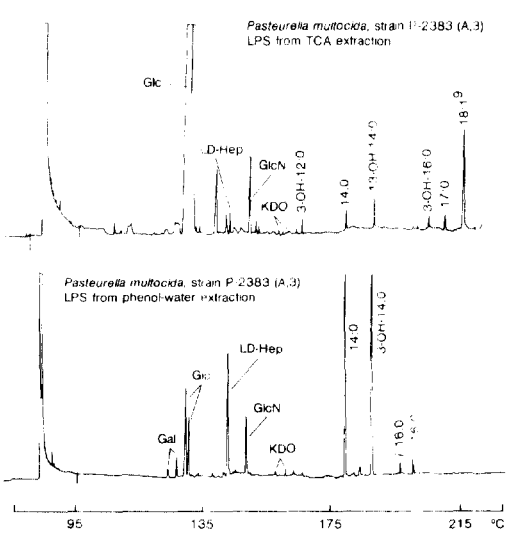

Fig 2. Gas chromatograms from a fused-silica capillary column (SPB-1) of *P multocida* LPS prepared by TCA and PW extractions. Abbreviations are listed in Fig 1.

serotypes 2 and 5 appeared to be similar in chemical composition to *P haemolytica* LPS (Fig 3). However, *P haemolytica* LPS contained a large quantity of dodecanoate, which was either not detectable or present in limited quantity in *P multocida* LPS. However, The PW-LPS from different strains of a common serotype of *P multocida* (capsular type A, 3 ; P-1059, P-1062 and P-2383) gave identical GC pattern. The same was true for *P haemolytica* (biotype A, 1 ; P-101 and D-80).

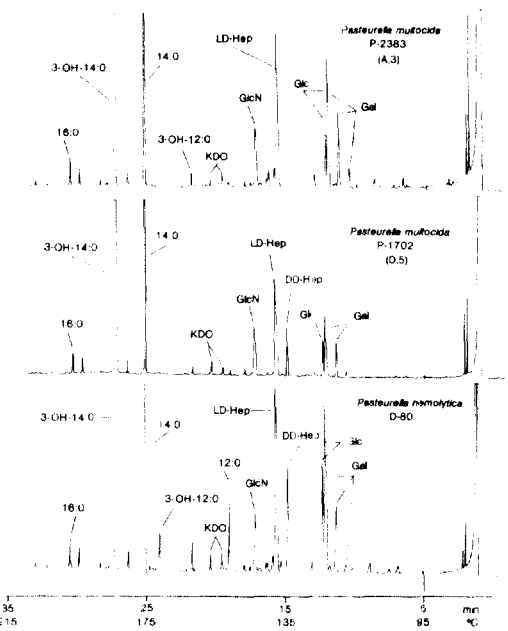

Fig 3. Gas chromatograms from a fused-silica capilaary column (GB-1) of LPS prepared from *Pasteurella* species. Abbreviations are listed in Fig 1.

Chemical relationship of PW-LPS between *P multocida* and other Gram-negative bacteria : The PW-LPS of Gram-negative bacteria closely related to *P multocida* possessed a chemical composition similar to that of *P multocida* LPS (Fig 4). The LPS of *A ligniersii*, a species of the genus *Actinobacillus* within the family *Pasteurellaceae*, contained the same chemical constituents as *P multocida* LPS with limited quantitative variations in glucose and galactose. The LPS of *H somnus*, a species of uncertain affiliation within the genus *Haemophilus* in the family *Pasteurellaceae*, also had similar constituents, but it contained a large quantity of

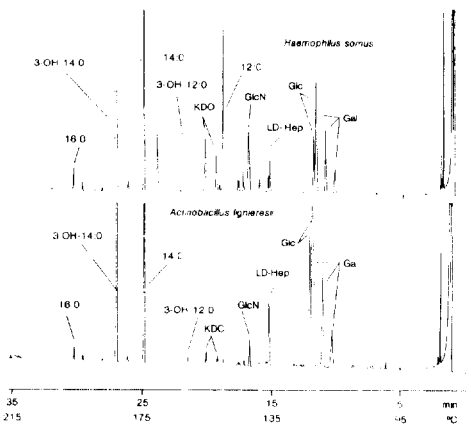

Fig 4. Gas chromatograms from a fused-silica capillary column (SPB-1) of *P multocida* LPS prepared from *H somnus* and *A ligniersii*. Abbreviations are listed in Fig 1.

KDO than other bacteria within the family. When PW-LPS of bacteria with a lesser relationship with *P multocida* were examined (Fig 1, 5 and 6), compositional variations were found in both the carbohydrate and fatty acid moieties. However, the LPS of enteric bacteria (*Salmonella* species and *E coli*) did share several typical LPS constituents with *P multocida* LPS such as LD-heptose, KDO was 3-hydroxy-tetradecanoate (Fig 1, 5 and 6). The LPS of *B abortus* also contained 3-hydroxy-tetradecanoate (Fig 5), but absence of LD-heptose and predominance of long chain fatty acids

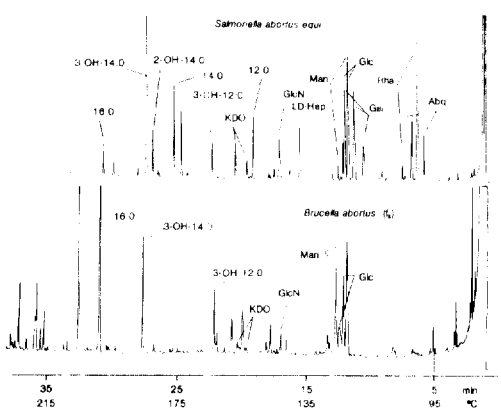

Fig 5. Gas chromatograms from a fused-silica capillary column (GB-1) of *P multocida* LPS prepared from *S abortus equi* and *B abortus* (f5). Abbreviations are listed in Fig 1.

made it uniquely different from other bacterial LPS. The LPS of *T hyodysenteriae* and *B fragilis* contained completely destinctive fatty acid moiety as compared to LPS of *Salmonella* species. *E coli* and genera of the family *Pasteurellaceae* (Fig 6). LPS of both bacteria contained 13-hydroxy-methyl-tetracecanoate and 3-hydroxy-hexadecanoate as main fatty acid constituents rather than tetradecanoate and 3-hydroxy-tetradecanoate[37]. *B fragilis* LPS contained additional fatty acids such as 3-hydroxy-pentadecanoate, 30 hydroxy-15-methyl-hexadecanoate and 3-hydroxy-heptadecanoate.

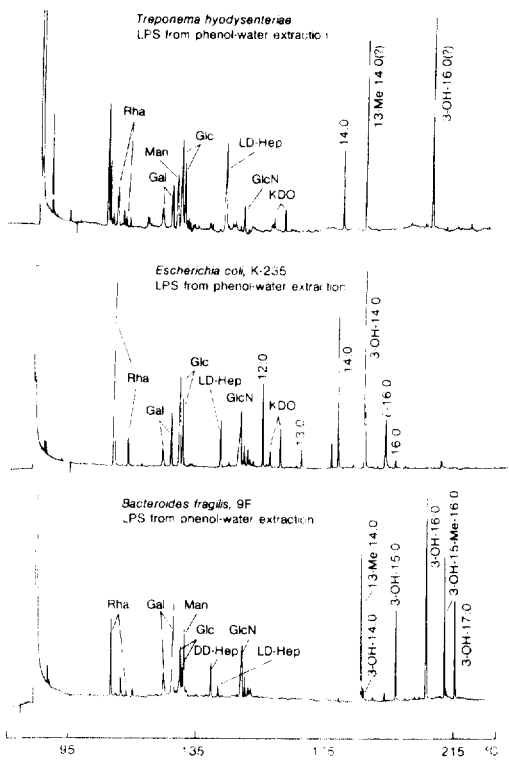

Fig 6. Gas chromatograms from a fused silica capillary column (SPB-1) of LPS prepared from *T hyodysenteriae*, *E coli* (K-235) and *B fragilis* (9F). Abbreviations are listed in Fig 1.

Discussion

Bacterial LPS is a chemically heterogeneous material which is present in the outer membrane of Gram-negative bacteria and has a variety of bacterial activities in a host: these activities may include toxicity, metabolic alterations or immunological effects[10,11,13,25]. LPS isolated from *Salmonella*

species has been characterized most extensively and structurally. These LPS are composed of three chemically distinctive regions including O-specific polysaccharides, core polysaccharides and lipid A[10,11,13,25]. Common components of the *Salmonella* LPS are LD-heptose, KDO and 3-hydroxy-tetradecanoate.

For many years, it was thought that the LPS contained a molecular component common to all Gram-negative bacteria. Variation in the structure resided in the polysaccharide moiety, while the remainder of the structure is the same in all LPS molecules regardless of origins. Earlier chemical studies on LPS of the family *Enterobacteriaceae* by Luderitz and coworkers seemed to support this premise[11,13], recently. Nowotny and his colleagues questioned the validity of this assumption based on their experimental evidience[15,16]. They found obvious differences in the chemical composition, particularly in fatty acid constituents, of LPS prepared from a bacterium by different methods and between LPS from different bacterial species.

The results in this study on chemical analysis of *P multocida* and several Gram-negative bacterial LPS by GC clearly demonstrated the compositional heterogeneity of various LPS preparations in support of the findings of Nowotny and his colleagues[15,16]. Compositional differences between LPS extracted by PW and TCA procedures from a *P multocida* organism (Fig 2) indicated that extraction methods influenced the chemical charactor of LPS. This contrasted with enterobacterial LPS that seemed to be similar in chemical composition irrespective of the methods utilized for extraction (Fig 1). An interesting observation is the finding that the PCP extraction of the non-capsulated serotypes of *P multocida* yielded LPS similar to the PW-LPS from the encapsulated serotypes. This observation may indicate that *P multocida* LPS basically similar in chemical composition irrespective of the serotype. The PCP method has been used for extraction of LPS from non-capsulated bacteria since LPS from these bacteria is more soluble in PCP than in water[6]. This is apparently due to the lack of O-specific polysaccharides in non-capsulated bacteria. This increases hydrophobicity of LPS of non-capsulated bacteria as compared with LPS from encapsulated bacteria. The reason is due to the different extraction methods. PW and TCA procedures, influenced the chemical character of LPS from an encapsulated *P multocida* is difficult to explain. However, this difference may result from the surface character of the encapsulated organism, particularly the mucoid nature of the capsule. The capsule may influence the effectiveness of TCA in dissociating LPS from the mucoid organisms.

The O-specific polysaccharides are reported to exhibit serotype specificity of individual bacteria and to be extremely heterogeneous in chemical composition[10,11,13,15,25]. Studies in *Salmonella* LPS indicated that the repeating unit of oligosaccharides in the O-specific polysaccharides are the most important factors for determination of serotype specificity; individual somatic polysaccharides may exhibit several serologic specificities[11,13,15]. Observation in this study indicated that the carbohydrate constituents of *P multocida* were very similar between the different serotypes although quantity of the individual constituents was variable and some serotypes contained additional sugars such as DD-heptose. Brogden and Rebers[2] reported that LPS isolated from 16 different serotypes of *P multocida* did not crossreact except for LPS of serotypes 2 and 5. Rimler *et al*[21] quantitated carbohydrate composition of *P multocida* LPS and found that LPS of serotypes 2 and 5 were markedly alike in chemical composition; both contained similar amounts of glucose, galactose, DD-heptose, LD-heptose and KDO. However, LPS of serotype 1 that was also quite similar in chemical composition with LPS of serotypes 2 and 5, except for the lack of DD-heptose, did not crossreact with the two serotypes. This indicated that, in general, LPS of *P multocida* contains O-specific polysaccharides uniuqe to the individual serotype and exhibits primarily one serologic specificity. This contrasts with findings on *Salmonella* LPS in which somatic polysaccharides may exhibit several serologic specificities[11,12,15].

In contrast to the O-specific polysaccharides, the constituents (LD-heptose and KDO) of the core polysaccharides have been reported to be common to many Gram-negative bacteria, certainly to all in the family of *Enterobacteriaceae*[10,13]. However, the results of this study indicated that these compounds were either absent or present in limited quan-

tities in some bacterial LPS such as *B abortus* and *B fragilis* (Fig 5 and 6). Also, the apparent quantity of these compounds is quite variable in individual LPS from unrelated bacteria and even related bacteria within the facultative anaerobic Gram-negative bacteria that comprise the families *Enterobacteriaceae* and *Pasteurellaceae* (Fig 1, 2, 3, 4, and 6). For example, LPS of *Salmonella, Shigella* and *Escherichia* may contain similar amounts of KDO as previously reported[11,15], however, LPS of *P multocida*, *P haemolytica* and *A ligniersii* from the family *Pasteurellaceae* contained a lesser quantity of KDO than LPS of *H somnus* from the same family and the family *Enterobacteriaceae* (Fig 3 and 4).

The fatty acid moiety of LPS, called lipid A, is known to represent the component of LPS, which is responsible for its endotoxic properties[10,11,13,15,25]. However, compositional differences in lipid A of various LPS preparations have been reported[12,15,16]. The results of this study confirmed the heterogeneity in fatty acid components of various LPS preparation. Also, the results indicated that LPS of members of the family *Pasteurellaceae* (Fig 3 and 4) contained almost identical fatty acid constituents while they were slightly different from the LPS of enteric bacteria (Fig 1, 5, and 6). Distinctive differences were noted in the fatty acid composition of LPS from unrelated bacteria (Fig 5 and 6). This indicates that the similarities and differences in the fatty acid composition of various Gram-negative bacterial LPS may depend on relationship between the bacteria. Differences in the relative endotoxicity of these bacterial LPS can be expected since compositional differences in lipid A of different origin and in synthetic lipid A analogues have been demonstrated to influence biological activities of LPS including endotoxicity[12,15,16].

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