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Evaluation of DNA Extraction Methods in the Detection of Nocardiaspp 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 STETbuffer(SoduimCholride –TRIS-EDTA –Triton)for extraction of Genomic DNA from 20 soil samples and from 300 milk samples of drily animalsto detect the presence of Nocardiaand Nocardia- like species in these samples.*

Materials and Methods: *The study subjects included 300milk samples and 20 soil samples from different farms in Khartoum State, which include HilatKoko, Ghandahar, Al SilateandAlmuaileh. 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 testsand Mycolic acid analysis were used for identification. Finally DNA extraction bySoduimCholride –TRIS-EDTA –Triton (STET) buffer and PCR products targeting 16SrRNAgene 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 Nocadial DNA was successfully detected in milk and soil samples using 16S rRNA sequence analysis.The extracted DNA had a high molecular weight in 2%agrose 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(Kämpfer*etal*., 2007 andKachuei*etal*., 2012)catalase and urease positive bacteria that belong to *Actinomycetes*group (Zakaria*etal*., 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*e and *Nocardia* –like growth.Afterwards some of the Phenotypic characteristics were

studied (Rodríguez *etal*., 2007 and Zhang*etal*., 2003)such as: Modified acid fast, Degradation tests (Casein, Starch Tyrosine and Xanthine,), Sugars fermentation(Mannitol , Rhaminose , Sorbitol and Arabinose) and Anti –microbial Susceptibility Test and Analysis of mycolic acids **(**Barrow *etal*.,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 usingSoduimCholride –TRIS-EDTA –Triton (STET) buffer according to(Millar*etal* ., 2000). Pure colonies were picked from nutrient agar plateand inoculated in 5mL of TrypticSoy 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 × 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 mMTris-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 ofsterile 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*etat*.,2006 and Ahsani*etal*., 2010).

Polymerase Chain Reaction

1-Universal primers

Amplification was done in Eppendorf China thermal cycler (Appendix IV- Figure 26). The DNA amplifies was done using Maxime PCR Premix kit (*I*-Taq) (iNtRON, Korea) (AppendixV). The Polymerase Chain Reaction Technique (PCR)for 16S rRNA gene, was used 1µLForward universal primers 243F5'GGATGAG CCCGC GGCC TA-'3 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 30sec, 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*etal*., 2007and Reddy *etal*., 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 (*I*-Taq) (iNtRON, 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' – GGTTGTAA CCTCT TCGA -3' in 1µL of DNA template and mixed well in 18 µL of water for injection (WFI).The amplification conditions included three step :heating 94ºC for 5 min 35 cycles at denaturation 94°C for 30s, annealing at 53°C for 30s, and extension at 72°C for 30 s and 5 min of final extension at 72°C according to (Brown*et.al* 2004).

III. Results and Discussion

Chromosomal DNA was extracted from 20 soil sample and 300 milk samples from drily 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%agraose gel electrophoresis [Figure 1].PCR amplification of 16s rRNA gene (universal primer)regions yielded 639 bp and 999bpdetails were shown in [Figure2].The described method was simple, fast, cost-effective, sensitive, and highly reproducible for DNA extraction from*Nocardia* and *Nocardia*-likeand there was no need for a skillful. This procedure was time –consuming and monotonous(Loeffelholz*etal*., 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 ratio; in goats 13 isolated (13%), cows 11 isolated (11%), sheep 0 isolated (0%) andsoil 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.

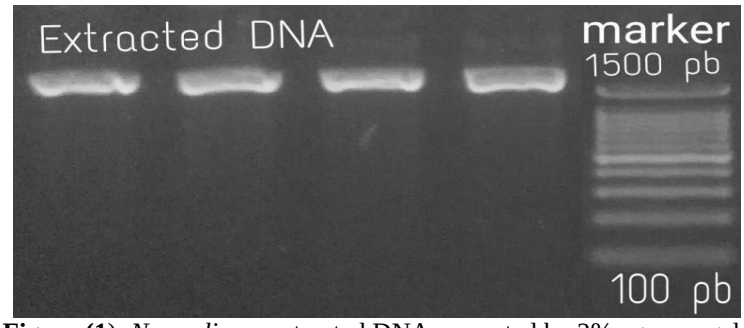

Figure (1): *Nocardia*sppextracted DNA separated by 2% agarose gel.

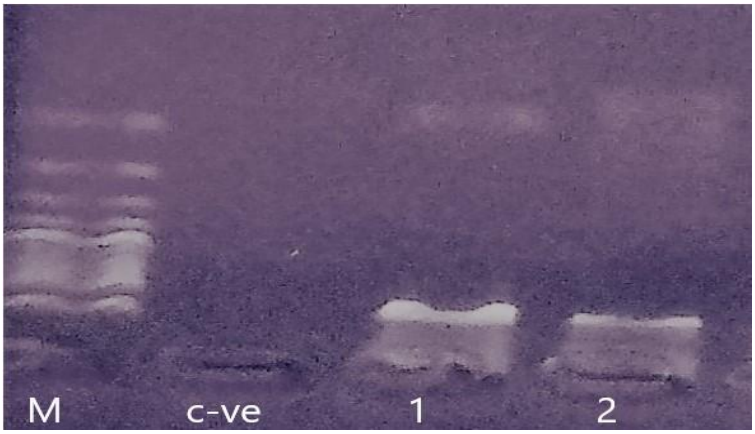

Figure 2:PCR products separated by 2% agarose gel, M ladder (100 bp), c-ve negative control, Lane1 positive control (999bp) and Lane 2 sample (1000 bp)

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