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THE USE OF MORPHOLOGICAL AND CELL WALL CHEMICAL MARKERS IN THE IDENTIFICATION OF STREPTOMYCES SPECIES ASSOCIATED WITH ACTINOMYCETOMA

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ABSTRACT
Most aerobic, filamentous, spore-forming *Actinomycetes* are saprophytes but some are considered pathogens of humans and animals, notable examples are the causal agents of mycetoma. The present study aimed to identify *Streptomyces* spp. isolated from actinomycetoma cases in Sudan by examining some morphological traits and analyzing the cell wall composition. Nineteen *Streptomyces* strains isolated from purulent materials of patients with mycetoma (human) or fistulous withers (donkeys) were included in the study. Isolates were tentatively identified as *Streptomyces* species based on morphological and cultural characteristics. Cell wall analysis of isolates yielded LL-diaminopimelic acid (LL-DAP) which authenticates that the isolates are members of genus *Streptomyces*. The isolates, though they are *Streptomyces*, but are variable phenotypes. The study concluded that using few selected criteria, as above, would allow identification of unknown actinomycetoma agent to the genus level. The study also assumes that apparently limitless, numbers of saprophytic *Streptomyces* enter human or animal skin tissue causing actinomycetoma and perhaps other complications in man and animals.

KEYWORDS: Actinomycetoma, *Streptomyces* species, Madura foot, Sudan

INTRODUCTION

Mycetoma is a slow destructive infection of cutaneous and subcutaneous tissues, fascia and bone, caused by fungi (eumycetoma) and actinomycetes (actinomycetoma). It is mainly prevalent in tropical rural areas in a belt that matches the *Acacia* belt in Africa, India, Central and South America (1, 2, 3). Mycetoma is a major health problem in Sudan notably among rural workers, particularly male farmers, peasant and shepherds. Thorns from *Acacia nilotica* and other tropical trees, which grow in most parts of tropical Africa, poses serious threat to health by predisposing to mycetoma through direct inoculation of contaminated soil and plant debris to skin.

Actinomycetoma is reportedly caused by *Actinomadura madurae, A. pelletieri, Nocardia brasiliensis, N. otitidiscaviarum, N. transvalensis, Streptomyces sudanesnsis* and *S. somaliensis* (4, 5). Most mycetoma cases in Sudan are attributed to *S. somaliensis* (1, 6) and *S. sudanesnsis* (5). Currently, the genus *Streptomyces* includes over 500 validly described species (7, www.ncbi.nlm.nih.gov/Taxonomy/). They form an integral part of soil microbial communities and making up approximately 10% of total soil microbial flora (8). The majority of research focused on the classification of these saprophytic strains (9, 10), albeit the genus contains few human and plant pathogens (4, 11). *Streptomyces* species are causal agents of diseases in man (*S. somaliensis* and *S. sudanesnsis);* animals (*Streptomyces* species) and plants *(Streptomyces scabies)* (6, 12, 13). The cultural and microscopic features of genus *Streptomyces,* which are commonly used for routine identification, include aerobic growth, gram-positive, non-acid-alcohol-fast, non-motile *Actinomycete* which forms extensively branched, light yellow substrate mycelia on a variety of media with or without aerial hyphae, with or without diffusible pigments on medium surface (7, 14). Cell wall components of *Actinomycetes* enable rapid qualitative identification of certain *Actinomycetes*. Such outcome has been believed as "completely satisfactory" (15, 16).

The present study was aimed to investigate some growth and morphological features and chemical markers for the identification of *Streptomyces* species isolated from patients with mycetoma and fistulous withers in Sudan.

MATERIALS AND METHODS

Clinical specimens

Purulent material (0.5 mL) was collected by needle aspiration from unopened parts of lesions from donkeys with fistulous withers. In case of human mycetoma, grains were taken from deep excision biopsy material of patients, stored in sterile containers and transported to the laboratory where they were either kept on ice for up to 24 hours or used immediately.

Isolation of *Streptomyces* species

Clinical specimens (needle aspirates, grains) were used to inoculate Tryptic Soy agar (TSA; Difco) plates which had been incubated at 37°C for up to two weeks. Plates were examined daily until *Streptomycete*-like colonies were seen, the latter were subcultured onto fresh TSA agar plates which were incubated at 30°C for up to 14 days to allow better morphological observation.

Nineteen (n = 19) *Streptomyces* strains have been isolated between 1998 and 2003 from various parts of Sudan from cases of actinomycetoma in human (madura foot) and actinomycetoma in donkeys (fistulous withers). In this study bacteriological and chemotaxonomic characterization was completed on the isolated *Streptomyces* strains as part of a project that had completed some parts **(**5, 17, 18**)** and other part are underway.

Strains

The 19 *Streptomyces* strains are labeled as *S. somaliensis* DSM 40738^T, *S. sudanensis* DSM 41923^T (SD504), D501, SD509, DSM41607, *Streptomyces* spp.: SD511, SD524, SD528, SD534 and DSM40760 (human isolates); SD551, SD552, SD559, SD572, SD573, SD574, SD575, SD576, SD579 (donkey isolates) and *S. somaliensis* DSM 40738T, *S. sudanensis* DSM 41923^T, SD509, DSM41607, DSM41608, DSM41609, *Streptomyces* spp.: SD511, SD524, SD528, SD534 and DSM40760 (human isolates). *S. somaliensis* DSM 40738T and . *sudanensis* DSM 41923^T served as controls.

Morphological characterization

Isolates were tentatively identified as member of genus *Streptomyces* based on selected morphologic criteria (7, 14). The clusters of the isolates were recognized based on colony color, substrate and aerial mycelia and the presence of diffusible pigments on TSA media.

Cell wall analysis

Biomass for chemotaxonomic studies was prepared by growing each strain for 2 weeks at 30°C in a 100 ml shake flask containing 25 ml of trypticase soy broth (Difco). The isolates were examined for the presence of the isomers of diaminopimelic acid (DAP) in whole-organism hydrolysates by thin-layer chromatography (TLC) of whole-organism hydrolysates following the procedure described by Staneck and Roberts (19). A standard solution (10 mM) of A_2pm (Sigma) containing a mixture of LL- and *meso*-DAP isomers was used as a reference. The following markers were also used to control the TLC analysis: *S. sudanesnsis* (DSM 41923^T (SD504) as it reveals LL-DAP; *Nocardia farcinica* ATCC 3318 which reveals *meso*-DAP and *Dermatophilus congolensis* DSM 44180 which reveals neither LL-DAP nor *meso*-DAP (19).

RESULTS

The isolates recovered from human and donkey's actinomycetoma cases exhibited different phenotypic features. The initial identification of isolates to cluster and phenotypic groups was done according to growth and colony features characteristics and microscopic appearance (Table 1). The isolates revealed colony morphology of various forms and colors that ranged from grey to blue to grey brown or grey white colonies (Fig. 1).

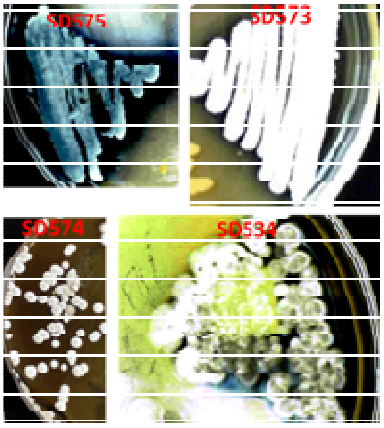

FIGURE 1. Growth of *Streptomyces* spp. isolated from actinomycetoma cases showing variations in colony morphology which ranged from grey to blue to grey brown or grey white in color.

These different phenotypic features triggered further studies so as to recognize new species among them. Overall, these isolates had common shared properties of *Streptomyces* i.e. these were aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycete that formed extensively branched substrate mycelium on standard media (Fig. 2). The resultant analyzed data revealed that most of the isolates were distinct from both *S. sudanensis* and *S. somaliensis*. These results are in line with the known description of *Streptomyces* spp. (4, 7).

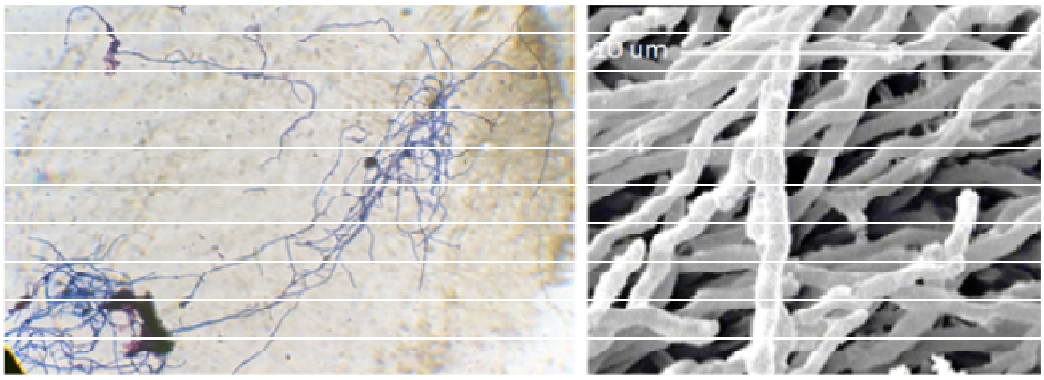

FIGURE 2. Microscopic features of isolated *Streptomyces* sp. (SD574) (left) and scanning electron micrograph of *Streptomyces* sp. (SD509) (right). The organism are gram-positive, non-acid-alcohol-fast, forms extensively branched mycelia that are none fragmenting.

In TLC analysis, all the strains were found to contain LL-DAP similar in chromatographic behavior to that produced by the marker species *S. sudanesnsis* (Fig. 3). Such chemical markers strongly support the identification of the isolates as members of the genus *Streptomyces* and in accordance with standard descriptions of the genus (7).

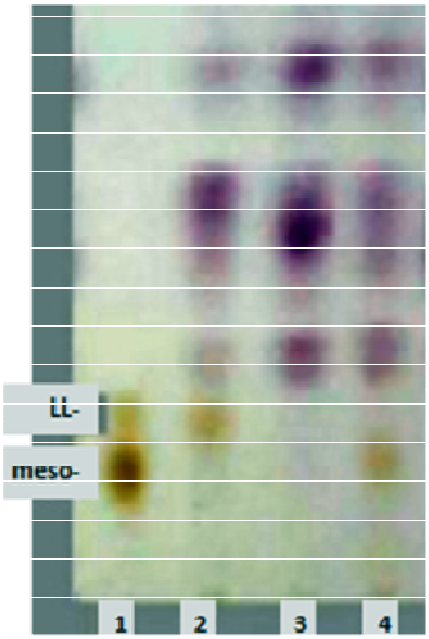

FIGURE 3. TLC analysis of whole cell hydrolysate of Streptomyces isolates. All test strains contain LL- A2pm (lane 2) similar in chromatographic behavior to that produced by the marker strain (lane 1) but distinct from Nocardia farcinica (lane 4) and the negative control (Dermatophilus congolensis; lane 3)

DISCUSSION

The isolated organisms were tentatively identified as *Streptomyces* specie on the basis of culture-morphological characteristics (Fig. 1 and 2). Nevertheless, a good level of support to this initial identification was achieved with the analysis of cell wall diaminopimelic acids, namely LL- and *meso*-DAP. This

chemotaxonomic feature is a robust technique in differentiating *Streptomyces* from *Nocardia* species and from many other species within the order *Actinomycetales* of the phylum *Actinobacteria* (7, 14).

TABLE 1. MORPHOLOGICAL CHARACTERISTICS OF *STREPTOMYCES* SPP. (N= 19) ISOLATED FROM HUMAN AND DONKEY

Species	Strain code	Colony colour	Reverse colony colour	Aerial hyphae
Streptomyces sudanensis (n = 4)	DSM 41923^T (SD504), D501, SD509, DSM41607	Light gray	Light yellow	No aerial hyphae
Streptomyces somaliensis (n = 1)	DSM 40738^T	Light gray	Light yellow	No aerial hyphae
Streptomyces isolates (n = 14)	SD 511	Light gray	Light yellow	No aerial hyphae
	SD 534	White	Yellow	White aerial hyphae
	SD 528	Light gray	Colorless	No aerial hyphae
	SD524	Light gray	Medium red brown	Light gray aerial hyphae
	DSM 40760	White	Light yellow brown	White aerial hyphae
	SD 551	Medium gray	Light brown gray	Medium gray aerial hyphae
	SD 552	Light gray	Light gray yellow brown	Light gray aerial hyphae
	SD 559	Light gray brown	Brown gray	Light gray brown aerial hyphae
	SD 572	White	Buff	White aerial hyphae
	SD 573	White	Medium yellow brown	White aerial hyphae
	SD 574	White	Medium yellow brown	White aerial hyphae
	SD 575	Light green gray	Gray green	Light green gray aerial hyphae
	SD 576	White	Buff	White aerial hyphae
	SD 579	White	Medium yellow brown	White aerial hyphae

Abbreviations: T, type strain; DSM, Deutsche Sammlung von ikroorganismen; Inhoffenstraße 7B, 38124 Braunschweig, Germany

Donkey's fistulous withers and human mycetoma share some pathological and ecological attributes. However, a question remained to be answered: why the infection mainly affects man and donkeys? Some isolates from these lesions have been previously identified as *Streptomyces* (5, 17, 19).

The 16S rDNA gene sequence analysis of some strains analyzed so far confirmed that the isolates falls within the phylogenetic clade, which encompasses the genus *Streptomyces* (data not shown). Studies are underway to further describe these bacteria and assign names to them. This report represents a good evidence to further implicate *Streptomyces* in the etiology of fistulous withers in donkeys and increases the rate of *Streptomyces* spp. as causal agents of actinomycetoma in Sudan (6).

Soil saprophytes cause considerable health hazard as demonstrated by a significant, apparently limitless, number of saprophytic phenotypes of *Streptomyces*. These *Streptomyces* spp. enter human or animal skin tissue through traumatic injuries, cause actinomycetoma and perhaps other complications in man and animals. DNA-DNA pairing and further phenotypic characterization of these isolates may enable descriptions of new species. This paper has achieved the view of seeking and endorsing the development of simple diagnostic appraoches especially in low income coutries or in laboratory with limited resources.

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