

Sudan University of Science and Technology

College of Graduate Studies

**Frequency of Actinomadura among Mycetoma Patients and
its Relevance to Soil Actinomycetes in Sudan**

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بالشعاعيات الترابية في السودان

A thesis Submitted for the Fulfilment of the Degree of

MSc in Medical Laboratory Science (Microbiology)

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2010

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﴿ اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ ﴾

صدق الله العظيم

سورة العلق الآية ١

Dedication

I dedicate this research to

My Parents.....

Who gave me the meaning of the life My brothers and sisters....

For their support and kindness

My friends and my colleagues.....

The persons whom I love, respect and appreciate.....

Everyone I love.

Acknowledgments

First of all, thanks for alMIGHTY Allah who gave me the power for preparation and completion of this study.

With a great deal of respect I would like to express my thanks to my supervisor Dr. Mogahid Mohamed Elhassan, Head of Microbiology Department, College of Medical Laboratory Science, Sudan University of Science and Technology for his advice, enthusiasm, help and endless guide.

Thanks were also extended to the staff members of the Microbiology Department for their help and encouragement.

Finally, I am grateful to Dr. Adil Mahjoub, College of Veterinary Medicine University of Kartoum, for his help and support.

ABSTRACT

The present study aimed to determine the frequency of actinomadura among Sudanese patients suffering from mycetoma in endemic areas in Sudan and correlate these infections with soil actinomycetes isolated from these areas.

One hundred clinical specimens were collected from mycetoma patients who attended the Mycetoma Research Center at Soba Teaching Hospital as also as clinics in different areas in Sudan including Um-Rawaba Western Sudan, White Nile State and Wad Madani Hospital during the period from November 2008 to August 2010. Grains were collected from sinuses and incision biopsies in sterile containers. They were washed with normal saline, then sent to laboratory for culture and further investigations. Primary identification of the clinical isolates was performed based on the color of the grains, colony morphology and Grams. It stain revealed that 12% were actinomycetes among which 6 were identified as *Actinomadura madurae*, while the other were considered as eumycetoma (50%+88%).

Further confirmation was done by subjecting the twelve isolated actinomycetes to conventional Polymerase Chain Reaction (PCR) and Real Time PCR (RT-PCR).

The results showed that 6 (50%) were *Streptomyces* while the others (50%) were negative to *stb1* gene.

On the other hand, one hundred soil samples were collected from different geographical areas including Um-Rawaba Northern Kordofan, Western Sudan, White Nile State and Khartoum State. Soil samples were collected in clear dry containers, and sent to the laboratory for culture and further investigations. Serial dilution from the soil were performed and

inoculated on Tryptic Soy Agar (TSA) and Actinomycetes Isolation Agar (AIA) media, and incubated aerobically at 37°C.

Soil isolates were identified by colony morphology, Gram Stain, modified Ziehl Neelson stain, biochemical tests and mycolic acids profile.

The results showed that 20% of the isolates were Gram positive branched bacilli and weak acid fast bacilli with distinguished growth on TSA and AIA. Among these 20 isolates 3 (15%) were *Streptomyces* species, while the other 17 (85%) were *Nocardia* and *Nocardia* like.

In conclusion that, actinomadura exist with high frequency among Sudanese patients with mycetoma. Since a number of bacterial agents which are known to cause actinomadura have been isolated from soil in target areas, the study suggests that soil may represent a source of the infection.

ملخص الاطروحة

هدفت هذه الدراسة لتحديد تردد الورم الفطرى الشعى ضمن مرضى الورم الفطرى وعلاقته بالشعيات التريبية الشعاعية والمسببة لمرض الورم الفطرى الشعاعى فى المناطق الموبوءة فى السودان.

تم جمع عدد مائة عينة من مرضى النبت الدين توافدوا لمركز ابحاث المايستوما بمستشفى سوبا التعليمى والمستشفيات فى كل من أم روابة شمال كردفان غرب السودان وولاية النيل الابيض ومستشفى ود مدنى فى الفترة ما بين نوفمبر ٢٠٠٨ الى اغسطس ٢٠١٠.

تم جمع حبيبات من الجروح المتقيحة ومن داخل الانسجة المصابة فى وعاء معقم، غسلت الحبيبات بملح الطعام ثم ارسالها للمعمل للزراعة ولمزيد من التقصى. تم التعرف المبدئى للعينات الاكلينكية اعتمادا على لون الحبيبات والشكل الظاهرى للمستعمرات وصبغة القرام.

اظهرت النتائج نسبة ١٢ % من انواع البكتيريا الشعاعية و ٨٨ % من انواع الفطريات. وسط ال ١٢ بكتيريا شعاعية عرفت ٦ منها عبارة عن اكتينو مادورة مادورى، ولمزيد من التقصى اظهرت نتائج تفاعل البلمرة السلسلى ان ٦ (٥٠%) بكتيريا شعاعية كانت موجبة المؤشر لجين البكتيريا الشعاعية (المتسلسلة الفطرية) والنسبة الاخرى ٦ (٥٠%) سالبة.

من ناحية اخرى تم جمع عدد مائة عينة من التربة جمعت من مناطق مختلفة والتى تشمل كل من أم روابة شمال كردفان غرب السودان، وولاية النيل الابيض ولاية الخرطوم. تم جمع عينات التربة فى حاويات جافة ونظيفة وارسلت للمعمل لزراعتها ولمزيد من التقصى.

عولجت عينات التربة بالتخفيف المتدرج وزرعت فى وسط التريبتك صويا والاكتاينومايست ايسوليشن اقرار وتحضينها فى درجة ٣٧ درجة مئوية.

تم التعرف على عينات التربة بالمظهر الخارجى للمستعمرات و صبغة الغرام والزيل نلسون المعدلة والاختبارات الكيموحيوية والاحماض الدهنية طويلة السلسلة.

اظهرت النتائج أن ٢٠ % من العزلات موجبة لصبغة الغرام واظهرت عصيات متفرقة انواع البكتيريا الشعاعية و ٨٠% من انواع البكتيريا الاخرى كما أظهرت النتائج ان من جملة ٢٠ بكتيريا الشعاعية،

٣ (١٥ %) من أنواع بكتيريا الشعاعية (المتسلسلة الفطرية) و ١٧ (٨٥ %) من نوع بكتيريا النوكارديا والشبيهة بالنوكارديا .

خلصت الدراسة الى ارتفاع معدل نسبة البكتيريا الشعاعية وسط مرضى الورم الشعاعى، ونسبة لعزل عدد من البكتيريا المسببة لهذا المرض فى مناطق توطن المرضى ممايدل على أن التربة تمثل مصدرا لمرض الورم الشعى.

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APPENDIX I

Bacteriological Media

Tryptic soya agar

Tryptic soya agar, 30 g; distilled water, 1 litter; pH 7.2 autoclaved at 121°C for 15 minutes.

Glucose Yeast Extract Agar GYEA

Glucose 10g, yeast 10g, agar 14g, distilled water, 1litre; pH 6 to 8 autoclaved at 15psl for 15 min.

Lowenstein-Jensen media (L.J. media)

Composition of L.J media

Mineral salt solution

Potassium dihydrogen phosphate($K_2H_2PO_4$ 2.4g, Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)0.24g, Magnesium citrate 0.6g, Asparagines3.6g, Glycerol12ml, Distilled water 600ml, Malachite green0.4g

Starch medium agar

Starch 10 gm, nutrient agar (oxoid) 28 gm, 1 liter distilled water sterilized by autoclaving at 121°C for 15 minutes.

Tyrosine medium

L Tyrosine, 4 gm, nutrient agar (oxoid) 28 gm, 1 liter distilled water sterilized by autoclaved at 121°C for 15 minutes.

Xanthine medium

Xanthine, 4 gm, nutrient agar (oxoid) CM3 28 gm, 1 liter distilled water sterilized by autoclaved at 121°C for 15 minutes.

Casein medium

100 ml of skimmed milk powder (10% W/V, oxoid) was autoclaved at 121°C for 15 minutes, and then was added to sterile molten GYEA to give a final concentration of 10% W/V.

Urea medium

4 gm urea agar base, 95 ml of distilled water was sterilized by autoclaving at 121°C for 15 minutes, and then 2gms of urea crystals were added to 5ml distilled water. The preparation was dispersed in a bottle and allowed to set in slope position.

Peptone water sugars

The reaction of 900 ml peptone water was adjusted to pH 7.1-7.3 so that the addition of 10 ml of Andrade's indicator will bring it to pH 7.5 the preparation was then sterilized by autoclaving at 121°C for 20 minutes.

This medium is pink when it is hot but the color fades out by cooling. Then 5-10 gms of appropriate sugar was dissolved in 90 ml of distilled water and steamed for 30 minutes. The sterilized peptone water with Andrade's indicator has a pink color in acid medium (Salicin, Sorbitol, Mannitol, Rhaminose).

Appendix II

Stains

1. Modified Ziehl –Neelson Stain (ZN stain)

(A)ZN carbol fuchsine

Basic fuchsine (powder)	10 g
Phenol (crystalline)	50 g
Alcohol (95% or 100% ethanol)	
Distilled water	1000mL

Dissolve the fuchsine in phenol by placing them in 1L flask over boiling water. Bass for about 1mint, shaking the content from time

to time when solution complete add the Alcohol and mix thoroughly.

Then add the distilled water. Filter the mixture before use.

(B) Acid Alcohol decolorize

Concentrated hydrochloric acid (HCL)	75ml
Industrial methylated spirit	2425ml

Pour the methylaed spirit in tow large flask. Place the flask 5-8 cm of cold water in the sink, add the HCL and cover the top of the flask to top fumed escaping. Leave for 10 minutes. Decant into a labeled bottle for use. The final concentration of HCL is 3%.

(C) Methylene counter stain

1% stock solution	40 ml
Distilled water	360 ml

Add the dye diluted to make the working solution to the distilled water in a bottle and shake to dissolve.

2. Gram 's stain

(A) Crystal violet stain

To prepare 1 liter

Crystal violet	29g
Ammonium oxalate	9g
Ethanol or methanol, absolute	95 ml
Distilled water	to liter

Weight the crystal violet on a piece of clean filter paper. Transfer to a brown bottle Premark to hold 1 liter.

Add the absolute alcohol and mix until the dye is completely dissolved.

Weight the ammonium oxalate and dissolve in about 200 ml of distilled water, add to the stain. Make up to 1 liter mark with distilled water, and mix well.

Label the bottle and store it at room temperature. The stain is stable for several months.

(B) Logo's iodine

To prepare 1 liter:

Potassium iodide	20g
Iodine	10g
Distilled water	to 1 liter

Weight the potassium iodide, and transfer to a brown bottle premarket to hold 1 liter.

Add about a quarter of the volume of water and mix until the potassium iodide is completely dissolved.

Weight the iodine, and add to potassium iodide. Mix until iodine is dissolved.

Make up to 1 liter mark with distilled water, and mix well. Label the bottle and mark it toxic. Store in a dark place at room temperature.

(C) Acetone-alcohol decolorize

To prepare 1 liter:

Acetone	500 ml
Ethanol or methanol, absolute	475 ml
Distilled water	25 ml

Mix the distilled water with the absolute alcohol. transfer the solution to screw-cap bottle of 1 liter capacity.

Measure the acetone and add immediately to the alcohol solution, mix well.

Label the bottle, and mark it highly flammable. Store at room temperature.

(D) Neutral red, 1g/L

To make 1liter :

Neutral red	1g
Distilled water	1liter

Weight the neutral red on a piece of filter paper and transfer it to a bottle of 1liter capacity Add about quarter of the volume of water, and mix until the dye is completely dissolved Add the remainder of the water, and mix w

ell.Laebottle and store at room temperature. The stain is stable for several months.

Appendix III

Questionnaire Form

Patient Name

Age

Occupation.....

Location.....

Trig

Duration of the Disease

Type of Grain Discard

CHAPTER ONE
INTRODUCTION

CHAPTER TWO
LITERATURE REVIEW

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER FOUR

RESLUTS

CHAPTER FIVE

DISCUSSION

CONCLUSIONAND RECOMMENDATION

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