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Nocardial Mastitis among Dairy Animals in Khartoum State; Probability of Acquisition the Infection from Soil

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ABSTRACT

Introduction: *Nocardia* spp are widely distributed group of actinomycetes that occur in a wide range of manmade and natural habitat including activated sewage sludge, soil, water and tissues of plants and animals including human. *Nocardia* causes many diseases, notably pulmonary infections in man and mastitis in animals.

Methods: The study subjects included 300 milk samples and 20 soil samples from different farms in Khartoum State, which include Hilat Koko, Ghandahar, Al Silate and Almuaileh. Distribution of the enrolled milk samples was as follows; 100 from goats, 100 from cows and 100 from sheep. Soil samples distribution was as follows; All target samples were cultured on TSA and GYEA and then Gram's stain and different biochemical tests were used for further identification. Finally, PCR targeting *16Sr RNA* gene was carried out for all *Nocardia* isolates.

Results: Dairy animals included in the present study were found to be infected with *Nocardia* species with different ratio; goats 13/87 (14.9%%), cows 11/77 (14.2%) and sheep 0/60 (0%). Other pathogenic bacteria were also identified in milk, these included Streptococcus, Dietzia, Rhodococcus and Mycobacteria.

Our findings proved also the existence of *Nocardia* spp. among 35% (7/20) of the soil samples, three samples isolated from farms of Hilat Koko, one sample from Ghandahar, two samples from Al Silate and one samples from Al muaileh.

Conclusion

In the present study, the *Nocardia* species were isolated and identified from the soil and milk samples of dairy animals of the same farms by conventional and molecular methods. This finding strongly suggest that soil could be the possible source for the infection of farm animals.

INTRODUCTION

Mastitis is the inflammation of the mammary glands which is manifested by changes in chemical, physical and bacteriological properties in the nature of the milk resulting from pathological abnormality in the glandular tissue.

Mastitis is caused by different members of pathogenic bacteria such as *Mycoplasma* spp, *Staphylococcus* spp, *Streptococcus spp (*Blood, 1983) and recently, Nocardia (Goodfellow, 1998).

Nocardia species are Gram - positive, partial to week acid - fast, catalase positive, aerobic actinomycetes. *Nocardia* species have extensive branched hyphae, that tend to fragment into coccoi and rod elements (Goodfellow, 1998). *Nocardia* spp is a widely distributed group of actinomycetes (Goodfellow, 1998). Most *Nocardia* produce carotenoid-like pigment that result in colonies with various shades of orange, pink, red or yellow, brown or yellowish pigments (Goodfellow 1998). Colonies may be smooth or granular and irregular, wrinked or heaped. *Nocardia* causes many diseases, notably pulmonary infections in man as well as in animals. Moreover, several species cause mastitis in animals. For instant, *Nocardia asteroids* causes granulomatous mastitis in cattle, *Nocardia faranica* causes mastitis in cattle and goats, and *Nocardia ateriodes* causes granulomatous mastitis (Hamid and Goodfellow 1998).

MATERIALS AND METHODS

Study design

In this study, three hundred milk samples (from goats, sheep and cows) and twenty soil samples were collected from different sites in Khartoum state.

Milk samples were collected from goats, sheep and cows. In each case, the udder was cleaned by disinfectant using a piece of cotton, the milk samples were collected in sterile falcon tubes and transferred in ice for culture.

Regarding soil samples, five samples from each study area were collected. Ten gram were collected in each case (five gram from surface and five gram from below the surface), then mixed well and stored in sterile containers at room temp and then transported to the laboratory for culture.

Identification of isolates

Nocardia and *Nocardia*- like colonies from the primary cultures were purified by sub-culturing on Trypticase Soya Agar (TSA) and Glucose Yeast Extract Agar (GYEA) media. Isolates were identifies on the basis of colony morphology and confirmed by microscopic examination, biochemical reactions (urease, catalase, casein degradation, tyrosine and sugar fermentation) and mycolic acid profiles.

Phenotypic characterization

The members of the genus *Nocardia* are aerobic growth, catalase positive, non-motile Gram-positive and with slightly acid-fast branched vegetative hyphae, these hyphae often fragment on mechanical disruption into rod-shaped to cocco-bacilli, the cell wall of *Nocardia* contains mycolic acids.

Most *Nocardia* spp. produce carotenoid-like pigment that result in colonies with various shades of orange, pink, red or yellow. Colonies of Nocardia may be smooth or granular and irregular.

Analysis of Mycolic acids

In the present study, phenotypic clusters of mycolic acid profiles was detected after the extraction of mycolic acids and thin layer chromatography (TLC) analysis of extracted mycolates (Minnikin, 1988). The mycolic acid appears as a single spot and it was chromatographed with reference *Nocardia* spp. (positive control) and *Streptomyces* spp (Negative control).

DNA Extraction and PCR

For DNA extraction from the genus *Nocardia*, several colonies from the new growth of bacterial isolates on nutrient agar - were suspended in 5 ml of H_2O, then were boiled for 10 minutes and centrifuged at 14000 g for 10 minutes, supernatant was stored at -20°C as a template DNA stock. The purity of the extracted DNA was determined by running the DNA on 2% agarose gel.

Polymerase Chain Reaction

Universal primers

In Polymerase Chain Reaction targeting *16S rRNA* gene, the universal primer consists of Forward primer 243 F 5'-GGATGAGCCCGCGGCCTA-'3 and Reverse primer A3R 5'-CCAGCCCCACCTTGAC -'3. The reaction adopted in 25 µl volume containing 0.5µl of each primer with 1µl of DNA template, 18 µl master mix and 5µl of water for injection. The PCR machine was programmed as follows: heating for initial denaturation at 94ºC for 5 min 35 cycles of denaturation (94°C for 30 s), annealing (at 53°C for 30s), and extension (at 72°C for 30s) and 5 min of final extension at 72°C. The PCR products (900bp) were electrophoresed on 1% agarose gel.

Specific primer for *Nocardia* spp.

In Polymerases Chain Reaction, specific primers for *Nocardia* spp were used as follows; 0.5µl Forward primer NG1 F 5' ACCGACCCAAGGGG -3'and 0.5µl Reverse primer R NG2 5'–GGTTGTAACCTCTTCGA -3' in 1µL of DNA template and mixed well in 18 µl of master mix and 5 µl of water for injection (WFI) in sterile tube. The PCR program was as the following steps: heating 94ºC for 5 min 35 cycles of denaturation (94°C for 30 s), annealing (at 53°C for 30s), and extension (at 72°C for 30 s) and 5 min of final extension at 72°C. The PCR products (1500bp) were electrophoresed on 1% agarose gel.

RESULTS

A total of three hundred of milk sample and twenty sample of soil were collected from different farms in Khartoum stats. Among the present study of 13/87 isolates (14.9 %) of milk samples of goats, 11/77 isolates (14.2%) of milk samples of cows and 7/20 isolates (35%) of soil samples showed *Nocardia* and *Nocardia*- like, all significant growth was identified by Gram stain and Modified Ziehl–Neelsen (MZN) stain, all isolates were Gram's positive and some isolates were acid fast [Figure1].

The isolates were cultured on Tryptic Soya Agar (TSA) medium and showed different colonial morphology ranging from irregular, raised, dry, to moist, the pigment ranged from yellow, orange, to pink [Figure 2].

The isolates were also examined by thin layer chromatography to detect the presence of mycolic acid [Figure 3]. To identify more precisely, PCR was performed for 16S rRNA gene [Figure 4].

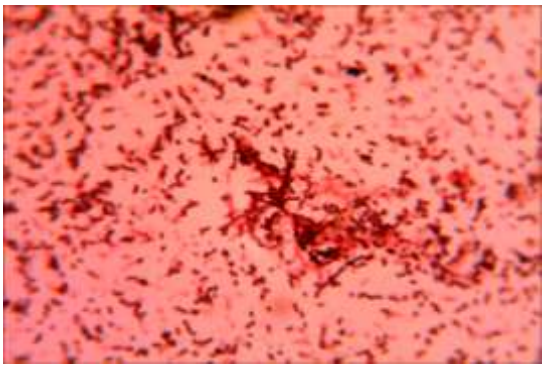

Fig. 1: Modified Ziehl –Neelsen stained of *Nocardia facinica* .Note the partial acid fast branching filamentous strain (x 100).

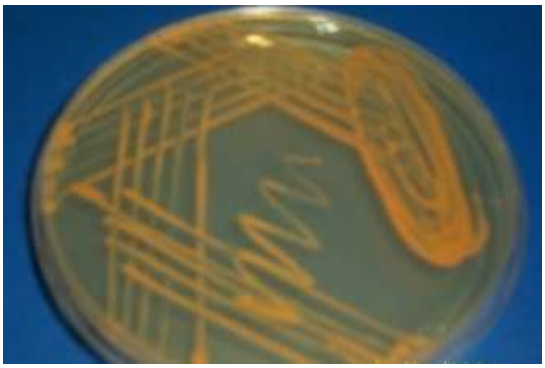

Fig. 2: Growth of *Nocardia Farcinica* on TSA medium after aerobic incubation at 37 ºC for 96 hours .Note the colonies shapes variation (orange, rough, smooth and extensive aerial.)

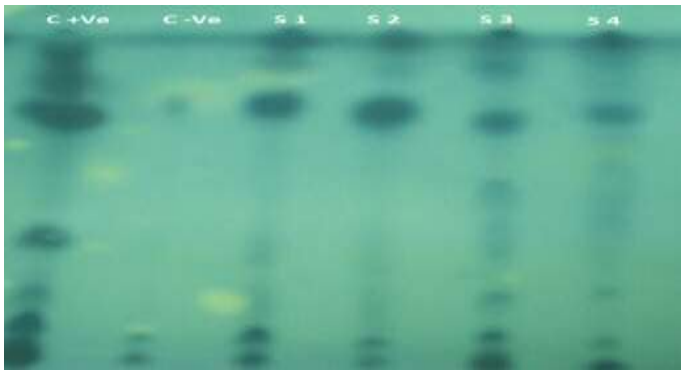

Fig. 3: Thin layer chromatography analysis of mycolic acids extracted from *Nocardia* isolate from cow and goats in (S1, S2 .S3 and S4) isolated (C + ve positive control) (C-ve negative control)

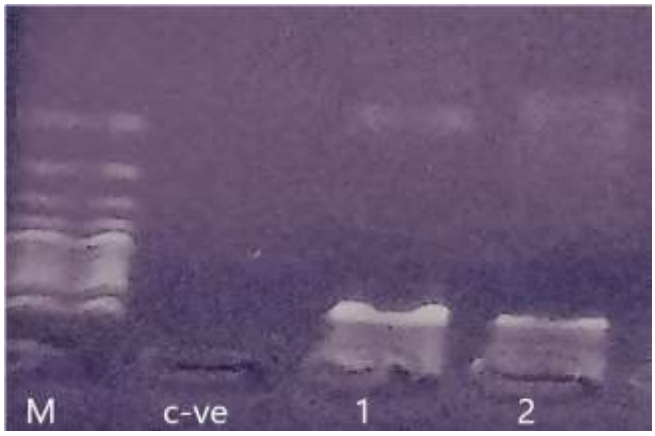

Fig. 4: M ladder 50 bp c-ve negative control: PCR products were separated by 2% agarose gel. of the 999- bp (lane 1) and 990 bp (lane 2).

DISCUSSION AND CONCLUSION

Mastitis is one of the major disease affecting dairy goats and cows. In the present study, the main objective was to determine the role of actinomycetes, mainly Nocardiae, in causing bovine and caprine mastitis. Little work was done in the Sudan regarding bovine mastitis due to actinomycetes infections (Shigidi and Mamoum, 1988; Hamid *et al*., 1998) .

Nocardia are widely distributed and abundant in soil (Good fellow, 1998). Isolation of *Nocardia* spp from Sudanese soil was firstly reported by Sid Ahmed (2001). One of the objectives of the present study was to find out the co-relation between soil actinomycetes and their clinical presentation among goats and cows with mastitis. The results revealed that among three hundred milk samples of goats, and cows, twenty four of them showed typical occurrence of *Nocardia*. Similar finding were shown by Maldonado (2004) who reported a number of cases of mastitis due to *Nocardiae* in 8% of goats' milk samples. On the other hand, among twenty soil samples, 35% of them (7 isolates) showed typical occurrence of *Nocardia*. Similar study was directed towards isolation and identification of *Nocardia* spp from soil samples collected from Khartoum state (Awatif, 2001). In this study, mastitis was caused by *Nocardia* spp in milk samples of cows (14.2°/o), in goats (14.9%) and isolated from soil samples (35%). some strains were confirmed to be *N*. *Farcinica* by analysis of the sequence of the gene 16SrDNA.

Most of the surveyed farms were small in size, so problem of disposal, ventilation and draining have been clearly observed. Moreover, most of the studied farms building material were traditional, made of mud and wood with old iron sheets for the doors. The uneven floor surfaces were clear hazards to the animals and were not suitable for cleaning and predispose for foot-rot infection. In addition, it was observed that, these traditional building materials cause injury to udder and teat of the dairy animals and hence predispose for mastitis occurrence. Hooves outgrowth and dirty grounds were common observation in much of these farms. Deep mud and excessive moisture in such barn yards increase coliform organism contaminating the udder (William, 1995).

In conclusion, Soil could be the major source of mastitis caused by Nocardia in the study area. Hygienic procedures in general are very important in controlling mastitis by ensuring a healthy environment. Moreover, milking management and milking technique have been shown to be important risk factors, with machine milking being more risky than hand milking and calf suckling (Hamann *et al*., 1991).

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