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Research Article

Analysis of Lipid a Isolated from *Brucella ovis* R-Lipopolysaccharide

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Abstract

The cellular fatty acids profile of *Brucella ovis* by gas-liquid chromatography GLC has the fatty acids 15:0, 16:0, 17:0, 17:0 cyclopropane, 18:0, 18:1 and 19:0 cyclopropane. Differences in the fatty acids GLC profile can be used for the identification of *Brucella ovis* and its differentiation from *Brucella* spp. The fatty acids from lipid A give a structural diversity, which influences of lipopolysaccharide biological activity. The lipopolysaccharides (LPS) of Gram-negative bacteria are amphiphilic macromolecules located in the outer membrane of the cell wall and consist a lipidic portion Lipid A linked to oligosaccharide core region with or without a polysaccharide O-antigen (S or R form LPS) .The lipid A anchor the molecule in the outer membrane and represents the toxic principle of LPS. It generally contains in its structure a disaccharide consisting of two hexosamines esterified with fatty acids and phosphate. The aim of this study was the analysis of fatty acids from de lipid A of *Brucella ovis*.

Methods: Rough lipopolysaccharide R-LPS was extracted by phenol, chloroform and petroleum ether from *B. ovis* REO198 strain was used to obtain of lipid A molecule. Mild acid hydrolysis and heat are sufficient to disrupt this linkage between the lipid A and the core oligosaccharide. Lipid A was processed to prepare the fatty acids methyl esters (FAMEs) and analyzed by gas-liquid chromatography (GLC) and gas-liquid chromatography mass spectrometry (GLC-MS). The monosaccharides present in lipid A were characterized by GLC and GLC-MS as the acetates of the corresponding methyl glycoside.

Results: The fatty acid composition of lipid A is complex as seen in the chromatogram of the corresponding methyl esters obtained by methanolysis of the product as fatty acids 16:0, 17:0, 18:1, 18:0 and 19:0 cyclopropane. A compound with elution time 18:76 min corresponds to a hydroxy acid. The carbohydrate composition of lipid A may correspond to an acetyl hexosamine, either acetyl glucosmine or acetyl galactosamine according to the mass spectra.

Conclusion: The fatty acid profile found in lipid A is very rich in palmitic acid. According to our results we have been able to identify a location of the cyclopropane carboxylic acids described for this bacteria. The fatty acids 16:0, 17:0, 18:1,18:0 and 19:0 cyclopropane with a hydroxy acid form part of lipid A of R-LPS with a disaccharide formed by glucosamine and/or galactosamine.

Keywords: *Brucella; Lipid A; LPS; GC; GC-MS*

Introduction

Brucella ovis is a Gram-negative coccobacillus. *B. ovis* cause an infection disease responsible for infertility and subsequent economic losses in sheep, causing genital lesions such as epididymitis in rams and abortion in ewes. The standard serological test to detect *B. ovis* infection in rams is the complement fixation test (CFT), which has imperfect sensitivity and specificity. The clinical detection of the disease is difficult because other bacteria, such as *Actinobacillus seminis, Histophilus ovis, Chlamydophila abortus* or *B. melitensis*, may cause similar symptoms [1]. In many parts of the world, *B. ovis* infection in sheep is a serious economic problem. *B. ovis* is not zoonotic; however, in areas where *B. melitensis* and *B. ovis* coexist, special care must be taken when handling and sending samples, since

B. melitensis is highly pathogenic for humans. The main problem of serological diagnosis in sheep is due to the absence of a technique capable of differentiating between diseased animals from those vaccinated or infected with *B. melitensis*. *B. ovis* is rough, serological techniques cannot be used to determine the generic identity of isolates. Care must be taken to differentiate *B. ovis* from *Alcaligenes* spp. as well as from other *Brucella* spp. On the basis of cellular fatty acids studies of *Brucella* spp, Dees., *et al*. [2] shown that the major cell wall fatty acids were: 19:0 cyclopropane (19:0 cyc), 16:0, and 18:1.GLC analysis of cellular fatty acids composition can be used for the rapid identification of *B. ovis* and its differentiation from *B. abortus*. This work was further extended by Coloe., *et al*. [3] and both species were characterized by the presence of cellular fatty acids 16:0, 17:0, 17:0 cyc, 18:0, 18:1 and 19:0 cyclopropane. *B. ovis* also contained some 15:0 on cellular fatty acids fraction. There were differences in the relative proportions of the cellular fatty acids presents, and it was possible to differentiate *B. ovis* from *B. abortus* on the basis of the absence of 15:0, lower concentration of 17:0 and 18:1 and higher concentrations of 19:0 cyc in *B. abortus*.

In the present study, we have applied GLC and GLC-MS to the methyl esters from the fatty acids from the lipid A of *B. ovis* R-LPS in order to determinate it structure. The characterization of the LPS structure can be apply to improve future *B. ovis* diagnosis by antigen assays.

The genus *Brucella* is characterized by its cellular envelope consisting of one inner membrane, one outer membrane and periplasmic space. The outer membrane contains unevenly distributed phospholipids, proteins and lipopolysaccharides (LPS) [4]. The following antigens have been identified inside *B. ovis*: a rough lipopolysaccharide R-LPS and -outer membrane, periplasmic and cytoplasmic- proteins [5].

LPS that are part of the outer membrane of gram-negative bacteria are important surface antigens and immune response modulators. These compounds are negatively charged macromolecules and have an amphipathic character. There are three well differentiated regions in their structure: lipid A, nucleus and O-specific chain. Lipid A is a glycolipid that is non-covalently bound to the outer membrane of *Brucella*. It generally contains in its structure a disaccharide consisting of two hexosamines esterified with fatty acids and phosphate.

The O-specific chain is the polysaccharide fraction of the LPS is outwardly directed and contains in its structure repeat units of the same oligosaccharide residue. The glycosidic nucleus, which binds to lipid A and the O-specific chain, consists of 2-Keto-3-deoxy-octonate (KDO) residues, among others. The O-specific chain is responsible for specific serological reactions while lipid A is responsible for the toxicity and pyrogenicity of Gram negative bacteria. In the S-form species, the S-LPS has a composition similar to that described above, whereas in the R-LPS rough mutants of said species the reduced length of the O-specific chain. Rough species such as *B. ovis* and *B. canis,* they also have a small O-specific chain [6,7]. LPS can be used to develop diagnostic and prevention methods. Lipid A anchors the LPS in the outer membrane and is primarily responsible for endotoxic activity. Biosynthetic modifications of Lipid A can be used to obtain LPS with reduced or no toxicity, which can be included in vaccines based on products derived from gram-negative bacteria [8].

Materials and Methods

Bacterial strain and growth conditions: *B. ovis* REO198 was cultured on plates of tryptic soy agar (TSA) supplemented with 0.5% of yeast extract and 0.5% bovine blood, for 48 hours at 37^0C. The cultures were tested for purity by Gram stain. Twelve slants of TSA with 0.5% sterile bovine serum were inoculated for 48 hours at 37^0C. Two slants per 4L Erlenmeyer flask x 6, with 1600 mL of Tryptic soy broth (TSB) with 0.5% of yeast extract and 5% de sterile bovine serum were grown in an orbital incubator at37^0C for 36 hours. About 9.6 liters of culture were prepared. The organisms were inactivated with phenol until a final concentration of 0.5%. The cells were harvested and washed with distilled water. Saline solution should be avoid as such treatment may result in a very poor lipopolysaccharide yield. Cells were packed by centrifugation and lyophilized.

R- LPS extraction: The rough lipopolysaccharide (R-LPS) was extracted by the method of Galanos., *et al*. [9]. Briefly, dried cells were suspended in a mixture of liquid phenol (90g dry phenol + 11 mL water), chloroform and petroleum ether PCE (2:5:8) by volume at ratio of 1g of dried cells to 4 mL of mixture. This mixture was a monophasic system if the phenol used was dry.

The cells were homogenized and the cellular debris was removed by centrifugation at 5000 rpm for 15 min, and re-extracted twice. The supernatant extracts were pooled, filtered and the chloroform and the petroleum ether were evaporated at 600C. The solution was transferred into a glass centrifuge tube Corex 30 mL capacity and distilled cold water added drop-wise until the lipopolysaccharide precipitated.

Addition of water was stopped when the lipopolysaccharide started settling down after the mixture was allowed to stand for 1 to 2 min. Although precipitation of the R-LPS is complete before the phenol is saturated with water, care must be taken not to add too much water as this causes formation of two phases. The suspension was centrifuged at 3000 rpm at room temperature for 10 min, the supernatant decanted and the tube allowed to stand for 2 to 3 min upside down. It was then wipen inside with filter paper. The precipitate was washed tree times with 5 mL of 80% phenol and centrifuged at 3000 rpm for 10 min. Finally, the precipitate was washed tree time with 2mL de ether to remove any remaining phenol and after was washed with distilled water and freeze-dried.

Isolation of Lipid A: R-LPS (10 mg) was suspended in 2 mL of acetic acid 1% v/v 90 min at 1000C. Lipid A was obtained from the aqueous solution by centrifugation at 7000 rpm for 15 min at room temperature. Lipid A was in the precipitate and the glicosidic core was in the supernatant [10].

Lipid A: Fatty Acids analysis: Lipid A was processed to prepare the fatty acids methyl esters (FAMEs) and analyzed by gas-liquid chromatography (GLC) and gas-liquid chromatography mass spectrometry (GLC-MS). To a lipid A 2 mg sample in a screw-capped glass test tube was added 1 mL HCI 1M enMeOH, and the mixture was heated at 80°C for 1h. After cooling, the reagent was removed by blowing down under a stream of nitrogen, and then FAMEs were extracted with 500 µL AcOOEt. FAMEs were analyzed with a HP 6890 Series Modelo Plus+gas chromatograph equipped with capillary column of HP-5 -5% fenil-metil-siloxano (30.0m x 0.25 mm x 0.25 µm) temperature program of 150°C to 320°C temperature gradient 4°C/min. For GC-MS/MS analysis, in the electron impact mode using HP 5890A-ll (GC) coupled to a mass selective detector (MSD). FAMEs were separated on a column DB-1 (30m x 0.25 mm x 0.25 m) using helium as the carrier gas (constant column flow, 1.0 ml/min). The oven temperature was 150 °C to 320°C with a temperature gradient of 4°C/min. FAMEs were identified using a EI-MS data base, analysis of their mass spectra fragmentation and comparison with similar derivatives prepared from authentic standard samples.

Analysis of monosaccharides present in Lipid A: The monosaccharides present in Lipid A were characterized by GLC and GLC-MS as the acetates of the corresponding methyl glycoside. The derivatives of the monosaccharide components of Lipid A were obtained as follows, in a screw cap tube a sample of Lipid A (2 mg) was placed and dissolved in 1 mL of 1M HCl in MeOH. The solution was heated at 80°C for 1h, the excess reagent was cooled and removed under nitrogen stream. 500 µL of pyridine and 500 µL acetic anhydride were added to the residue, capped and heated at 120°C for 1 hour. The excess pyridine-acetic anhydride was removed under a stream of nitrogen, the product was taken up with 500 µL of ethyl acetate and analyzed by gas chromatography.

Results

Fermentación de *Brucella ovis* REO198 y extracción del R-LPS: Five grams of cell pellet were obtained from 9.6L culture medium. Ten milligrams of R-LPS was extracted from 1g of the *B. ovis* lyophilized cells.

Isolation of Lipid A: Isolation of lipid A take advantage of the labile linkage between the lipid A backbone and the KDO in the LPS core. Mild acid hydrolysis and heat are sufficient to disrupt this linkage. The lipid A is insoluble in water and forms a precipitate that can be readily extracted by centrifugation.

Lipid A: Fatty Acids analysis: The fatty acid composition of Lipid A is complex as seen in the chromatogram of the corresponding methyl esters obtained by methanolysis of the product. Figure 1 shows the TIC -Total Ion Chromatogram- obtained in the GC analysis by mass spectrometry of the corresponding methyl esters. The compounds with elution time 15.83, 17.77, 19.29, 19.64 and 21.21 were identified by comparison of their retention times and/or their mass spectra with standards, such as fatty acids 16:0, 17:0, 18:1, 18:0 and 19:0

cyclopropane, respectively. A compound with elution time 18:76 corresponds to a hydroxy acid but the length of the chain could not be determined. Table 1 shows the respective retention times and relative abundances of the fatty acids obtained.

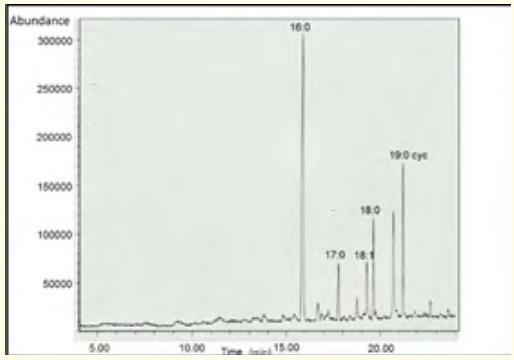

Figure 1: *GC-MS TIC chromatogram of FAMEs from Brucella ovis lipid A.*

Fatty Acid	t_R(min)	Relative Abundance
16:0	15.83	5
17:0	17.77	1
18.1	19.29	1
18:0	19.64	2
19:0 cyc	21.21	3

Table 1: *Fatty acids from lipid A of Brucella ovis.*

The identity of these components was confirmed by the analysis of the mass spectra, in which M+ ions were used as diagnostic ions, $[M-31]^+$. , $[M-59]^+$. and $[M-74]^+$. among others. All the methyl esters have a characteristic fragmentation due to the oxygen of the carbonyl group and the ester that act as sites of initiation of the fragmentation, obtaining the diagnostic ions by α and β fragmentation or by a McLafferty rearrangement involving the migration of a H atom located on the γcarbon [11] (Figure 2-3).

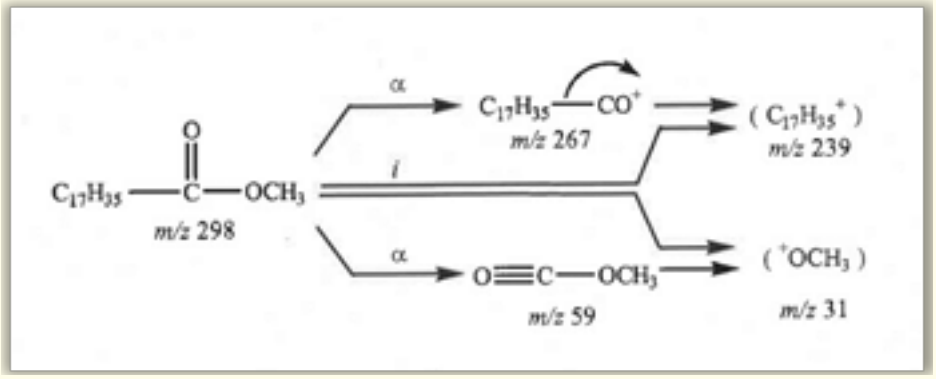

Figure 2: *Fragmentation pattern of FAMEs.*

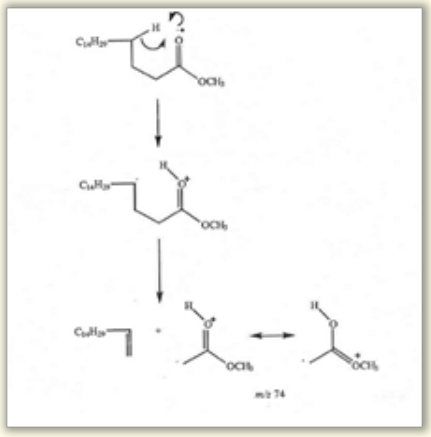

Figure 3: *Fragmentation pattern of FAMEs generated by a McLafferty rearrangement mechanism.*

Analysis of monosaccharides present in Lipid A: The carbohydrate composition of Lipid A may correspond to an acetyl hexosamine, either acetyl glucosmine or acetyl galactosamine according to the mass spectra of the acetates of the corresponding methylglycosides and the diagnostic ions corresponding to the fragments with loss of acetyl $[M-43]^+$, acetoxy $[M-59]^+$, acetic acid $[M-60]^+$, acetic anhydride $[M-102]^+$., and ketene $[M-42]^+$. of the mass spectrum (Figure 4).

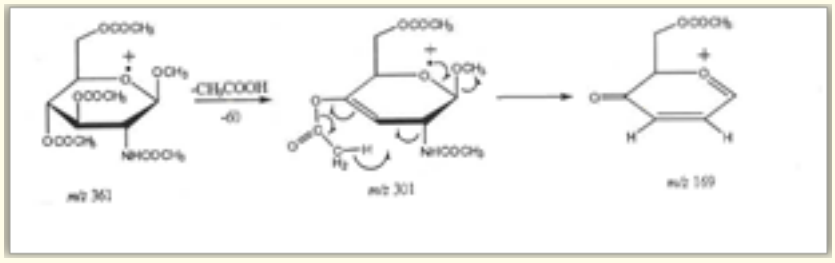

Figure 4: *EI-MS fragmentation pattern of per O-acetylated hexosamine methyl glycoside.*

Discussion

The fatty acids identified in the R-LPS are among those reported by Coloe., *et al*. [3] for the total cellular fatty acids of *Brucella ovis*. The presence of 19:0 and 18:1 would indicate that *B. ovis* has the ability to synthesize CFA-cyclopropane carboxylic acids from unsaturated fatty acids UFAs. This type of synthesis is frequent in bacteria and its occurrence depends in addition to the physiological conditions of the organism. Culture conditions that enhance the formation of CFAs are low pH, incubation at high temperatures and high concentrations of Mg^{+2}. Their presence confers increased chemical stability to the membrane because CFAs are more resistant to oxidative degradation.

Conclusion

The fermentation and purification process allowed obtaining the R-LPS with good yield. The extraction of R-LPS by the method of Galanos allows the production of a product essentially free of nucleic acids and proteins.

From the analysis of the fractions obtained by controlled hydrolysis of the R-LPS it is observed that it presents a singular composition. First, the fatty acid profile found in Lipid A is very rich in palmitic acid. According to our results we have been able to identify a location of the cyclopropane carboxylic acids described for this bacteria. The palmitic acids, stearic, 18:1, heptadecanoic acid, cyclopropane-

nonadecanoic acid together with a hydroxy acid form part of Lipid A of R-LPS together with a disaccharide formed by glucosamine and/or galactosamine.

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Disclosure

No potential conflict of interest relevant to this article was reported.

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