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Cellular Fatty Acid Profile of *Corynebacterium pseudotuberculosis* and its Relation to Pathogenicity

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Abstract: Cellular Fatty Acid Methyl Esters Profile (FAME) of a reference strain and three Sudanese field isolates of *Corynebacterium (C) pseudotuberculosis* was characterized by gas liquid chromatography. The Cellular Fatty Acids (CFAs) were liberated from whole cells by base hydrolysis, methylated and analyzed by gas-liquid chromatography. The principal fatty acids in *C. pseudotuberculosis* whole cell methylates were methyl laurate, methyl myristate, methyl palmitoleate, methyl palmitate, methyl oleate and unknown with retention time 23.825. The FAME profile of *C. pseudotuberculosis* isolates which were highly pathogenic in mice revealed high total area of palmitoleate (39.053%0) and oleate (36.973%) while the least pathogenic showed high content of myristate (68.506%).

Key words: *Corynebacterium pseudotuberculosis*, gas liquid chromatography, cellular fatty acid methyl esters

INTRODUCTION

C. pseudotuberculosis, a gram positive facultative intracellular pathogen, is the etiological agent of Caseous Lymphadenitis (CLA) in sheep and goats. CLA is a chronic disease characterized by adenitis of one or more of superficial and/or internal lymph nodes[1]. There have been numerous attempts to characterize strains of *C. pseudotuberculosis* the most reliable of which has been the nitrate reduction where two biotypes have been identified[2].

Analysis of cellular fatty acid composition is now used routinely to characterize, differentiate and identify genera, species and strains of bacteria[3,4]. It has been well established that the total fatty acid composition of a microorganism is an important taxonomic character and that fatty acid data can be analyzed quantitatively to provide useful taxonomic information at the species level and, in some studies, the subspecies level[5,6].

The objectives of this study have been to analyze the cellular fatty acid compositions of *C. pseudotuberculosis* and to determine if different isolates have a unique fatty acid profile that could be used to differentiate the species of the this genus. The relationship between the FAME profile and pathogenicity to mice was also studied.

MATERIALS AND METHODS

Gas-liquid chromatography: The qualitative and quantitative analysis of whole cell Fatty Acid Methyl Esters (FAME) was done according to the method described by Heitefuss[7].

Bacteria: *C. pseudotuberculosis* strains 3450, 24 (natural goat isolate), 226 and 26 (natural sheep isolate) were obtained freeze dried from the Department of Microbiology, Faculty of Veterinary Science, University of Khartoum, Sudan. Cells for CFA analysis were grown in a fermenter as a continuous culture (pH 8.0, temperature, 31°C, dilution rate 0.02 h^{-1} in modified Burrell's liquid medium).

Saponification: 20 mL fermenter sample of each strain were washed twice in normal saline by centrifugation at 2150 g 15 min^{-1}. The pellet was suspended in 1 ml of reagent 1(45 g NaOH, 150 mL methanol (99%) in 150 mL distilled water), vortexed for 5-10 sec and incubated

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at-100°C 30 min^{-1}. The preparation was allowed to cool to room temperature.

Methylation: Samples were methylated by the addition of two ml of regent 2 (325 mL of 6 N HCL plus 275 Hexan) and incubation at 80°C 10 min^{-1}.

Extraction: The fatty acids methyl esters were extracted by the addition of 1.25 mL of reagent 3 (Hexan 500 mL in 500 mL ethyl ether (supplemented with 2% ethanol)), gentle shaking for 10 min and centrifugation at 2150 g 5 min^{-1}. The sub-phase was discarded.

Neutralization: 3 mL of reagent 4 (10.8 NaOH in 900 mL double distilled water) was added to the above supernatant, shaked for 5 min and finally centrifuged at 2150 g 5 min^{-1}. The upper two thirds of the supernatant in the last centrifugation was used for gas liquid chromatography analysis.

Analysis: Samples were analyzed in a gas chromatograph (PERKIN-ELMER Sigma 2000). Condition of analysis were: detector 330°C; injector 350°C; oven 100°C to 220°C at 4°C per min and then at 30°C per min to 280°C and temperature. The carrier gas was nitrogen. The sample size was 5 μL. A mixture of commercial fatty acid methyl esters was used as standard.

Pathogenicity *C. pseudotuberculosis* in mice

Mice: NMRI-mice were purchased from Charles River Company-Germany. Mice were inoculated with live bacteria, fermenter culture supernatants.

Bacteria: A dose of 10^8, 10^7, 10^6,10^5 and 10^4 CFU mL^{-1} of *C. pseudotuberculosis* strains NCTC 3450, 24, 26 and 226 were injected Subcutaneously (SC) to a group of five mice each. Mice were observed for up to ten days postinoculation for deaths and abscess development. Dead as well as surviving animals were autopsied and their internal organs (lung, liver, spleen and kidneys) were inspected, smeared and gram stained and cultured on brain heart infusion agar plus 0.1% Tween 80.

RESULTS

Cellular fatty acid profile: The percentage composition of the fatty acids from the four strains examined is shown in Table 1. The princpal fatty acids in *C. pseudotuberculosis* whole cell methylates determined by gas liquid chromatography were methyl laurate, methyl myristate, methyl palmitoleate, methyl palmitate and methyl oleate. An unknown compound with a retention time of 23.825 was detected in all strains. Major peaks were considered as those contributing to > 3% of the total peak area in one or more strains, whereas those to < 3% were reported as trace amounts.

Pathogenicity to mice

Bacteria: The four strains differed in their pathogenicity to mice. Strain 24 was the most pathogenic. 108 and 107 CFU mL^{-1} doses caused acute disease and death within 3-5 days. Lower doses of 106, 105 and 104 CFU of the same strain caused no acute disease but a subcutaneous abscess at the site of inoculation. The organism was demonstrated in Gram-stained smears and isolated from the liver, spleen, lung and kidneys. Strain 26 at doses of 108 and 107 CFU caused only a subcutaneous abscess at the injection site but no acute disease. Lower doses of strain 26 caused no abscess. Strains NCTC 3450 and 226 caused neither acute disease nor SC abscesses at all dose levels during the study period, but organisms were isolated from internal organs.

Relationship between FAME profile and pathogenicity: Strains NCTC 3450 and 226 which were non-pathogenic to mice, had a high percentage of myristic acid and its hydroxyl and methyl derivatives. They constituted 68.505 and 41.360% of the total peak area for the two strains, respectively. Strain 26, which was moderate in pathogenicity, had 15.83% peak area for myristic acid and its derivatives. The highly pathogenic strain 24 had the least percentage of myristic acid, representing only 1.099% of the total area. Instead it had a high percentage of palmitoleic and oleic (39.054 and 36.97%, respectively) Table 1.

DISCUSSION

Variation in growth conditions and the stage of growth were found to influence the cellular fatty acid composition in *Clostridia*[8] and *Nocardia*[9], to overcome this difficulty and to ensure homogeneity; samples for gas liquid chromatography of *C. pseudotuberculosis* were obtained from continuous culture under the same optimal fermentation parameters.

In the present study we attempted the use of gas liquid chromatography of cellular Fatty Acid Methyl Esters (FAME) to qualitatively assess the lipid types among the four C. pseudotuberculosis isolates and the possible correlation between the FAME profile of a particular isolate and its pathogenicity to mice. The FAME profile of the strains contained methyl laurate,

Table 1: Fatty acid composition of *C. pseudotuberculosis* NCTC 3450 and three field isolates, bolded numbers = major peaks

		Total area (%)			
Methyl fatty acid	Common name	24	26	226	3450
M.dodecanoic	Lauric	-	-	1.625	-
M.2-OH-dodecanoic		0.1179	-	2.789	-
M.3-OH-dodecanoic		1.774	1.192	2.632	1.631
M. tetradecanoic	Myristic	-	0.522	8.019	17.878
M. 13-M- tetradecanoic		0.470	15.309	15.370	47.288
M. 12-M- tetradecanoic		0.629	-	4.785	0.394
M.3-OH-tetradecanoic		-	0.509	13.186	2.946
M. 14-M-pentadecanoic	-	3.111	0.576	4.286	1.230
M. cis-9-hexadecanoic	Palmitoleic	39.053	8.920	3.774	2.421
M. hexadecanoic	Palmitic	11.280	12.077	2.957	4.094
M.13-M-hexadecanoic		-	-	0.350	1.429
Unknown, RT (23.825)	-	2.755	22.220	0.755	8.470
M. cis-9-octadecanoic	Oleic	36.973	10.695	3.535	7.474
M. octadecanoic	Stearic	0.626	0.596	0.722	0.221
M.eicosanoic	Arachidic	-	4.823	0.661	1.670

methyl myristate, methyl palmitoleate, methyl palmitate, methyl oleate, methyl arachidate and unknown fatty acid with retention time 23.825 as the major peaks, each representing more than 3% of the total area in at least one strain or more. The unidentified fatty acid, which was detected in the four strains probably, constituted mycolic acid characteristic of the MNCR group. Methyl Lauric and methyl stearic are also present but none of them as a major peak. Results of the study clearly demonstrated that the different isolates have different FAME profiles and this fact could be used as a tool to subtype the species of the *C. pseudotuberculosis*.

This investigation is the first known direct comparison of cellular fatty acid profiles of *C. pseudotuberculosis* isolates and the results worth further study to confirm the use of FAME profiling as a useful tool for sub-typing the species.

The white mouse is the most suitable experimental animal for investigating disease caused by *C. pseudotuberculosis*. The none pathogenic strains have a high content of methyl myristate while the highly pathogenic strain 24 has methyl palmitoleate and methyl oleate as the major fatty acids, representing 39 and 36% of the total area, respectively. In view of that, we put forward, the fact that the content of these two fatty acids have a direct influence on the pathogenicity of *C. pseudotuberculosis* and its ability to form abscess. It is worthwhile to conduct further studies in this area deploying a substantial number of isolates.

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