CHEMICAL AND BIOCHEMICAL PROFILE OF FILAMENTOUS NON-TUBERCULOUS MYCOBACTERIA ISOLATED FROM CASEOUS LESIONS IN SUDAN

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المستخلص

اجريت هذه الدراسة بغرض عزل الانواع المختلفة من المتفطرات التي يمكن ان تسبب افات الابقار القيحية المتجبنة ثم التعرف على مسببات جقيل البقر باستخدام خصائص التصنيف الكيميائي. تم العزل و التعرف على 59 عزلة كمتفطره خيطية صامده للاحماض من الافات المتجبنة للابقار في جنوب دارفور باستخدام الاختبارات المزرعية والمجهرية. منها (32) عزلة اخضعت للتحليل البيوكيميائي بينما (24) عزلة اخضعت للاختبارات الكيميائية. استخدمت تقنية الترسيب بالتولوين والاسيتونيتريل للقياس الكمي لحمض الميكوليك. لاحقاً تم تطبيق تقنية الرسم اللوني الرقيق لراسب الحمض بغرض التحليل النوعي لحموض الميكوليك. بينما استخدم الرسم الوني الرقيق لمستخلص الكلورفورم\الميثانول لتصنيف الدهون السكرية الفينولية لجدار المتفطرات.

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اظهرت نتائج التحليل البيوكيميائي والكيميائي لـ 32 عزلة من المتفطرات الخيطية سريعة النمو أن 26 عزلة عرفت كمتفطرة جقيلية بينما *6* عزلات عرفت كمتفطرة سنغالية كما خلصت الدراسة الى ان للتحليل الكيميائي الكمي والنوعي لحمض المايكوليك دور كبير في التعرف والتصنيف على جنس المتفطرات وتفريقه من النوكارديا والجراثيم المشابهه الاخرى. بينما تحليل الدهون السكرية الفينولية مكن من تصنيف مسببات جقيل البقر الى المتفطرات الجقيلية والسنغالية.

Abstract

The aims of this study are to isolate the possible mycobacterial causes of caseated bovine tissues and subsequently identify the filamentous farcy agents using chemotaxonomic profile. A total of 59 acid fast filaments were isolated from bovine caseous lesions in South Darfur State. The isolates were conventionally identified using cultural and microscopic tests. For biochemical analysis 32 isolates were investigated while, the chemical profile applied for 24 isolates. Toluene-Acetonitrile precipitation method was applied to determine the quantitative mycolic acid precipitation. Subsequently thin layer chromatography was done from the precipitates for qualitative mycolates. Chloroform / methanol method was applied for typing phenolic glycolipids.Rapidly growing filamentous mycobacterial isolates (n = 32) were identified as *M. farcinogenes* (n =26) and *M. senegalense* (n =6) by their biochemical profiles, glycolipids and mycolic acid patterns. Mycolic acid analysis has a reliable role in differentiation and taxonomy of Mycobacteria from Nocardia and other related bacteria. However glycolipids test could differentiate the farcy agents into *M. farcinogenes* and *M. sengelenses*.

Key words: *Mycobacterium farcinogenes*, *M. senegalense*, filamentous non tuberculous mycobacteria, Mycolic acid and glycolipids.

Introduction

Bacteria of the genus *Mycobacterium* are acid fast Actinomycetes have a high content (61-71 %) of guanine plus cytosine (G+C) in the

genomic deoxyribonucleic acid (DNA), and high lipid content in the cell wall, probably the highest among all bacteria (Palomino *et al.*, 2007).Theses lipids include mycolic acids and glycolipids (GL) which have great role in acid fastness reaction, identification, classification and differentiation between Mycobacteria and Nocardia (Morris, 2003). Filamentous acid fast Mycobacterium; *M. farcinogenes* and *M.sengelense* are found the main causes of Bovine farcy (BF) which is chronic infectious disease affecting zebu cattle, endemic to East and Central Africa, (El Sanousi *et al.*, 1979, Chamoiseau 1974 and Hamid *et al.*, 2002).

Biochemical tests of farcy agents showed variable reactions, some were strongly catalase positive, urease negative and arylsulphatase negative (El Sanousi *et al.*, 1979 and Tag Eldin *et al.*, 1988) while Ridell and Goodfellow (1983), reported that *M. farcinogenes* strains are catalase negative in contrast to *M. sengelenses*. Shigedi *et al.*, (1980) reported that most of bovine farcy isolates ferment fructose and glucose, while few strains ferment galactose, lactose, maltose, manose, raffinose, sorbitol and xylose. Chamoiseau (1979) and Goodfellow and Wayne (1982) recorded that both *M. farcinogenes* and *M. sengelense* were catalase and nitrate positive and urease negative. Riddell *et al*, (1985) found that some Sudanese *M. farcinogenes* strains were catalase negative in contrast with the findings of Chamoiseau, (1979, El Sanousi *et al.* (1979). Viencet *et al.* (2009) recorded that *M. sengelenses* reduced nitrate, urease and arylsulphatase.

The chemical profile of Mycobacterium cell wall lipids distinguished the genus member from other Actinomycetes either by the presence of C22 to C26 products from Gas Liquid Chromatography (GLC) Pyrolysis (Minninkin, D.E., 1982; Minninkin *et al.*, 1985 and Kumar *et al.*, 2010) or by precipitation of mycolic acid ether solution by addition of alcohol whereas, other genera mycolates remained soluble (Kirschner *et al.*, 1993). Hence chemical profile of mycobacteria designated the farcy agents as mycobacterium and not nocardia (El Sanousi *et al.* (1977). Mycolic acid TLC of bovine farcy agents revealed α-, ά mycolates and epoxymycolates (Hamid *et al.*, 1994). The farcy agents mycolates co-chromatographed with methyl mycolates and non-

hydroxylated fatty acid methyl esters similar to those found in *M. fortuitum* and. *M. porcinum* also had a- a'-mycolates similar to *M. farcinogenes*, *M. fortiutum*, *M. peregrinum*, *M. senegalense*, *M. smegmatis* and *M. chitae* (Loquin *et al*., 1987).
Quantitative mycolic acid precipitation results in extraction of more than 8mg of mycobacteria mycolic acid from 1 g of wet colonies in melting point of 50-52C, while *Nocardia* mycolates were less than 5mg/ g wet colonies and did not melt in less than 150 Ċ (El Sanousi *et al.*, 1977). Later Hamid (1994) precipitated 2-4 mg and 1-2mg mycolic acids from 50mg dry weight of *M. farcinogenes* and *M. senegalense* respectively.

Glycolipids chromatography has a significant role in classification of farcy agents as reported by Hamid *et al.*, (1993). They reported that *M. senegalense* strains could produce pink and brown lipid spots when sprayed with α naphthol Hamid, (1994). El- Hussein (2001) reported that *M. farcinogenes* could not produce any glycolipids in contrast to *M. senegalense*. Besra *et al.*, (1994) also proved that Chadian farcy agent had contained glycolipids and accordingly classified as *M. senegalense* strain.

The current study aimed to isolate the possible mycobacterial agents which may cause bovine suppurations and subsequently identify the farcy agents using chemotaxonomic profile.

Materials and Methods

Reference strains

The reference strains *M. farcinogenes* (M39) and *Nocardia africana* strains *(*NRs) used in this study kindly donated by Dr. Mohamed Elamin Hamid, King Khalid University. and by Dr. Mugahid M. Alhassan Sudan University of Science and Technology (SUST) respectively.

Identification and Propagation of Mycobacterium cultures.

The Mycobacterium cultures were identified with conventional microscopy and biochemical reactions such as Catalase, nitrate reduction, Urease and Arylsulfatase activity (Konemann *et al.,* 2005, Palomino, 2007, Murray *et al.* 2007 and Viencet *et al*. 2009).

For propagation; strains were grown in Modified Souton´s medium, Glucose Yeast Extract Agar (GYEA) and Bennett's agar according to Hamid *et al*. (1995).

Quantitative analysis of Mycolic acids

Extraction and Precipitation method of mycolic acids using toluene-Acetonitrile mixture (0.2: 0.3 ml v/v) was developed from the methods described by Kantesuna and Bartoli (1972), El Sanousi *et al.,* (1977) and Hamid, (1994) for 32 filamentous isolates

Qualitative Mycolic acid chromatography

Methanolysis method was applied according to methods described by Kantesuna and Bartoli (1972) and Ridell *et al.,* (1982) with some modification in temperature of extraction (90C̊).

Chloroform / methanol method

Five mg biomass of propagated cultures was processed according to methods of Minninkin and Goodfellow (1980). Colonies were washed in normal saline, the supernatant was discarded and the pellet dissolved in 4ml of chloroform and methanol (2:1 by vol.), shaked for 1 hr, the mixture was centrifuged and the supernatant was collected in capped tubes. The pellets were extracted again in 2ml of an extraction solvent and the combined extract dried and chromatographed. From the extract 5µL was chromatographed in silica gel sheet (10 X10 cm, Merck, silica gel 60 F254, MERCK, and D-6100 Darmstadt, Germany) developed by Chloroform: methanol: water (100:14; 0.8 v/v) three times and the sheet

stained with 5% ethanolic molybdophosphoric acid and heated at 150C for 15 minutes. Yellow to brown spots indicate the sugar containing lipids. PGL Thin layer chromatography (TLC) of farcy field isolates, *M. farcinogenes* reference strain (M39) and Nocardia africana strain followed the method described by Dobson *et al*., (1985).

Results

Propagated cultures

Modified Souton´s broth revealed luxuriant growth during the first weeks of colony incubation at 37C° when compared with reference strains of *M. farcinogenes* (M39). All the strains cultured in GYEA and Bennett's agar produced good growth (Fig.1).

Biochemical tests

Biochemical investigation of (32) filamentous mycobacterium revealed (6) isolates as *M. sengelenses* and (26) isolates were designated as *M. farcinogenes* Table (3).

Quantitative analysis of mycolic acid precipitates

Toluene-acetonitrile precipitation method of mycolates derived from field isolates revealed 7.2 mg mean values mycolates precipitins from 113.9 mg mean values of total weights of 24 farcy tested strains.

Thin layer chromatography (TLC) Mycolic acids

TLC of methanolysates of farcy strains (24 strains) in aluminum sheet revealed production of mycolates typical to those produced by reference strain of *M. farcinogenes* (M39), which consist of α,ά mycolates (A&A') and epoxy-mycolates (M). The Developing solvents of petroleum ether (60-80) and acetone (95:5) revealed clear spots of Mycolic acids when compared with diethyl ether and petroleum ether developing solvent

(15:85). Tlc of Mycolic acids pattern could not distinct between *M. farcinogenes* and *M. sengelenses* Fig.(2).

Thin layer chromatography (TLC) of glycolipids

Glycolipids TLC chromatography of 24 farcy isolates showed different patterns of free lipids in comparison with the reference strains, *M. farcinogenes* reference (M39) and *Nocardia africana* (NRs). Eighteen lipids (75%) patterns of the tested strains resulted in dark black spots arranged in three major bands identified as *M. farcinogenes*. While 6 (26.1%) strains represented (MF; 305, 310, 314, 319, 320, 321) revealed free lipids pink in color when heated, later the color changed to dark after cooling theses strains were identified as *M. sengelense* (Fig. 3).

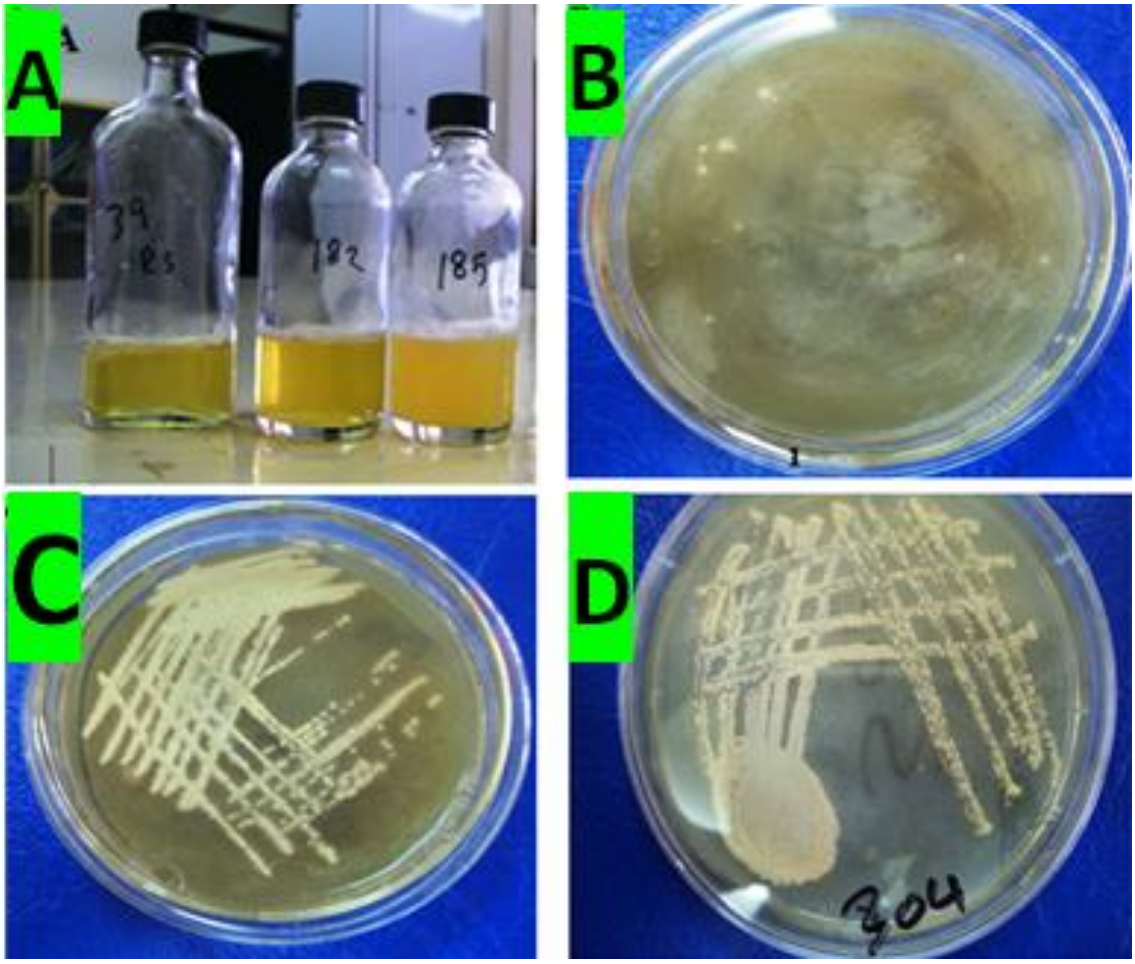

Fig: (1).A: (39 RS). *M. farcinogenes* reference strain, Mb 182: *M. bovis* & MF 185: *M. farcinogenes* post 14 day incubation. **B&C:** *M. farcinogenes* (MF1& MF13) grown in GYEA. D: *M. farcinogenes* (MF 304) grown in Bennettś agar.

Table (3): Biochemical tests of farcy agents**.**

Test	+ Ve (%) total	- Ve (%)	total
Catalase 68C°	32 (100)	0(0)	**32**
Nitrate	31 (96.9%)	1 (3.1)	
Ureiase	21 (65.6%)	11(34.4%)	
Arylsulphatase	26 (81.3%)	6 (18.7%)	

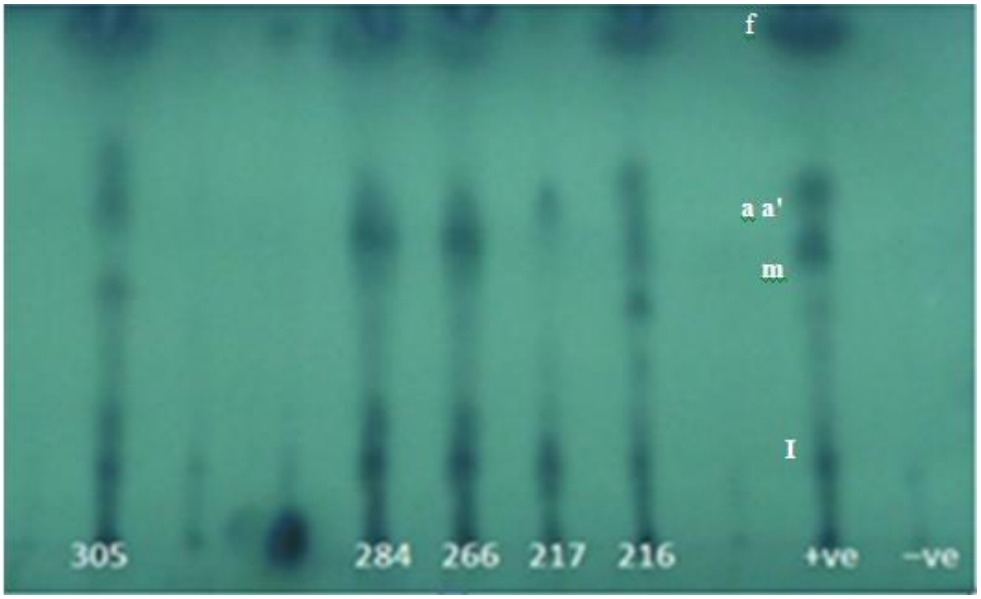

Fig. (2): Thin layer chromatographic analysis of mycolates extracted from farcy field strains, compared with M39 reference strain mycolates (+ve control) and Staphylococcus spp. (-ve control), developed by petroleum ether (60-80) and acetone (95:5) solvents three times for 20 minutes, stained by molybdenum phosphoric acid and heated for 15 minutes. Accordingly the strains MF216, MF217, MF266, MF284, and MF305 considered as farcy agents.

Key: (f, free fatty acids, a a', alpha- and apha'- mycolates, m, epoxy-mycolates and I short chains
Derivatives of epoxy-mycolates)

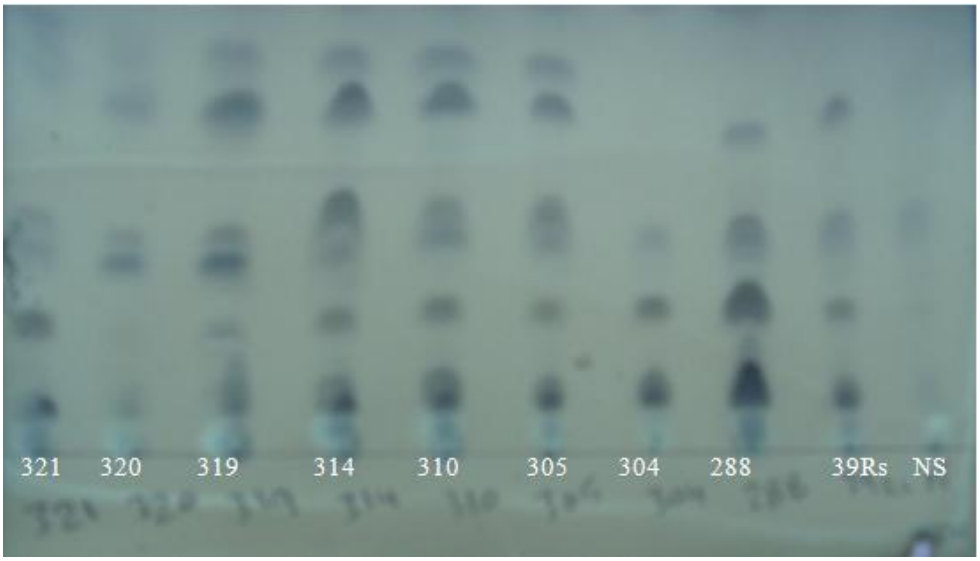

Fig. (3): Thin layer chromatographic analysis of phenolic glycolipids (PGL) extracted from farcy field strains, compared with *M. farcinogenes* reference strain (M39) glycolipids and Nocardia africana (NRS). The Developing solvent was Chloroform: methanol: water (100:14; 0.8 v/v).

Accordingly strains MF288 and MF304 were identified as *M. farcinogenes* while strains MF305, MF310, MF314, MF319, MF320 and MF321 were typed as *M. sengelenses*.

Discussion

It has been found that chemotaxonomic methods have a reliable role in characterization and taxonomy of Mycobacteria and Nocardia from other related bacteria (Minninkin, 1982 and Minninkin *et al.*, 1985). In Africa

Chamoiseau, (1979) and El Sanousi *et al.*, (1979) designated *M. farcinogenes* and *M.sengelenses* as the causative agents of bovine farcy. Later, Ridell *et al.*, (1985) supported the designation of *M. farcinogenes* as the causative agent of bovine farcy in the Sudan. The present result could designate Sudanese farcy agents to *M. farcinogenes* (18) and *M. sengelenses* (6) isolates using TLC of glycolipids. This result agrees Hamid *et al.* (1993) and disagrees with El - Hussain, (2001) who reported that glycolipids were not extracted from *M. farcinogenes* (typed strain) while, *M. sengelenses* showed clear spot of lipid when compared with the reference strain. In this study, the glycolipids were stained by molybdenum phosphoric acid; however the previous studies mentioned alpha-naphthol stain produced pigmented glycolipids which enable classification of farcy agents and related organisms such as *M. fortiutum* and related taxa.

Quantitative mycolates analysis precipitated (7.2mg) mean values of mycolic acids from (113.9mg) mean values gross biomass weights of colonies. The present results disagree with El Sanousi *et al.*, (1979) who reported that 1g of dry colonies could produce about 8mg mycolic acids and this minor variation may be due to methanolysis extraction method.

Qualitative mycolic acids methanolysates chromatography produced mycolates of alpha and epoxy types similar to those obtained by Hamid et al. (1993) from *M. farcinogenes*. On the basis of mycolic acid analysis, *M. farcinogenes* and *M. senegalense* cannot be separated as each contains a-mycolates and characteristic polar mycolates previously only found in *M. fortuitum* and *M. smegmatis* (Ridell and Goodfellow 1983). However, Biochemical tests and TLC of glycolipids identified (6) strains as *M. sengelense* and (26) stains as *M. Farcinogenes* agrees with (Ridell and Goodfellow 1983 and Hamid *et al.*, 1993).

In this study thin layer chromatography of mycolates using developing solvents; petroleum ether (60-80) and acetone (95:5) three times for 20

minutes produced clear dark spots better than diethyl ether and petroleum ether developing solvent (15:85).

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