Evaluation of DNA Extraction Methods in the Detection of Nocardia spp Isolated from Soil and Milk Samples

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Background: Nocardiaspecies have a complex cell wall structure similar to that of mycobacteria, and the extraction of DNA from this bacterium is extremely difficult. Currently, to identify Nocardiaspecies particularly, it is essential to utilize molecular techniques. Amis: In the present study we investigated STET buffer (SodiumChloride – TRIS-EDTA – Triton) for extraction of Genomic DNA from 20 soil samples and from 300 milk samples of dairy animals to detect the presence of Nocardia and Nocardia-like species in these samples.

Materials and Methods: The study subjects included 300 milk samples and 20 soil samples from different farms in Khartoum State, which include HilatKoko, Ghandahar, Al SilateandAlmualeh. Distribution of the enrolled milk samples was as follows: 100 from goats, 100 from cow, 100 from sheep and 20 Soil samples all target samples were cultured on TSA and GYEA and then Gram’s stain and different biochemical testand Mycotic acid analysis were used for identification. Finally DNA extraction by SodiumChloride – TRIS-EDTA – Triton (STET) buffer and PCR products targeting 16S rRNA gene was carried out for all Nocardia and Nocardia-like isolates for sequencing in Coria.

Results: The animals included in the present study were found to be infected with Nocardia and Nocardia-like with different ratio: in soil 7 isolated (35%), goats 13 isolated (13%), cows 11 isolated (11%) and sheep 0 isolated (0%). Other pathogenic bacteria were also identified in milk and Soil samples included: 8% Dietzia from milk of cow, (3%) Rhodococcus from milk of goats and (1%) Mycobacteria and (5%) Dietzia in soil samples.

The extracted DNA had high molecular mass, and its concentration and purity was suitable when tested in 2% agarose gel, and using UV spectrophotometry. Amplification of different genes was successfully performed.

Conclusion: The Nocardial DNA was successfully detected in milk and soil samples using 16S rRNA sequence analysis. The extracted DNA had a high molecular weight in 2% agarose gel electrophoresis (Figure 1). The study concluded that using STET method was simple, fast, cost-effective, sensitive and highly reproducible for DNA extraction from Nocardia and Nocardia-like and there is no need for a skillful specialist.

Keywords: 16S rRNA, DNA extraction, Nocardia, PCR.

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I. Introduction

The genus Nocardia belongs to the family Nocardiaceae, and members of the genus are all aerobic, Gram-positive, modified acid fast and non-motile actinomycete that form filamentous branched cells which fragment into pleomorphic rod-shaped or coccoid elements, non-spore-forming(Kämpferet al., 2007 and Kachueiet al., 2012)catalase and urease positive bacteria that belong to Actinomycetes group (Zakariaet al., 2008) Members of the genus Nocardia cause many diseases, notably pulmonary infections in man and mastitis in animals (Brown and McNeil, 2003).

II. Materials and methods

Isolation methods: Milk samples were collected from cow sheep and goats. The udder of the animals were cleaned by disinfectant in a piece of cotton with alcohol (70%) then milk was collected in sterile containers, the milk samples were prepared by centrifugation, and the deposited part was used for culture.

Soil Selection Ten gram of soil (five gram from surface and five gram from below the surface), then mixed well 1 g of soil to 10 ml of sterile 25% (V/V) strength Ringer's solution. The 0.1 dilution was shaken for 30 minutes then was let to precipitate. 0.1 ml soil suspension on to TSA plates which containing 5µg/ml tetracycline and 50µg/ml nystatine. The plates were incubated at 37˚C and examined after 7, 14 and 21 days for the presence of Nocardia and Nocardia-like growth. Afterwards some of the Phenotypic characteristics were
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studies (Rodriguez et al., 2007 and Zhang et al., 2003) such as: Modified acid fast, Degradation tests (Casein, Starch Tyrosine and Xanthine), Sugars fermentation (Mannitol, Rhamnose, Sorbitol and Arabinose) and Anti-microbial Susceptibility Test and Analysis of mycotic acids (Barrow et al., 1993).

DNA extraction techniques
DNA Extraction by STET buffer
In this report we describe a simple protocol for DNA extraction from the Nocardia and Nocardia-like using Sodium Chloride – TRIS-EDTA – Triton (STET) buffer according to (Mill et al., 2000). Pure colonies were picked from nutrient agar plate and inoculated in 5 mL of Tryptic Soy Broth (TSB). The tube was incubated at 37°C and shaken until the turbidity of the bacterial suspension was adjusted to match 1.0 McFarland standard (approximately 3 x 108 bacterial cells). Bacterial suspension was pelleted via centrifugation at 13000 rpm for 5 min. The pellet was washed with sterile distilled water and re-suspended in 200 of μL STET buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 5% [v/v] Triton X100, pH 8.0), and the cell suspension was vortexed vigorously. The cell suspension was boiled at 100°C for 30 min and then centrifuged at 10000 rpm for 10 min. Supernatant fluid was transferred into a sterile Eppendorf tube. Subsequently, cold 95% ethanol was added to the supernatant and kept at −20°C for 60 min. After this stage, the solution was centrifuged at 13000 rpm for 10 min, the supernatant fluid was discarded, and DNA pellets were dried. DNA template was dissolved in 50μL of sterile distilled water and stored at −20°C until the PCR amplification. The extracted DNA had a high molecular weight in 2% agarose gel electrophoresis [Figure 1]. For PCR amplification, 5μL of PCR products were electrophoresed in 2% agarose gel at 70 V for 100 min in TBE buffer (Tris-HCl, Boric acid, EDTA), and stained with ethidium bromide (EtBr).

Recently, STET buffer solution has been used for other bacteria, including Lactobacillus species, Clostridium perfringens, and Listeria monocytogenes (Gevers et al., 2006 and Ahsani et al., 2010).

Polymerase Chain Reaction

1-Universal primers
Amplification was done in Eppendorf China thermal cycler (Appendix IV - Figure 26). The DNA amplifies were done using Maxime PCR Premix kit (iNTiRON, Korea) (Appendix V). The Polymerase Chain Reaction Technique (PCR) for 16s rRNA gene, was used 1μL Forward universal primers 243F5'GGATGAG CCCGC and 1μL Revers universal primers A3R5'- CCAGCC CCACC TTGAC -3' in 1μL of DNA template and mixed well in 17 μL of water for injection (WFI). The amplification conditions included three steps: heating at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 3 min; and the final extension at 72°C for 3 min (Chakravorty et al., 2007 and Reddy et al., 2009).

2-Specific primer for Nocardia spp
The amplification was done using CLASSIC K960 China thermal cycler (Appendix IV - Figure 25). DNA amplifies was done using Maxime PCR Premix kit (iNTiRON, Korea) (Appendix V). The PCR assay was carried out in a total volume of 20 μL of mixture containing 0.5 μL of each of the specific primers (1 μL), 2 μL of template DNA and 17 μL of water for injection (WFI). In Polymerases Chain Reaction assay was used 0.5μL for specific primers Forward NG1F5' ACCGACCCAAGGGG -3' and 0.5μL Revers primers NG2 R 5’ – GGTTGTAAA CCTCT TCGA -3’ in 1μL of DNA template and mixed well in 18 μL of water for injection (WFI). The amplification conditions included three steps: heating at 94°C for 3 min 35 cycles at denaturation 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 3 min, and the final extension at 72°C for 3 min according to (Brown et al., 2004).

III. Results and Discussion
Chromosomal DNA was extracted from 20 soil sample and 300 milk samples from dairy animals. Using this method, the extracted DNA gave an A260:A280 ratio and concentration of DNA was 141.35 μg/ml. The extracted DNA had a high molecular weight in 2% agarose gel electrophoresis [Figure 1]. PCR amplification of 16s rRNA gene (universal primer) regions yielded 639 bp and 999bp details were shown in [Figure 2]. The described method was simple, fast, cost-effective, sensitive, and highly reproducible for DNA extraction from Nocardia and Nocardia-like and there was no need for a skillful. This procedure was time-consuming and monotonous (Loeffelholz et al., 1989). Recently STET buffer solution has been used for other bacteria, including Lactobacillus species, Clostridium perfringens, and Listeria monocytogenes. The animals included in the present study were found to be infected with Nocardia and Nocardia-like with different ratios; in goats 13 isolated (13%), cows 11 isolated (11%), sheep 0 isolated (0%) and soil 7 isolated (35%). Ther pathogenic bacteria were also identified in milk and soil samples included (8%) Dietzia in milk of cow, (3%) Rhodococcus in milk of goats and (1%) Mycobacteria and (5%) Dietzia in soil samples.

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Figure 1: Nocardiaspp extracted DNA separated by 2% agarose gel.

Figure 2: PCR products separated by 2% agarose gel, M ladder (100 bp), c-ve negative control, Lane 1 positive control (999 bp) and Lane 2 sample (1000 bp)

References