Mansoura Journal of Biology Vol. 35 (2) December, 2008.

DIAGNOSIS OF BOVINE TUBERCULOSIS BY ZIEHL NEELSEN STAINING, CULTURE AND MYCOLIC ACID ANALYSIS

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ABSTRACT

Ziehl Neelsen acid fast staining and lowein stain Jensen cultures were carried out on 200 caseated lymph nodes collected from slaughtered cattle on suspicious of bovine tuberculosis at Omdurman abattoir. Identification was made according to the characters detailed by **[Runyon *et al.*, (1974)]**.

The results showed detection of acid fast bacteria in 24 samples (12%) by Ziehl Neelsen staining compared to 36 (18%) for isolation on cultures. Identification revealed II Mycobacterium bovis, 18 Mycobacterium farcionogews, 3 M.avium, 1 M.fortititum, and 2 M.pheli,

Mycolic acids of these identified Mycobacterium species were analyzed using acid Methanolysis and thin layer chromatography as stated by **[Minnikin *et al.*, (1975 & 1980)]**. The different mycobacterial mycolic acid methyl esters pattern on one dimensional thin layer chromatography of the general composition of the tested isolates were exemplified. M.bovis contained α mycolate, methoxy mycolate and ketomycolate. M. farcionogenes contained a mycolale, Ketomycolate, w-carboxy mycolate and 2 eicosanol homologies. M.fortuitum contained α mycolate, α mycolates and unknown components. M.pheli contained α mycolate, ketomycolate, w-carboxy mycolate, eicosanol homologies and unknown components.

Mycolates of *M. bovis* has been-well separated on the two dimensional thin layer chromatography and their pattern was compared to reference *M.bovis*. This confirmed their identity as typical *M.bovis*.

It is concluded that thin layer chromatography of the whole organism methanolysates provides a reliable method for confirmation of *M.bovis* identification and hence diagnosis of bovine tuberculosis.

Key words: Diagnosis – Bovine – Tuberculosis – Mycolic – Analysis.

INTRODUCTION

Bovine tuberculosis is a chronic disease of cattle caused by Mycobacterium bovis, The disease can infect all warm blooded vertebrates and is of great importance for its effect on animal production as well as for public health concern.

Diagnosis of bovine tuberculosis is not easy since clinical signs were seldom becoming apparent and that some infected liver stock show no evidence of pathological lesions on postmortem inspection. Beside that there are some diseases and conditions with tubercle-like lesions that confuse diagnosis.

Cassation in lymph nodes of the slaughtered cattle is one of the affections that lead to suspicious of bovine tuberculosis. Several laboratory methods are used for diagnosis; of bovine tuberculosis from postmortem lesions. Microscopic and microbiological examinations of the involved tissues are required before a definite diagnosis can be made **[Alhaji (1976)]**, The detection of acid fast bacteria in suspected material by direct smear stained with Ziehl Neelsen's, although highly suggestive, is not. absolutely diagnostic, **[Merchant & Paker (1971)]**. The difference in morphology is not a sufficient basis upon which to differentiate one type of tubercle bacilli from another, **[Hagan & Bruner (1961)]**. *Mycobacterium bovis* can be visualized only if at least 5 x 104 of the organism/ml of material is present. Different culture media have been used for cultivation of Mycobacterium species from clinical or postmortem materials with variable efficacy and advantages.

Loweinstein Jensen medium was proved by **[Jezierski (1937)]** to be the most effective medium for cultural diagnosis compared with some other media. **[Coper & Cohn (1933)]** reported 10-100 organisms per ml suffice for the detection of tubercle bacilli by the cultural method. Mycobacteria possess a complex, lipid rich cell well, with high content of mycolic fatty acid. Analysis of lipid composition is known as a useful tool for differentiating Mycobacterium species and other taxa containing mycolic acid, **[Smith *et al.*, (1957); Jenkins *et al.*, (1971); Lechevalier *et at.*, (1971); Marks *et al.*, (1971); Minnikin & Goodfeilow (1980); Minnikin (1982) and Hamid *et al.*, (1993)]**.

The aim of the present work is to identify and confirm *Mycobacterium bovis* and other Mycobacterium species responsible for caseation of lymph nodes of the slaughtered cattle on a criteria based on the standard method of Ziehl Neelsen staining, culture on Loweinstein

Jensen medium and mycolic acid analysis.

MATERIAL AND METHODS

Collection of the caseated lymph nodes:

A total of 200 caseated lymph nodes were aseptically collected from slaughtered cattle at Omdurman abattoir. The samples were taken following the method described by **[Lepovetsky *et al.*, (1953)].**

Microscopic & Microbiological Investigation For Acid Fast Bacteria:
Preparation of the samples:

Aseptic removal of the node was made by repeated searing of the fat capsules using a red hot spatula. The node was dipped in 95% ethyl alcohol then held in a flame until alcohol was burned off. This procedure was repeated twice.

The caseated lymph node was incised by a sterile scalpel blade and the caseated material was exposed.

Ziehl Neelsen staining:

Direct smear from the caseated material taken was made and left to dry. The film was fixed by heal then stained by the standard method of Ziehl Neelsen's stain.

Culture on Lowcinstem Jensen medium:

The caseated material was prepared for primary isolation according to the method describe by **[Jensen (1932)]**, the method based on treatment of the caseated material with 4% NaOH followed by centrifugation for 30 minutes and then culture was obtained from the deposit. The sediment for each sample was inoculated on dublicate of screw capped bottles containing slopes of Loweinstein Jensen medium. The inoculated medium bottles were incubated at 37°C for 8 weeks.

Identification of acid last bacteria:

The cultural and biochemical characters used in identification of acid fast bacteria were those detailed by **[Runyon *et al.*, (1974)]**. These include: Type and time for growth. Colony form, Pigmentation and response to light, Catalase, Growth on Mac Conkey agar without crystal violet, Urease, Nitrate reduction, Aryl sulphatase. Tolerance of 5% NaCl, Tween 80 hydrolysis, Niacin production, Deamination of pyrazinamide.

Mycolic acid analysis:

Mycolic acids of some identified Mycobacterium species were analysed using acid methanolysis and thin layer chromatography as follows:-

Acid methanolysis [Minnikin *et al.*, (1975)].

Two colonies from Loweinstein Jensen medium growth were placed into a glass tube containing 1ml of extraction mixture (methanol: toluene: cone. H_2So_4; 30: 15: 1, v/v). The tubes were incubated at 80°C for 12 hours and left to cool. One ml of petroleum ether was added to each tube. The tubes were shacked well for extraction of mycolic acid methyl esters, followed by centrifugation. The upper layer that formed on each lube was[1] aspirated with a sterile disposable syringe and then each was transferred to another clean tube. The tubes were left for drying and stored at 4°C to be ready for thin layer chromatography.

Thin layer chromatography (TLC) [Minnikin *et al.*, (1980)]

- The extracted lipids that was prepared by acid melhanolysis was dissolved in 10 ml petroleum ether. Five ml of the extracted lipids with petroleum ether were spotted onto small sheets cut from aluminum backed sheets (20x20 cm, Merck; silica gel 60 F_{254} TLC aluminum sheets, No. 5554).
- For single dimensional thin layer chromatogeaphy of whole-organism acid methanolysates of the tested strains, 10x10 cm aluminum sheets were used. The spotted sheet was put vertically in a glass jar containing light petroleum-diethyl ether (85: 15 v/v) and left to develop for 20 minutes. This was repeated after drying.
- For two dimensional thin layer chromatography of whole organism acid methanolysates of the tested strains 6.6x6.6 cm aluminum sheets were used. The spotted sheet were placed in a glass jar containing petroleum ether (b. p. 60-80°C)/ acetone (95: 5 v/v) for three development in the first direction. This was followed by single development with toluene/ acetone (97/3, v/v) in the second direction.
- The thin layer plates (either one or two dimensional) were sprayed with 5% ethanolic niolybdophosphoric acid. They were then heated in an oven at 100-180°C for 15 minutes.

RESULTS

Microscopic & Microbiological Findings:

Twenty four caseated lymph nodes revealed acid fast by direct smears stained with Ziehl Neelsen's stain whereas thirty-six acid fast bacteria were isolated on Loweinstein Jensen from the various caseated lymph nodes. Acid fast bacteria were obtained in both the direct smear method and the cultural method on Loweinstein Jensen medium from 21 caseated lymph nodes.

On identification the species of acid fast bacteria isolated were 11 *M. bovis*, 18 *M.farcinogenes*, 3 *M.aviwn*, 2 *M.fortuitum* and 2 *M.pheli*. Cultural growth of *M.bovis* on Loweinstien Jensen medium was illustrated in fig. (1).

Mycolic acids analysis

One dimensional thin layer chromatographic analysis of whole organism acid methanolysates revealed the presence of multispot pattern of mycolic acid methyl esters. The mycolic acids were dark on a green background (Fig. 2).

The different nncobacterial mycolic acid methyl esters pattern on one dimensional thin layer chromatography of some of the tested isolates were exemplified in (Fig. 3). *M.bovis* isolated from sample 4 contained α mycolate, methoxymycolate and ketomycolates. *M.farcinogenes* isolated from sample 83 contained α mycolate, α¯mycolale and cpoxymycolate. *M.avium* isolated from sample 124 contained α mycolate, ketomycolate, co-carboxvmycolatc and 2 eicosanol and homologues. *M.fortuitum* isolated from sample 131 contained α mycolate, α mycolates and unknown components. *M.pheli* isolated from sample 167 contained α mycolate, Ketomycolate, ώ-carboxymycolate, eicosanol homologues and unknown components.

Mycobacterium bovis isolated from sample 4 revealed typical pattern which consisted of α mycolate, methoxymycolate and ketomycolate. These mycolates has been well separated on the two dimensional thin layer chromatography (Fig. 4). The pattern of the tested isolate from sample 4 was compared to reference *M.bovis*. The pattern of α mycolate, methoxymycolate and ketomycolate in the tested isolate confirmed their identity as typical *M.bovis*.

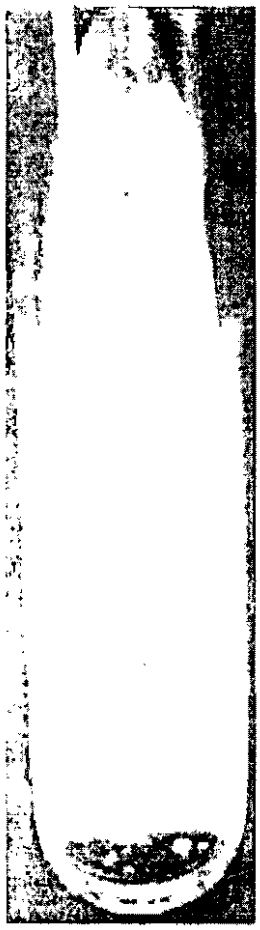

Fig. (1): Culture of *M. bovis* on Lowcinstein Jensen medium

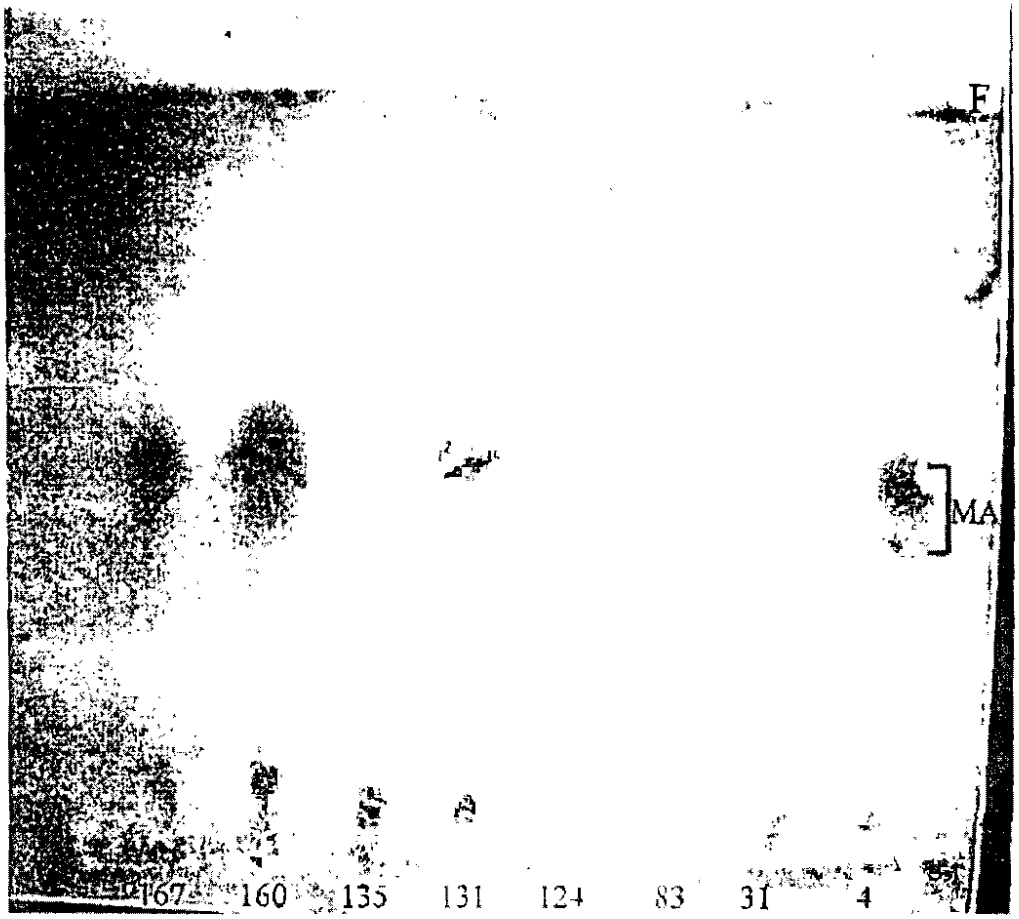

Fig. (2): One dimensional T.L.C. pattern of mycolie acid methyl ester of the whole organism acid methanolysis of some of the isolated Mycobacterium species. Abbreviations; MA, Mycolie acid; F, non hydroxylaled fatty acid methyl ester.

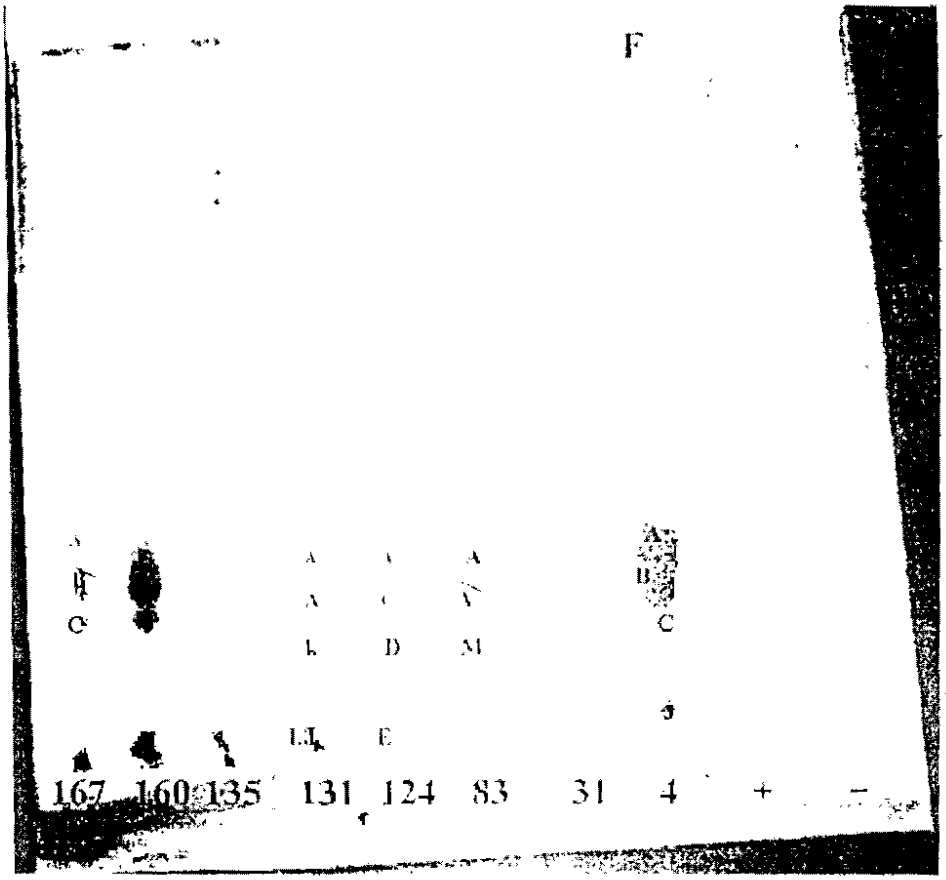

Fig. (3): One dimensional T.L.C. pattern of mycolic acid methyl ester of whole-organism acid methanolysis of *M.farcinogenes* isolated from samples 31, 83, 160; *M.bovis* isolated from samples 4, 135; *M.avium* isolated form sample 124; *M.fortuitum* isolated from sample 131; *M.pheli.* isolated from samples 167.
Abbreviations: A, α mycolate; A', α mycolate; B, methoxy mycolate; C, keto mycolate; D, ώ-carboxy mycolate; E, eicosanol and homologues; F, non hydroxylated fatty acid; I, J,

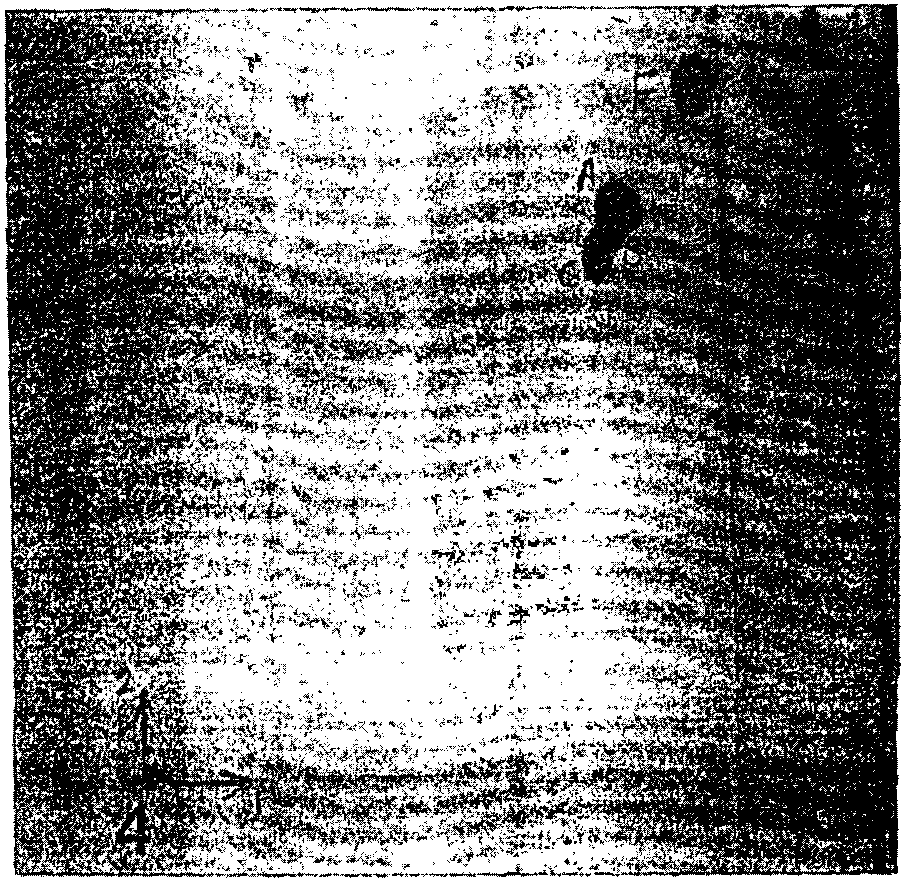

Fig. (4): Two dimensional T.L.C. pattern of mycolic acid methyl ester of the whole organism acid methanolysis of *M.bovis* isolated from sample 4. Abbreviations. F, non-hydroxylated fatty acid; A, α mycolate; B, methoxy mycolate; C, keto mycolate; 1, development in the first direction; 2, development in the second direction.

DISCUSSION

Acid fast bacteria were detected in 12'% of the examined samples by the direct smear method. Meanwhile, using the cultural method on Loweinstein Jensen medium, it was possible to isolate acid fast bacteria from 18% of the same samples. This indicated that negative results of acid fast bacteria by the direct smear method can be positive by the cultural method. Such finding was in conformity with the comparative study made by **[Murphy *et al.*, (1938)]**, for demonstration of tubercle bacilli by different methods. They reported that from 171 different specimens which were negative in direct smears, 31 were positive by culture. In the present study, three of the samples which showed acid fast bacteria in the direct smear could not yield a positive result on Loweinstein Jensen medium. This could be attributed to, either death of the acid fast bacteria during decontamination process, or due to the growth of inhibtor acid fast bacteria.

In the present study, *Mycobacterium bovis* is isolated from 11 (5.5%) of the caseated lymph nodes, whereas other Mycobacterium species isolated from the caseated lymph nodes included 18 M.farcinogenes , 3 M.avium ,2 M.fortuitum and 2 M.pheliL **[Pritchard (1988)]** stated that Mycobacterium species other than *M,bovis* may be isolated from the bovine tissue since members of this genus are widely distributed in the environment. Some of these species occasionally produce lesions in cattle, whereas the presence of others may be incidental. **[Pallaske (1935)]** mentioned that the type of bacilli present in different tuberculous organs of cattle can't be determined by macroscopic examination of the lesion. **[Barton (1972)]** stated that histopathology can't reliably differentiate bovine tuberculosis from other infections of Mycobacterium species. These statements showed that the caseation produced by these Mycobacterium species may mimic tuberculosis and that the bacteriological examination is necessary to determine the aetiological agents. Isolation of *M.farcinogenes*, in the present study, is in conformity with the reports of several authors, **[Awad & Mustafa (1974); Salih *et al.*, (1978); El sanousi *et al.*, (1979) and Hamid *et al.*, (1991)]**.

Runyon *et al.*, (1974) stated that *M.avium* is less frequently found in lesions of the lymph nodes of cattle, whereas *M.fortuitum* was isolated from the lymph glands of cattle and that *M.pheli* was isolated from hay and grasses and that these organisms are present in soil. In the present

study, isolation of *M.avium*, *M.fortuitum* and *M.pheli* was attributed to ingestion and/or to contamination of wounds with soil.

It has been well established that lipid analysis notably mycolic acids are a good chemotaxonomic markers. Chemical markers derived from lipid analysis have been widely used in classification and identification of actinomycetes **[Goodfellow & Cross (1984); Kroppenstedt (1985); Schaall (1985) and Suzuki *et al.*, (1993)].**

The interpretation of mycolic acid analysis by thin layer chromatography of methanolysates of the tested isolates of acid fast bacteria indicated a number of characteristic patterns of myeobacterial mycolates. The distribution of mycolic acid contents of the tested isolate belonging to *M.farcinogenes*, *M.bovis*, *M.avium*, *M.fortuitum* and *M.pheli* provide valuable markers for identification of these mycobacterium species. This comes m agreement with **[Minnikin *et al.*, (1975); Minnikin *et al.*, (1980); Minnikin (1982); Minnikin *et al.*, (1982); Ridell *et al.*, (1982); Minnikin *et al.*, (1984); Hamid *et al.*, (1991) and Hamid *et al.*, (1993)]**. Thin layer chromatography of whole organism methanoly sates provides a reliable method for confirmation of *Mycobacterium bovis* identification and hence diagnosis of bovine tuberculosis. It is concluded that the criteria based on the standard method of Ziehl Neelsen staining, culture on Loweinstein Jensen medium and mycolic acid analysis is of great value in diagnosis of bovine tuberculosis.

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تشخيص الدرن البقرى باستخدام صدفة ينهل نيلسن وتحليل حمض الميكوليك

على بابكر عثمان

قسم الصحة العامة – كلية الطب وعلوم الصحة- جامعة شندى- السودان

لقد استخدمت صدفة ينهل نيلسن وتحليل حمض الميكوليك على ٢٠٠ عقدة لمفية من الأبقار المذبوحة فى مجزر أم درمان والتى بها شك بوجود درن بقرى .

أوضحت النتائج وجود ١٢% من الحالات الموجبة باستخدام طريقة ينهل نيلسن و١٨% من الحالات الموجبة باستخدام تحليل حمض الميكوليك والتى بينت وجود ١١ نوع من الميوباكتيريم بوفيس و١٨ من الميوباكتيريم فاركيو نوجوس و ٣ أنواع من الميوباكتيريم أفيم ونوع من الميوباكتيريم فورتيتم ونوعين من الميوباكتيريم فيلى.

وبالتحليل الكروماتوجرفى لهذه الأنواع باستخدام التحليل الميثانى وجد أن الميوباكتيريم بوفيس يحتوى على ألفا ميكولات وميثوكبسى ميكولات وكيتوميكولات أما الميوباكتيريم فاركيو نوجوس يحتوى على ميكولات وكيتوميكولات وكربوكسيل ميكولات ومتناظرى إكوسانول. أما الميوباكتيريم فورتيتم يحتوى على ألفا ميكولات ومركبات غير معروفة. أما الميوباكتيريم فيلى يحتوى على ألفا ميكولات والكيتوميكولات والكربوكسيل ميكولات ومتناظر إكوسانول ومركبات غير معروفة.

ونستنتج أن التحليل الكروماتوجرافى يعتبر طريقة موثوق بها فى تشخيص الدرن البقرى.