Pulmonary Nocardiosis; Similarity to Tuberculosis (A Bacteriological and Proteomics Study)

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ABSTRACT

Objective: The aims of the present study were to decide the occurrence of nocardia spp. among Sudanese patients suspected with tuberculosis and to investigate all proteins expressed by the genome of Nocardia africana (formerly isolated from patients with pulmonary infection misdiagnosed as MDR and their structures and functions compared to Mycobacterium tuberculosis.

Materials and Methods: Three hundred and twenty-nine patients, presented with pulmonary infection were included in this study. Those patients were examined for the presence of acid- fast bacilli. Two tubes of Lowenstein- Jensen (L.J) medium were inoculated with 20 µl of the neutralized sputum sample. All cultures were incubated at 37°C for 8 weeks before being discarded. Phenotypic characterizations were performed. For nocardia proteom poly acrylamide gele lectrophoresis (PAGE)-based analyses of the four nocardia strains N. farcinica SD1828, N. africana SD 925, and N. asteroides N317 are discussed. In-gel tryptic digestion of these isolates was also performed, then the resulting peptides were introduced to MALDI-TOF peptide mass fingerprints were searched using MASCOT software.

Results: Ten isolates showed rapid growth pattern within 2-3 days after inoculation, further conventional methods suggested that all these isolates were belonging to the family nocardiacea. Two Dimentional Poly Acrylamide Gel Electrophoresis (2D-PAGE) using pH strips 3-10 revealed that the soluble proteins were visible in a much smaller pI range. All strains exhibited similar protein distributions. A similarity analysis revealed that mycobacterium sequences are of high relevance for the investigated strains.

Conclusions: Nocardia revealed considerable occurrence among patients with pulmonary infections (3.3%) giving clinical symptoms similar to those occur by M. tuberculosis infection, this may be due to similarities in functional proteins expressed by their genomes. This finding suggested that pulmonary nocardiosis might occur in patients who suffer from chronic lung disease in Sudan. It is important, therefore, that clinicians in Chest Units should consider this condition, especially when patients with respiratory infections fail to respond to antitubercular therapy.

Nocardiae are Gram-positive aerobic actinomycetes, which are predominantly saprophytic (Orchard, 1981) but also include species forming parasitic association with animals and plants (Goodfellow, 1992). They are in the same family as clinically and industrially important genera such as Mycobacterium, Streptomyces,
Corynebacterium and Rhodococcus and they are known to cause a variety of infections in humans and animals. Nocardiae cause a variety of suppurative infections of humans and animals (Ishikawa, 2004; Mogahid et al., 2007). Human infections may be distinguished clinically into cutaneous, subcutaneous, and lymphocutaneous nocardiosis; extrapulmonary nocardiosis; pulmonary nocardiosis; and systemic nocardiosis involving two or more body sites. The incidence of such infections is not known, although nocardiosis has been reported in most regions of the world. Nocardial infections of the internal organs in nontropical countries are mainly caused by Nocardia asteroides, N. farcinica, and N. nova; relatively few are caused by N. brasiliensis, N. otitidiscaviarum, N. pseudobrasiliensis, and N. transvalensis. There have been isolated reports of pulmonary nocardiosis from tropical countries caused by N. asteroides, N. brasiliensis, N. farcinica, N. otitidiscaviarum, and N. transvalensis, (Koltzscher et al., 2003; Bauer et al., 1966; Gang et al., 2005). According to information from the Center of Disease Control and prevention 80% of cases present as invasive pulmonary infection, disseminated disease, or brain abscess, and 20% present as cellulitis. Nocardia asteroides causes at least 50% of invasive infections. In the United States, estimated 500-1,000 new cases of nocardia infection occur annually. The number of cases has increased with the overall rise in the number of severely immunocompromised persons. Diagnosis is a major challenge. N. farcinica frequently is resistant to antimicrobial agents, including the drug of choice trimethoprim-sulfamethoxazole, and has been demonstrated to be more virulent in an animal model. A new combination drug therapy (sulfonamide, ceftriaxone, and amikacin) has shown promise for infections difficult to treat. The hope is that the application of newer molecular diagnostic and subtyping methods may assist in earlier diagnosis and outbreak investigation (www.cd.gov).

Recently, proteomics have been introduced as a new field of research for ultra study of protein structure and function. Proteomics is the study of all proteins expressed by a genome. It involves the identification of proteins in the body and the determination of their role in physiological and pathophysiological functions. By proteomics, many new proteins have been identified in different microorganisms and their functions have been well understood (Cutler et al., 1999, Gorg et al., 2000).

Two-dimensional (2-D) gel electrophoresis of pulse-labeled proteins revealed a global analysis of protein synthesis and turnover in Escherichia coli (Weichart et al., 2003). Comparative proteomics of the human pathogen Campylobacter jejuni revealed an important first step in characterizing strain differences potentially responsible for different disease outcomes associated with this organism (Brubacher et al., 2003). Several studies subjected Mycobacterium tuberculosis to comprehensive proteomic analysis. Culture supernatant proteins of virulent Mycobacterium tuberculosis H37Rv and attenuated Mycobacterium bovis BCG were comprehensively analyzed using proteomics techniques (Mattow et al., 2001). In another study, proteomics revealed open reading frames in Mycobacterium tuberculosis H37Rv not predicted by genomics (Peter et al., 2003). This clearly showed that, analysis of proteins directly may come up with new knowledge which may completely alter the approaches to disease diagnosis, control and treatment. Definitive diagnosis of nocardiosis depends on the isolation and identification of the causative organism from clinical materials. These procedures are not straightforward; hence the true incidence of the disease is masked, a problem which is compounded by poor documentation. Generally, nocardia cases are difficult to diagnose (Garcia-Benitez et al., 2001).
MATERIALS AND METHODS

Collection of the Samples

Three hundred and twenty-nine patients, who were attending Abu-Anga Teaching Hospital, El-Shaab Teaching Hospital and the National Health Laboratory, in the Sudan during the period from October 2004 to January 2006, were examined for the presence of acid-fast bacilli. They were suspected of having tuberculosis infection according to the symptoms. Most of the patients had either not responded to treatment with antitubercular drugs or had responded and then relapsed. Sputum samples were collected according to WHO criteria in sterile, plastic wide-mouthed, strong leak-proof containers. Following treatment with the digestion-decontamination procedure of Roberts et al., 1991, the sputum samples were concentrated by centrifugation and the resultant preparations were used to inoculate Lowenstein-Jensen (LJ) slopes, which were incubated at 37°C for 14 days and then used to make smears, which were examined with a standard Ziehl-Neelsen acid-fast stain.

Phenotypic Characterization

Ten of the LJ slopes supported the growth of small orange filamentous colonies, which were considered to be typical of nocardiae. The isolates, which were designated SD1001, SD1002, SD1002, SD1003, SD1004, SD1005, SD1006, SD1007, SD1008, SD1009 and SD1010, were subcultured and maintained on glucose-yeast extract agar (GYEA) slopes at room temperature. The ten isolates were examined for a range of phenotypic properties described by Isik et al., 1999. Standard procedures were also used for the extraction and analysis of mycolic acids as described by Minnikin, et al., 1975 and Nocardia africana strains used as controls.

Preparation on Nocardia Lysate

The test strains were cultivated on GYEA medium (Appendix II) for 2-3 days at 37°C then checked for purity. Nocardia species were then harvested, inactivated in the water path at 90°C for one hour and the cells were washed with sterile phosphate buffer saline (P.B.S) and then centrifuged at 15000 rpm for 5 minutes. Supernatants were discarded and the pellet was washed by resuspending in MilliQ water and centrifuged again at 10000 rpm for 10 minutes, and the supernatant was discarded.

Then, 1ml of urea lysis buffer 8 M (Appendix IV) was added to each sample in eppendorf tubes in addition to 100 µl of protease inhibitor (CALBIOCHEM, Cat No 539134), 20 µl of (500 U) benzonase (Novagen Lot N 62211-1) and 0.5gm of silica beads (0.1mm, Cat No 11079101 Z- Biospec products, Inc). The mixture was then vortexed for one minute (Vortex Genie 2 Model G.560E Bohemia USA).

Samples were then lysed by freezing in liquid nitrogen and thawing in 37oC in waterpath, this step was repeated for 5 times. Samples were then put on a shaker (Type MM 300 cat No 85720 Gmbh and co KG, Germany) with a frequency of 3000 per minute for 30 minutes. Step of freeze-thawing was repeated again for other 5 times and the shaking step was also repeated as previously described. Samples were then spin down at 15000 rpm for 5 minutes, supernatant (which contain the soluble proteins) was collected and protein concentration was measured with spectrophotometer (Model 8024-0600, From Pharmacia Biotech, England).

Two Dimensional Gel Electrophoresis

Seventy (70) µg of protein were loaded on immobilized pH gradients (IPG) dry Strip (pH 4-7, 7 cm) together with IPG buffer (pH 3-10) and 20 mM DTT, overnight passive rehydration was performed for the dry strip then Ettan I PG phor (Amersham Bioscience) was used to a achieve First dimension Iso Electrical Focusing. A second dimension SDS – PAGE
was done by using Hofer miniv E8 ×9 cm gels device according to Laemmli.1970

**Tryptic Digestion and Mass Spectrometric Sequencing**

In-solution tryptic digestion of nocardia isolates lysate was also performed; a total of 4μl (containing approximately 250 μg) of the lysate was dry with vacuum centrifuge, the dry protein was redissolved in 250 μl of 8 M urea, 0.4 M NaHCO₃ followed by 25μl of 45 mM dithiothereitol the mixture was incubated at 50 °C for 15 minutes, 25 μL of 100 mM Iodoactamide were added and the mixture was incubated at ambient temperature in darkness for additional 15 minutes, 700 μL of deionized water was added together with 5% (w/w) trypsin (12.5 μg). The digestion was performed at 37° C overnight in darkness. Resulting peptides were then desalted by using Zip Tip column (microbed C₁₈; Millipore, USA), then the peptides were introduced to mass spectrophotometer (QSTAR from Applied Biosystem), peptide mass fingerprints were searched using MASCOT software.

**RESULTS**

**Bacteriology Results**

All positive smears showed positive growth on LJ medium after 2-21 days post inoculation. The colonial morphology of 319 (97%) appeared as rough, friable, warty, granular and grey in color with irregular margins and showed the appearance of AFB when stained again (indirect smear) with ZN staining procedure for more confirmation. The 319 isolates were initially identified as members of the *Mycobacterium tuberculosis* complex.

Ten (3%) of the LJ slopes reveled the growth of small orange filamentous colonies, which were tentatively considered to be nocardiae (Fig. 1).

The 10 nocardia isolates, which were then designated SD1001, SD1002, SD1003, SD1004, SD1005, SD1006, SD1007, SD1008, SD1009 and SD1010 were subcultured and maintained on glucose-yeast extract agar (GYEA) slopes at room temperature and as suspensions of mycelial fragments in glycerol (20% [vol/vol]) at −70°C. The 10 isolates were studied phenotypically.

**Biochemical tests for nocardia isolates**

Selected biochemical tests were performed. The result for these tests showed that all the strains utilize glucose by oxidation pathway and that they were all catalase positive and also positive for urea. Concerning growth at 45°C, 7 out of the ten were positive (70%). Only 2 (20%) were positive for manitol and rhaminose as well as starch whilst all the isolates were negative for xanthine, casein, tyrosine, sorbitol, arabinose and citrate. Regarding mycolic acids, all tested isolates showed the standard patterns of mycolic acid components using thin layer chromatographic technique. The tested strains were found to have phenotypic properties typical of members of the genus nocardia (Figs 1 and 2).
Proteomic Results

The results of 2-D gel electrophoresis revealed a total of hundreds of protein spots for nocardia lysate. The molecular weights range between 25-75 kDa and the Iso-electric point (pI) of these range between 4–7 (Figs 3 and 4).

On the other hand, different peptides were detected by mass spectrometer. Alignment of those peptides was performed on Swiss-Prot. The resulting data revealed highly significant homology with *Mycobacterium tuberculosis* (set of Table 1).

**CONCLUSION**

A relatively large sample size (329) was analyzed in this study compared with previous studies in Sudan conducted in the same field and nocardia species has constituted 3% of all isolate. A significant resistant pattern (40%) were observed through these ten (10) isolates, hence more attention should be drawn towards patients who did not respond to anti-tuberculosis therapy, as other pathogens, including *Nocardia* spp. may be the cause of the infection.

Hundreds of nocardia protein spots were captured on stained gel. Some proteins were identified in a gel digest using Q-STAR instrument analysis of the results showed significant similarities between nocardia and *Mycobacterium tuberculosis*; this may provide evidences for best understanding of the pathogenesis and increase the knowledge with respect to disease treatment and vaccination.
Table 1: Elongation factor Tu of Mycobacterium leprae EFTU_MYCLE (P30768)

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### Table 1/2: Chaperone protein dnaK, HSP70 of *Mycobacterium paratuberculosis* DNAK_MYCPA (Q00488)

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Table 1/3: 60 kDa chaperonin 1 of *N. farcinica* CH601_NOCFA (Q5Z1F9)

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<td>Mycobacterium sp. strain KMS</td>
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<td>-9MCCO</td>
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<td>Mycobacterium sp. 335-409</td>
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<td>CH60_NOCAS</td>
<td>Q9AFC5</td>
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<td>MYCMIR</td>
<td>Q0G5X0</td>
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Sequence comparison CH602_NOCFA and CH60_NOCAS and CH602_MYCPA

Query: 1 MAKTAYDEARRGLERGLNSLADAVKVTLGPKGRNVVLEKKGAPITGNDGVSIACHEI6E 23
MAKTAYDEARRGLERGLNSLADAVKTGPKGRNVVLEKKGAPITGNDGVSIACHEI6E

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Query: 2

Sbjct: 2 AKTYDEARRGLERGLNSLADAVKTGPKGRNVVLEKKGAPITGNDGVSIACHEI6E

Query: 61  LEDPYKGIAELKVEAKXXXXXVVVVVVVVVVVVVVVVVVVVVNEGRLENVAAANPLGKLRGIE 120

Sbjct: 61  LEDPYKGIAELKVEAKXXXXXVVVVVVVVVVVVVVVVVVVVVNEGRLENVAAANPLGKLRGIE 120

Query: 62  AKTIAYDEEARRGLERGLNSLADAVKVTLGPKGRNVVLEKKGAPITGNDGVSIACHEI6E

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Query: 121  KAVEAVTAKLDTAKEVTEKIQAAAATAGISDAGSAGSEELIGEAEAMDKVKGVEGTVIESNFT 180

Sbjct: 121  KAVEAVTAKLDTAKEVTEKIQAAAATAGISDAGSAGSEELIGEAEAMDKVKGVEGTVIESNFT 180

Query: 122  AVEVTIELKGKEVETKDETTIVEGAGDAEAIKGRVAQIRTEIENSDSDYDR 181

Sbjct: 122  AVEVTIELKGKEVETKDETTIVEGAGDAEAIKGRVAQIRTEIENSDSDYDR 181

Query: 181  FGLQLELTEGMRDFKGYISGYFVTDFPDREEQAVLEDYPLLIGVSVSTKVDLLPLEKLQI 240

Sbjct: 181  FGLQLELTEGMRDFKGYISGYFVTDFPDREEQAVLEDYPLLIGVSVSTKVDLLPLEKLQI 240

Query: 182  EVGLQLELTEGMRDFKGYISGYFVTDFPDREEQAVLEDYPLLIGVSVSTKVDLLPLEKLQI 240

Sbjct: 182  EVGLQLELTEGMRDFKGYISGYFVTDFPDREEQAVLEDYPLLIGVSVSTKVDLLPLEKLQI 240

Query: 241  AGKPLLAIADVEGAESTLVLVNNKGFTKSVAVAKPGDFDDKQLADAILTGEVIS 300

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Query: 242  GKPLLAIADVEGAESTLVLVNNKGFTKSVAVAKPGDFDFKQLADAILTGEVIS 300

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Sbjct: 301  EEVGLSLETAGIELQGQARKVVTDEDDITIVEGAGDAEIAKGRVAQIRTEIENSDSDYDRE 360

Query: 302  EVGLSLETAGIELQGQARKVVTDEDDITIVEGAGDAEIAKGRVAQIRTEIENSDSDYDRE 360

Sbjct: 302  EVGLSLETAGIELQGQARKVVTDEDDITIVEGAGDAEIAKGRVAQIRTEIENSDSDYDRE 360

Query: 361  EKLQERLXTKAKDQGIDLGDEATGANIVRVALSAPLKKQIAFNAGLEPGVVAEKVSNLEAGHGL 420

Sbjct: 361  EKLQERLXTKAKDQGIDLGDEATGANIVRVALSAPLKKQIAFNAGLEPGVVAEKVSNLEAGHGL 420

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Sbjct: 481  EYEDLLAAGVADPKVTRSLQNAASIALLFTTEAVADKPEKAAPADGTDGMGMG 539

Query: 540  DF 541

Sbjct: 541  DF 542
Table 1/5: Transkriptase alpha chain of *N. farcinica* RPOA_NOCFA (Q5Z1K9)

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<th>Description</th>
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<th>Organism</th>
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<td>Q083E7</td>
<td>Rhodococcus sp. strain RHA1</td>
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<tr>
<td>_MYCSS</td>
<td>Q1BD08</td>
<td>Mycobacterium sp. strain MCS</td>
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<tr>
<td>_9MYCO</td>
<td>Q1TRC7</td>
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<td>Mycobacterium sp. JLS</td>
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<tr>
<td>_MYCFV</td>
<td>Q27E10</td>
<td>Mycobacterium flavescens PYR-GCK</td>
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<td>Mycobacterium vanbaalenii PYR-1</td>
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<td>RPOA_MYCO</td>
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<td>Q73S43</td>
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<tr>
<td>RPOA_MYCLE</td>
<td>Q9X798</td>
<td>Mycobacterium leprae</td>
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Table 1/6: Enolase of *N. farcinica* ENO_NOCFA (Q5YQ30)

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<th>Description</th>
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REFERENCES


