# CHAPTER ONE

# INTRODUCTION

Mastitis means the inflammation of the mammary glands. It is characterized by physical , chemical and bacteriological changes in the milk and by pathological changes in the glandular tissue. The most important changes in the milk include discoloration, the presence of clots and large numbers of leukocytes (Blood *et al* .,1983). Mastitis causes by different numbers of pathogenic bacteria such as *Mycoplasma spp* , *Staphylococcus spp, Streptococcus spp* ( Blood *et al*., 1983). Bacterial contamination of milk from affected quarter may pass for human consumption and provide a mechanism of spread of disease to human such as *Tuberculosis* ( Blood *et al*., 1983). *Nocardia spp* are widely distributed group of actinomycetes they occur in a wide range of man made and natural habitat including activated sewage sludge , soil, water and tissues of plants and animals including human (Goodfellow *et al*.,1998 ). Nocardia species are associated with opportunistic infections in human and animals that might become fatal. Anyway, some species can infect both immune-compromised and immune-competent individuals (Brown *et al*., 2006 ). 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 *et al* ., 2007 and Kachuei *et al*., 2012). *Nocardia* spp causes pulmonary nocardiosis in human (Abubakar *et al* 2017). *Nocardia* causes many diseases notably pulmonary infections in man *Nocardia* and causes mastitis in animals (Brown and McNeil ,2003).

Infection may occur by inhalation, contaminated wounds and traumatic implantation (Quinn *et al*.,1999). *Nocardia africana* was isolated from patients with pulmonary infection in Sudan ( Mogahid *et al*., 2005 and Hamid *et al*., 2001 ). *Nocardia faranica* causes mastitis in Goats in Sudan (Omyama *et al*., 2007 and Maldonado *et al*., 2004). *Nocardia faranica* causes mastitis in cattle (Hamid *et al*., 1998). In sheep *Nocardia* causes arthritis (Nclikuwera *et al*., 1990). *Nocardia brasillinsis* was isolated from various kind of infection , including *Mycetoma* of foot (Goodfellow and Lechevalier .1989 ). Various *Nocardia* infections were reported in humans after surgical operation by (Vandime *et al*., 2001).

The **aim** of this study was to isolation and characterization of *Nocardia* and *Nocardia* -like as causal agents of mastitis in cattle , goats and soil sample.

### 1.2 Hypothesis of the Study

PCR is a branch of molecular biology with promising provision understanding to pathogens and host-parasite relationship, may provide important information to our understanding of udder pathogens and actinomycetes pathogenicity in general. The isolates of *Nocardia spp* may be of similar patterns or different from each other.

### 1.3 Rationale

Mastitis became a major problem and has been declared by the government as an urgent priority for control. The best indicator of the extent of mastitis problem is the average of annual risk infection (ARI) which is the proportion of the cattle and sheep

that likely infected rapid. On the other hand Nocardiosis also becomes a main problem that increasing day by day and early diagnosis and treatment. The strategies PCR is required to facilitate diagnosis, prevention and therapy of mastitis as well as infections due to nocardiae

### 1.4 Objectives

The objectives of this study were:

#### 1.4.1 General Objective

To isolate and identify the status *Nocardia* and *Nocardia*-like of causative agent of mastitis in goats ,cow ,sheep and soil sample in Khartoum state .

#### 1.4.2 Specific Objectives

1- To identify the isolates using culture and microscopic appearance.

2- To identify the isolates by biochemical test .

3- To the isolates by TLC test .

4- To carry out PCR technique for conventional of identification

5- To make phylogeny tree based on sequence of 16 S rRNA

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Mastitis

The term mastitis refer to the inflammation of the mammary glands. It is characterized by physical , chemical and usually bacteriological changes in the milk and by pathological changes in the glandular tissue. The most important changes in the milk include discoloration, the presence of clots and large numbers of leukocytes (Blood *et al*., 1983). Mastitis causes by different numbers of pathogenic bacteria such as *Mycoplasma spp* , *Staphylococcus spp, Streptococcus spp* ( Blood *et al* .,1983). Bacterial contamination of milk from affected quarter may pass for human consumption and provide a mechanism of spread of disease to human such as *Tuberculosis* ( Blood *et al*., 1983).

### 2.1.1Mastitis in General

Mastitis continues to be one of the major disease problems facing dairy farmers ( Funk *et al* ,1982) in spit of control effort (including intensified treatments with antibiotics and extensive research), mastitis is still the most important disease in the dairy industry (Dodd, 1985, Fetrow and Mann,1991). For this reason more and exact knowledge from extended epidemiology analysis of mastitis. Mastitis may be classified as clinical or subclinical depending on the degree of inflammation ( Philpot *et al*., 1991 ) ,Clinical mastitis is defined by the diagnosis of abnormal change ( acute ,local and systemic) in the body , udder and milk ,with concerned disease of at least 25% in daily milk production (Shigidi *et al* 1981). The

occurrence of mastitis involves the complex interaction among three major factors: the agent , host, and environment (Radostits *et al* ., 1996). Host factors include stage of lactation , anatomy of the udder. parity, intramammary defense mechanism and periparturient disease (Shook *et al*.,1989 and Radostitis et al .,1996) at the pathogen level , factors such as the nature of organism , its virulence and numbers ,toxins and antimicrobial resistance are import ant , whilst management ,climate ,feeding ,housing milking technique and malfunction of milking machines are environmental risk factors (Anon *et al* .,1987 ) .

### 2.1.2 **Mastitis in the Sudan**

Mastitis was first reported in the Sudan in (Annual Report of the Sudan Veterinary Service , 1953) . Since then it had been described as being fairly common (Annual Report of the Sudan Veterinary Service ,1953-1955 and Annual Reports of the Department of Animal Production, 1956-1957). The prevalence of it in dairy herds in the Sudan was the first time thoroughly investigated by (Wakeem *et al*.,1962 ). The investigation was carried out determine the incidence , prevalence rate of infection , the causative agents and response to control efforts which include treatment. Bagadi (1970) investigated the etiology of bovine mastitis in seven herds of cattle in three provinces in the Sudan both clinically and bacteriologically. He found that *staphylococcus aureus* was the most common causative agent representing 92.2 % of the isolates from clinical cases and 44.2% of the isolates from subclinical cases. Ibrahim (1975) surveyed 18 dairy herds in Khartoum and Gezira provinces for mastitis using the leucocytic count as an indicator of the disease. He found that 19.38% of the pool milk samples had counts cows in three herd . 32.4% of the single milk samples had leucocytic count

at level which indicate udder infection. A high incidence rate and persistent mastitis in dairy breeding center promoted the investigation of the various factors which contributed to the problem (Ibrahim and Habiballa ,1978) Mastitis was considered among the most important causes of culling of cows in dairy herds in the Sudan (Ibrahim *et al.*, ,1988).

### 2.1.3Nocardiae and Diseases

*Nocardia* causes many diseases notably pulmonary infections in man and causes mastitis in animals (Brown and McNeil ,2003). Infection may occur by inhalation, contaminated wounds and traumatic implantation (Quinn *et al.*,1999).

*Nocardia spp* causes pulmonary nocardiosis in human (Abubakar *et al* 2017,Mohamed *et al.*, 2013 and Mogahid 2005) . *Nocardia faranica* causes mastitis in cattle (Hamid *et al.,* 1998). In goats *Nocardia ateriodes* causes granulomatous mastitis ( Megid *et al* .,1990) .In sheep *Nocardia* causes arthritis .(Nclikuwera *et al.,*1990). *Nocardia faricana* was isolated from patients with pulmonary infection (Hamid *et al.*, 2001). *Nocardia brasillinsis* was isolated from various kind of infection , including mycetoma of foot (Goodfellow and Lechevalier .1989 ). Various Nocardia infections were reported in humans after surgical operation (Vandime *et al.*, 2001 ) .

### 2.1.4 Diseases in Human

Primary pulmonary nocardiosis may be subclinical or pneumonic; it may be chronic or acute with secondary involvement of other organs, mainly the brain . In human *Nocardia* cause pulmonary infection isolated by (Abubakar *et al.*,2017, Mohammed *et al.*, 2013

and Mogahid *et al.*,2005). In non-tropical countries, most infections are caused by *N. asteroides, N. farcinica* and *N. nova,* relatively few by *N. brasiliensis, N. Otitidiscaviarum* and *N. transvalenses. N. farcinica* shows a greater degree of virulence than *N. asteroide*s (Schaal and Lee 1992). Co-infection by *N. asteroides* and Mycobacterium tuberculosis was diagnosed in sporadic cases, which were treated by combined therapy for both diseases (Mogahid *et al.*,2005 and Shimokubo *et al.*, 2002 ). Nocardiae can cause brain abscesses, which may need surgical intervention (Brown *et al.*, 2003).

**2.2.1 Mastitis in Cow**

Sources indicate that 80% to 90% of all mastitis infections are caused by four types of organisms Streptococcus agalactiae ,others Streptococcal species and coliforms (Radostits *et al* ., 1996). These major pathogens are mainly spread from cow to cow through milker 's hand ,udder cloths residual milk in teat cups.Major pathogens causing environment mastitis for example coliforms and the other streptococci , the most prevalent pieces being *Escherrichia* coli ,*streptococcus* spp and streptococcus dysgalactiae ,gain access to the failure of proper hygienic procedures . the other environmental infection are an opportunistic nature ,namely those caused *Psyeudomonas spp .,* yeast *Prototheca* spp and *Nocardia* (Watts *et al.*,1988) . Environmental pathogens are a minor pathogens and include coagulase negative *staphylococci*, Actinomyces bovis ,Bacillus cereus , and *Serratia marcescens*. Minor pathogens are rarely associated clinical changes and often include only a moderate cell response ( Brooks *et al* ., 1983).

### 2.2.2 **Mastitis in goats**

Mastitis is common in lactating goats. Both acute and chronic forms my be encountered. The diseases is caused by number of different types of pathogenic bacteria such as *Mycoplasma spp, Staphylococcus spp and Streptococcus spp* (Blood *et al*., 1983). *Staphylococcus aureus* and *Streptococcus agalactiae* are most commonly involved but *coliform* mastitis can occur sporadically or sometimes as a herd problem. It is important to remember that milk from goats can appear relatively normal even with severe inflammatory changes in the udder. The diagnosis , control and treatment of mastitis in goats are similar to those for dairy cows .The a etiology of mastitis in goats and cows is similar but goats are more frequently affected .The reported causal agents of goats mastitis in the Sudan are S*taphylococci*, *Corynobacteria*, *Coliform*, *Streptococci* and *Mycoplasma spp* (Yassin and Hussein 1985, Ibrahim *et al*.,1968). In other parts of the world several pathogenic bacteria were associated with goats mastitis including *Staph-aureus* , *Streptococcus*, *Eschernichia coli Corynobacterria*, *Actinomyces* and *Nocardia spp* (Tripathi *et al* .,1993**).** *Staphylococcal* mastitis in goats is characterized by gangrenous and rapid necrosis of mammary tissue. In severe non gangrenous mastitis lobular changes consist of degeneration of the end inflammatory reaction leading to extensive fibrosis. Abscess formation also frequently occurs (Derby *et al*.,1958). *Staphylococcus spp* has been isolated from normal and mastitic milk of goats (Al Said *et al*.,2001). Atrophy of the udder is often associated with *Mycoplasma* mastitis (Prasad *et al*.,1985). *Nocardia* mastitis is

characterized by anorexia, fever , changes in size of the udder and lungs (Megid *et al*.,1990). *Nocardia asteroides* and *Nocardia farcinia* are resistant in vitro to the most common antibiotics (Hamid and Goodfellow ,1997). *Nocardia asteroids* was found to be sensitive to gentamicin, neomycin and streptomycin and resistant to ampicillin, chloro- amphenicol and tetracycline (Savalia *et at*., 1990).

### 2. 2.3 Diagnosis of Mastitis

Complete mastitis surveillance requires a combination of clinical examination of Ruminant and laboratory tests.

### 2.2 .4 Ruminant Site tests

Several simple tests performed on the farms (goats and sheep side). Clinical mastitis may be detected by examining the udder for warm, swollen quarters, which are indicative of acute mastitis. Abnormal milk is usually discolored , watery or contain flakes ,shreds or clots ,but in chronic mastitis the milk is not always macroscopically abnormal (Anon *et al*., 1987).Several simple tests on the farm (cow side) ,or more complicated laboratory tests are used to diagnose mastitis in individual cows or on a herd basis (Anon *et al*,.1987).Clinical l mastitis may be detected by examining the udder for warm ,swollen quarters , are indicative of acute mastitis ,or for misshapen ,hard ,atrophied and fibrotic quarters , indicating permanent damage caused by chronic mastitis . The strip cup test is a practical and effective method of identifying cows with clinical mastitis .Abnormal milk is usually discolored ,watery ,or contains flasks ,shreds or clots ,but in chronic mastitis the milk is not always

macroscopically abnormal . The strip cup test in these cases is not sufficiently sensitive to detect subclinical mastitis .

### 2.2.5California Mastitis Test( CMT)

The California Mastitis Test (CMT) ,or Rapid Mastitis Test (RMT) which used for detection of mastitis (Buss and Moller , 1934) this test can be used in the fields or in the laboratory tests and it is based on the quantity of DNA in the milk and hence of number of leukocytes and other cells which presence in the milk(Quinn *et* al .,1994) . The California ( Rapid mastitis test ) was commonly used for detection of mastitis and has proved to be highly efficient ( Blood *et al* ., 1983 ) .This is an indirect test that grossly measure the od DNA , primarily a function of the number of nucleated white blood cells in the milk ( Quinn *et at* ., 1994 ) it is more sensitive than the strip cup test and enables subclinical mastitis to be detected .The basis of the CMT is the reaction which occurs when the reagent comes into contact with the increased quantity of cells in the milk ,somatic cell number tend to increase during milking and remain high for several hours there after ,even in infected quarters (Anon *et at* ., 1987).For reliable result ,the CMT therefore ,should be conducted just before milking , after stimulating the cow ,and having discard the fore milk .Based on the amount of gelling that occurs as equal amounts of milk and reagent interact , the test ( reaction ) is subjectively read (scored) as 0(negative) ,t(trace) ,(slight) ,2(moderate) 3(heavy).These scores equate well with somatic cell levels (Philpot and Nickerson 1991). The CMT is most helpful in detecting subclinical mastitis and serves little purpose in acute clinical mastitis and serves little purpose in acute clinical mastitis ACMT tends to have a high score

in recently fresh cows and in cows at the end of lactations just prior to dring off. ACMT is also elevated in secretions from cows whose milk production has dropped precipitously due to illness (William *et at* .,1995).

### 2.2.6. PH indicator papers

These test strips detect the more alkaline pH in the udder with mastitis. Normal has a pH of approximately 6.5 to 6.7, where as mastitic milk pH often approaches plasma pH of 7.4 (William *et at* .,1995).

### 2.2.7 Laboratory tests

#### 2.2.7.1Culture tests

Most of bacterial pathogens causing mastitis grow on sheep blood agar ,MacConkey agar to detect *Enterococcus faeacalis* and any Gram–negative bacteria that able grow on the medium. Edward medium is highly selective for streptococci. Sabroud dextrose agar plate can be inoculated if fungal pathogen is suspected (Quinn *et at* .,1994)

### 2.2.8Clinical Syndromes of Mastitis

The main clinical types of mastitis are :Subclinical: the infection in the mammary gland is detected only by bacterial culture or test to demonstrate high leukocyte count in the milk. The Peracute form : is characterized by swelling , pain, heat and abnormal secretion in the mammary gland, these are accompanied by signs of systemic disturbance such as fever, depression, anorexia, weakness and a rapid, weak pulse. The signs are those of severe a toxaemia or septicemia .The Acute form: is characterized by in mammary

gland, it is similar to those of peracute mastitis but the signs are less severe. The Subacute: there is no systemic reaction and the change are less marked. The Chronic: there are no systemic signs and very few external signs of changes in the udder, with abnormal secretion in the gland (Quinn *et at* .,1994).

### 2.2.9 **Actinomycetes**

The *actinomycetes* comprise a heterologous group of prokaryotes that have the ability to form Gram-positive, branching filaments of less than 1 um in diameter . The main animal pathogens in the *actinomycetes* are in the genera *Actinomycetes*, *Nocardia* and *Dermatophilus*. *Nocardia* is closely related to *Corynobacterium* , *Mycobacterium* and *Rhodococcus* but A *ctinomycetes* differs from these in its DNA guanine / cytosine ratio and in the chemical composition of its cell wall ( Quinn *et at* .,1999).

#### 2.2.9.1 **Taxonomy of Actinomycetes**

The systematic of the aerobic actinomycetes began as a largely intuitive discipline based on microscopic morphology but has become increasingly objective with the introduction and application of modern taxonomic procedures, notably chemosystematics molecular systematic and numerical taxonomic methods (Murray *et at* ., 2003).The medically important genera are *Actinomadura, Corynobacterium* D*ermatophilus, Gordonia, Mycobacterium, Nocardia, Rhadococcus, Streptomycetes saccharomonospora, Saccharopolyspora, Thermoactinomyce Tropheryma, tsukamurella* (Murray *et at ., 2003).*The mycolic acid containing *actinomycetes* or mycolata, notably *corynebacteria,* mycobacteria, and *nocardia* , have a set of

chemical markers in common (The cell wall components meso-diaminopimelic acid [meso-DAP] arabinose and glatactase and mycolic acids) that characterizes them as the mycolata genera (Murray *et at* ., 2003).

### 2.2.9.2.Natural habitats

The aerobic *actinomycetes* are ubiquitous in the environmental have been isolated world wide from soil, fresh water, marine water and organic matter . *Nocardia spp* and related bacteria are considered saprophytic soil microorganisms, primarily responsible for the decomposition of organic plant material. Although *Nocardia asteroids* appears to be geographically wide spread, most cases of *Nocardia brasiliensis* infection in the United States have originated in the Southeast or Southwest (Murrray .*et at* ., 2003).

## 2.3. Description of the Genus Nocardia

*Nocardia are* widely distributed group of *actinomycetes* which are predominantly saprophytic (Orchard and Goodfellow 1980, and Williams *et al*.,1983) but also include species forming parasitic association with animals and plants (Goodfellow 1992, and Beaman 1994). They occur in a wide range of man made and natural habitat including activated sewage sludge, soil, water and tissues of plants and animals including human. *Nocardia* species are gram-positive, partially acid-fast and non-motile bacteria that often form branched hyphae in both tissues The genus *Nocardia* belongs to the family Nocardiaceae, and members of the genus are all aerobic, Gram-positive, partially acid fast and non-motile actinomycete that form filamentous branched

cells which fragment into pleomorphic rod-shaped or coccoid elements, non-spore-forming (Kämpfer *et al*., 2007 and Kachuei *et al*., 2012), catalase and urease positive bacteria that belong to *Actinomycetes* group (Zakaria *et al*.,2008). The only constant morphological feature of *Nocardia* is their ability to form filamentus. Which fragment into pleomorphic, rod-shape and coccobacilli (Conville *et al*., 2011 ,Rodríguez *et al*., 2006 and Locci *et at* ., 1976). Most *Nocardia spp* produce caratenoid -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 ,wrinkled or headed (Goodfellow *et at* ., 1998) .*Nocardia spp have* an oxidative type of carbohydrate metabolism. They can used diverse range fatty acid, hydrocarbons and sugars as source of carbon for energy (Goodfellow *et at* .,1998). Most strain grow on media containing simple nitrogen sources such as amino acid , ammonium nitrate on media and supplemented with casein , meat extract , soyia or yeast peptones and hydrolysates .They grow well between 25 – 37°C, same strains reach stationary phase in 3-7 days . Other grows more slowly (Goodfllow *et at* ., 1998). Member of the genus *Nocardia* form extensively branched hyphae that fragment into rod-shaped to coccoid , non motile elements and usually form aerial hyphae. Nocardia are characterized by the presence of meso-DAP, arabinose , and galactose in their wall peptidoglycan (wall chemo type IV ), having muramic acidin the N-glycolated form having diphosphatidyl glycerol phosphatidyl ethanolamine, phosphatidylinositol and phosphatidylinositol mannosides as their major phospholipids, having major

amounts of straight chain , unsaturated, and tuberculostearic acid, having mycolic acid with 40 to 60 carbon atoms and having DNAs that are rich in guanine-plus-cytosine (G+C) content (Murray *et at* ., 2003).

### 2.3.1. Differentiation of the genus *Nocardia* from closely related genera

*Nocardia* are member of the family *Nocardiaceae* (Castellani and Chalmers, 1919). The latter currently accomodates aerobic, Gram-positive, *actinomycetes* that have a wall chemotype IV and form rudimentary to extensive substrate mycelium which usually fragments into bacillary and coccoid elements (Goodfellow and Minnikin, 1981). It's clear that the family is markedly heterogenous (Goodfellow and Cross, 1984; Goodfellow and Minnikin, 1984), and can be divided into aggregate groups centered around the genera *Nocardia* and Micropolyspora. The first group is characterized by the presence of mycolic acids, and major amounts of straight-chain and unsaturated fatty acids, where are Micropolyspora and related taxa lack mycolic acids, but contain large amounts of branched chain iso and antesio-acids (Goodfellow and Minnikin, 1984). In general, nocardiae and related organisms are either amycelial (*Caseobacter, Corynebacterium and Mycobacterium*) or reproduce by mycelial fragmentation (*Nocardia,Rhodococcus*), whereas the genera in the *micropolyspora aggregate* show a greater differentiation of sporing structures (Locci *et at.*,1976). Morphology is of little use in distinguishing most members of the genius *Nocardia* from the taxa in the Micropolyspora group since among the nocardiae there is a

spectrum of morphological complexity going from undifferentiated (and often un fragmented) mycelium to a highly developed system of short conidial chains formed on the aerial and vegetative hyphae (e.g. *N. brevicatena* and some *N. asteroides*) to long chains of well formed spores on the aerial hyphae (*N. carnea, N. vaccinii*), all morphological type found within the *Micropolyspora aggregate*. *Nocardia spp* can be distinguished from the other mycolic acid-containing taxa, and from strains previously assigned to the genus, using a combination of chemical and morphological properties (Table 1).

**Table 1: Differential characteristics of the genus *Nocardia* and some related wall chemotype IV taxa containing mycolic acids*.**

| CHARACTERISTICS | NOCARDIAE | CASEOB-ACTER | CORYNE-BACTERIUM | MYCO-BACTERIUM | RHODOCOCCUS |
|---|---|---|---|---|---|
| SUBSTRATE MYCELIUM | + | - | - | $D^-$ | $D^+$ |
| AERIAL MYCELIUM MICROSCOPIC | D | - | - | - | - |
| AERIAL HYPHAE MICROSCOPIC | + | - | - | D | D |
| CONIDIA | $D^-$ | - | - | - | - |
| ENTIRE COLONIES | - | + | + | D | D |
| PHOSPHATI-BYLETHANOLAMINE | + | ND | - | + | + |
| MYCOLIC ACIDS: | | | | | |
| NUMBER OF CARBONS | 46-60 | 30-36 | 22-38 | 60-90 | 34-64 |
| NUMBER OF DOUBLE BONDS | 0-3 | 0-2 | 0-2 | 1-3 | 0-4 |

* Data from Goodfellow and Minnikin(1981, 1984) and Good fellow and Cross (1984). Symbols: +, 90% or more of the strain are positive. -, 90% or more of the strains are negative.

D: Different reactions occur in different taxa $D^-$: Uncommonly, $D^+$ more commonly than not ND, not determined

### 2.3.2. Pathogenicity of Nocardia

Pathogenic *Nocardia* survive within phagocytic vacuoles by preventing phagolysosome formation. This is attributed to a surface lipid. Other cell wall lipids may trigger granulomatous reactions. Variations between strains and growth phases in cell envelope constituents are paralleled by changes in virulence and infectivity (Dwight and Yuan, 1999). Superoxide dismutase and lysosomal enzyme inhibition protect *N. asteroides* against phagocytosis killing. Nocardiosis is a predominantly suppurative process with variable granulomatous features. Lymph Nodes are consistently involved. Hematogenous dissemination may result in osteomyelitis and widespread abscess formation. Central nervous system involvement is rare in animals. Infections can be regional or disseminated. Local wound infections may extend to regional lymph nodes (Dwight and Yuan, 1999).Pathogenesis of mastitis is explained in term of the three stages invasion, infection, and inflammation. Invasion is the stage at which organisms pass from the exterior of the udder teats to the milk inside the teats canal . Infection is the stage in which the organism multiplies rapidly and invades mammary tissue. After invasion a bacterial population may be established in the teat canal and using this as a base, a series of multiplication and extensions into mammary tissue may occur, with infection of mammary tissue occurring frequently or occasionally depending on its susceptibility. This in turn causes inflammation, the stage at which clinical mastitis appears

or a greatly increased leukocyte count is appears in the milk (Blood *et at* ., 1983).

### 2.3.3. Epidemiology

Members of the genus *Nocardia* occur in a wide range of the man –made and natural habitats including activated sewage , sludge , soil water and tissues of the plants and animals, including human (Good fellow *et at* .,1998). Pathogenic *Nocardia* had been isolated from soil sample around Khartoum State (Awatif et at ., 2001). The most commonly isolated species in human include N. *asteroides*, N. *farcinica*, N. *cyiriacigeorgica*, N.*nova*, N. *brasiliensis* (Larruskain *et al* ., 2011) .Higher levels of production, larger herd size, and a large percentage of goats treated with dry goats products are factors which increase the risk of *Nocardia* mastitis in herd (Ferns et *al*., 1991). Dry goats therapy with intramammary products containing neomycin and the use of multi - dose vials of dry goats medication were the predisposing factors identified as being significantly associated with *Nocardia* mastitis (Ollis *et al*, 1991). *Nocardia* species had been isolated from 21.54% of soil samples and 23.33% of milk samples from goats with mastitis (Abdel-Fattah *et al*.,1996).

## 2.4. Treatment

The importance to recognize mastitis due *Nocardia* lies in the fact that *Nocardia* especially *N.asteroides* and

*Nocardia farcinica* are resistante in vitro to the most common antibiotics (Hamid and Good fellow ,1997) *N.steroids* was found sensitive to gentamicin ,neomycin and streptomycin and resistant to ampicillin, chloroamphenicol and tetracycline (Savalia *et at*,1990).

### 1.5. Control Measures

The following routine as indicated by Quinn (1994 ) will reduce the proportion of infected goats and clinical mastitis by at least 70% if used regularly at each milking . Mastitis caused by *Streptococcus agalactiae* will be reduced to very low levels and is frequently eradicated. This includes the following:

1. Adopt good goat management practices as the essential basis for a mastitis control routine (e.g. feeding, housing, hygiene).

2 - Reduce exposure to pathogens.

3- Reduce the chances of pathogens penetrating the teat duct

4- Reduce mastitis in non-lactating growing goats in the dry period.

### 2.6. Diagnosis of *Nocardia* Infections

The improved classification of the genus *Nocardia* provides a sound base for the continued search for better diagnostic methods to distinguish between members of clinically significant taxa (Good Fellow *et al*., 1998 ).

#### 2.6.1. Preparation of smear

Smear from clinical speciments is and stained with modified Ziehl – Neelsen stain and with Gram s᾽stain ( Quinn *et al* ., 1999).

The positive *Nocardia* specimens are Gram positive slightly acid fast filaments .

### 2.6.2. Collection, transport and storage of specimens:

The general principles of collection ,transport and storage are applicable to most aerobic *actionmycetes*, However, if samples are suspected of containing *Nocardia*, should not be refrigerated or placed on ice before being transported, some strains of *Nocardia* lose their viability after exposure to near - freezing temperatures (Murray *et at* .,2003).Specimens for *Nocardia asteroides* should include exudates aspirate, mastitic milk samples, tissue from granulomas and thin sections from granulomas in 10 percent formalin for histopathology (Quinn *et al*., 1999). Normally sterile fluids and tissues should be transported to the laboratory promptly, especially if their collection required an invasive procedure (Murray *et at*., 2003).

### 2.6.3. Morphology and Cultural appearance

The only constant morphological feature of *Nocardia* are their ability to break into plemorphic, rod - shaped and coccoid elements. The growth and stability of both aerial and substrate hyphae often depends on the condition of culture (Beaman *et at* ., 1994).Scanning electron microscopy has been used to study the morphogenesis of *N. asteroides* fragments (Locci *et at* ., 1976) and showed that both lateral buds and terminal extension occur when fragments germinate in agar or in soil . The well known acid fastness of *Nocardia* is often more pronounced in clinical than cultural material. Other morphological features include a well developed conidia in *Nocardia*

*brevicatena* and less well differentiated spores in some strains of *N. asteroides* (Good fellow and Lechevalier, 1989). Most Nocardia produce carotenoid - like pigment that result in colonies with various shades of orange , pink , red or yellow, brown or yellowish pigments may be produced (Goodfellow *et at* ., 1998). Colonies may be smooth or granular and irregular, wrinked or heaped. Mesosomes are common L-form of *Nocardia asteroides* and *Nocardia otitidiscaviarum* may be important in pathogenesis (Beaman *et at* ., 1994). *Nocardia* often form a thick dry scaly or leathery pigmented surface pellicle in broth culture . Aerial hyphae may develop on the surface of the pellicle and may extend up the sides of the tube. Broth tends to be clear although some cultures produce a fine granular turbidity , either ropy or membranous sediment that might be pigmented . Growth may occasionally start at the bottom of the tube giving rise to fluffy masses of mycelium. (Erikson *et at* ., 1955).

**2.6.4.. Biochemical reaction**

Hydrolysis tests were performed for casein , xanthine tyrosine, ureas. *Nocardia brasilliensis* strains hydrolyzed casein and tyrosine but not xanthine . *Nocardia otitidiscavarium* strains hydrolyzes only xanthine (Brown and McNeil , 2003) . Members of the genus *Nocardia* are catalase – positive hydrolyse aesculin . They can also metabolize a diverse range of carbon compound for energy and growth (Tsukamura , 1969 ; Good fellow 1971 ) .

**2.6.5. Antimicrobial sensitivity testing of *Nocardia africana***

*N. africana* strains were tested for their in vitro sensitivity against 34 different antimicrobial agents. The antimicrobial susceptibility was determined by the disc diffusion method using Mueller-Hinton agar medium. The zone of Inhibition was read after 36-48 hour of incubation at 37˚C. The results indicated that all *N. africana* isolates were sensitive to ciprofloxacin (5µg/ml). clindamycin (10µg/ml) . Gentamycin (10 µg/ml), tobramycin (10µg/ml). amikacin (20µg/ml), doxycycline (30µg/ml) and vancomycine (30µg/ml). They were resistant to compound sulfonamides (300µg/ml). Sulphafurazole (10µg/ml). Metronidazole (50µg/ml), Aztereonam (30µg/ml), Cefotetan (30 µg/ml). Nalidixic acid (30µg/ml) and penicillin G (10 units) . *N. africana* revealed distinct, susceptibility and resistance profiles to antimicrobial agent's testes. The study underlines the importance of antimicrobial susceptibility testing for clinical isolates of *Nocardia spp*. (Tsukamura , 1969 ; Good fellow 1971 ) .*N. africana* has recently been found to cause a significant number of pulmonary infections among Sudanese patients attending respiratory disease clinics with symptoms simulating tuberculosis ( El Hassan and Hamid, 2005) .

**2.7. Selective Media**

Different method and media have been used for isolation of *Nocardia spp*. Tryptic soya agar supplemented with 5%(v/v) defibrinated bovine blood and Gentamicin Sulphate (25µg /ml )was reported as a good selective medium for isolation and identification *Nocardia spp* present in milk sample (Lynch *et at* ., 1990).

*N .farcinica* strain isolated on Lowestien Jensen slants which were incubated at 37◦C under aerobic condition (Hamid *et al* ,1998 ). *N.asteroides* was isolated from milk on 5% sheep blood agar at 37◦C under aerobic condition (Wendit ,et *al* .1969 ).

**2.8. Cell wall composition**

The walls of Nocardia consist of peptidoglycan lipid constituents and other polysaccharide or polypeptide fraction (Michel and Bordet, 1976). Sugars were reported in the *Nocardia* wall during early investigations on a*ctinomycetes* wall composition (Cummins and Harris, 1956 and Romano and Sohler , 1956).The presence of a rabinose and glactose in the cell wall was later found to be common feature of all *Nocardia.* Glucose has also been detected in the *Nocardia* wall (Michel and Bordet, 1976). as have amino acids that are not part of the peptidoglycan (Sohler *et al* .,1958) .Mycolic acids , α -branched , - hydroxylated long chain fatty acid the most characteristic component of the walls of nocardiae and related actinomycetes (Minnikin ,1993; and Goodfellow ,1980; Chun et al ., 1996).Mycolic acids with between 44 and 64 carbon atoms have been isolated from N.astroides ((Minnikin *et al*.,1988), N.*farcinica* (Yano et al.,1990) N.*nova* (Yano *et al* ., 1990) and N. seriolae (Kudo *et al* ., 1988) .Some nocardiae have also been found to contain shorter chain mycolic acids (Pommier and Michel ,1985). Several method are viable for the detection and characterization of the different structure type of mycolic acids (Minnikin and Goodfellow ,1980 ;Minnikin 1993; Embley and Wait ,1994) .Mycolic acids can be positively identified on chromatograms by their characteristic immobility when plates are washed with methanol :water ( 5:2 $^{v}/_{v}$ ). Methanolysates of

Mycobacteria typically give a multisport pattern, tsukamurellae a characteristic 2-spot configuration and nocardiae and other mycolic acid containing actinomyceres signal spots whose motilities reflect the chain length structure of the constituent mycolic acids ( Minnikin *et al* 1993 and Yassin *et al*., 1993).

### 2.9. Identification and differentiation at genus and species level

*Nocardia* are most easily distinguished from *actinomadura* , *sreptomycetes* and other *sporactinomycetes* Qualitative evaluation of mycolic acids can be easily and quickly achieved by the thin layer chromatography . A combination of chemical , morphological , and physiological tests are necessary to distinguish between the mycolata genera.The phenotypic identification tests recommended by a number of investigators are inadequate and only give presumptive identifications( Good fellow *et at* ., 1998 ).The use of polymerase chain reaction (PCR) coupled with restriction endonuclease analysis of PCR product has been the focus of recent interest for the separation of *Nocardia spp.*This approach promises to provide a rapid , sensitive and effective way of identification of clinically significant *Nocardia* (Good fellow *et at* .,1998 ).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study Approach

The study is a qualitative study, aimed to highlight the importance of using conventional and sequencing technique in detecting *Nocrdia* spp

### 3.1.1 Study design

This study was designed as a cross-sectional studies and experimental studies.

### 3.1.2 Study Area

In the following farms in Khartoum state were included in the study: Koko , Ganndahar , Al Silate , Almualih and South of Omdurman (Table 2).

### 3.1.3 Number of Samples

Three Hundred milk samples and twenty soil samples were collected from the farms sites in Khartoum state (Table 2).

### 3.1.4 Data Collection

Data were collected by using a standard data questionnaire consisting of basic demographic data. Additional information was also included history of clinical infection, (Appendix V).

### 3.1.5 Collection of Milk

Milk samples were collected from goats, cow and sheep. The udder of the target animals disinfected by alcohol 70%, then milk was collected in sterile containers, and transported in ice to the microbiology laboratory, 100 samples from each animals species.

### 3.1.6 Soil Selection

This was collected as ten gram of soil (five gram from surface and five gram from bellow the surface) ,then mixed well and stored in sterile containers at room temperature and the samples were transported to the microbiology laboratory for culture.

### 3.1.7 Culture Media

Tryptic Soy Agar medium (TSA) and Brain Heart Infusion medium(BHIA) (Appendix I) were used for milk samples and T.S.A supplemented with a combination of tetracycline (5µg/ml) and nystatine (50µg/ml) was used for soil samples.

## 3.2 Diagnostic Approach

## 3.2. Preparation of Samples

### 3.2.1 Preparation of milk Samples

The three hundred milk samples were prepared by centrifuged at 1000 rpm for 10 min, the deposited part was used for culture.

### 3.2.2 Preparation of Soil Samples

One gram for soil samples was added to 10 ml of sterile 25% (v/v) normal saline solution . Tubes were shaken for 30 min was let to precipitate, 0.1ml of the supernatant solution was transferred to another sterile tube by sterile pipette, the supernatant was used for culture.

## 3.3. Culture Methods

#### 3.3.1.1 Primary Culture for Milk Sample

Cultures were streaking by a loop full of each milk samples on TSA and BHI plates. The plates were incubated at 37°C and

examined after 7,14 and 21 days for the presence of *Nocardia spp* growth.

### 3.3.1.2 Primary Culture for Soil Sample

The soil samples were cultured by spreading 0.1 ml soil suspension on 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 spp* growth**.**

**Table 2: Different locations of enrolled samples for isolated of Nocardia from Milk and Soil**

| Sample site | No .of Soil | No.of Cows | No. goats | No.of sheep |
|---|---|---|---|---|
| Koko | 2 | 5 | 5 | 7 |
| Koko | 2 | 7 | 5 | 8 |
| Ganndahar | 2 | 8 | 10 | 10 |
| Gandahar | 2 | 12 | 15 | 12 |
| Amiualih | 2 | 20 | 15 | 15 |
| Amiualih | 2 | 15 | 10 | 10 |
| AL Silate | 2 | 15 | 20 | 15 |
| Al Silate | 2 | 10 | 10 | 15 |
| Jabal Awlela | 2 | 5 | 5 | 5 |
| Jabal Awlela | 2 | 3 | 5 | 3 |
| Total | 20 | 100 | 100 | 100 |

### 3.3.2. Purification of the Culture

Typical and well isolated *Nocardia* colonies from the primary culture were picked with a wire loop and streaked on the surface of a fresh plate of the corresponding medium. Pure cultures were obtained by repeating the subcultures on Tryptic Soy Agar medium (TSA) and Brain Heart Infusion medium(BHIA). Samples were processed according to (Quinn et al., 1999)

### 3.3.3 Preservation of Culture

#### 3.3.3.1 Slants

Growth of the isolates on Tryptic Soy Agar medium (TSA) was maintained in TSA slants (Bijoux, or Universal bottle) which remain viable for several months at -4 ºC. Sub- culturing every three months was done to ensure no loss of strains (Barrow and Feltham, 1993).

#### 3.3.3.2 Frozen Glycerol Suspension

Well-grown isolated strains were kept in sterile 20% glycerol Heavy cell biomass from young culture was transferred onto sterile tubes containing 1-2 ml 20% glycerol. The cell suspension was kept at-20˚C for preservation (up to 10 years) according to (Michel, Bordet, 1976., and Williams 1983)

### 3.4. Identification

### 2.4.1. Microscopic Examination

The isolated strains examined during this study were stained with Gram's stain and Modified Ziehl –Neelsen Stain (Appendix II). A smear was spread on a microscopic slide, fixed by gentle flaming

and stained with Gram's stain as described by Quinn (1999). . Gram positive organism had a purple, violet or blue colour. Another fixed slide was stained with Modified Ziel-Neelsen as described by Quinn (1999). MZN positive organism appeared red in a blue background.

#### 3.4.1.1Staining Technique

##### 3.4.1.1.1Gram Stain

Gram stain was essential technique for initial identification of bacterial isolates. The procedure was carried out according to (Cheesbrough, 2007) as follows; smear was prepared from overnight culture on a clean and dry slide. The smear was left to air dry. Fixation was done by rapid pass of the slide three times through the flame of a Bunsen burner then allowed to cool before staining. Crystal violet stain was added to smear for 30–60 seconds, and then washed by tap water. Lugol’s iodine was added for 30-60 minutes then washed by tap water and decolorized rapidly (few seconds) with acetone alcohol and washed immediately by tap water. Finally, the smear was covered with Saffranin stain for 2 minutes and washed by tap water. The back of slide was wiped clean and placed in a draining rack for smear to air dry. Drop of oil was added to the dried smear and examined under the light microscope by oil lens 100X.

##### 3.4.1.1.2 Modified Ziehl –Neelsen Stain ( ZN stain)

The smear was prepared from overnight culture on a clean and dry slide was mixed with drop of normal saline .The smear was left to air dry. Fixation was done by rapid pass of the slide three times through the flame of a Bunsen burner then allowed to cool before staining, Smear from each suspected milk sample was stained with

modified Ziehl Neelsen method according to (Quinn et al., 1999). The slide was flooded with dilute carbolfuchsin for 15 min, then washed thoroughly under running water, decolorized with 5% acetic acid for 15 s, and counter-stained with methylene blue for 1 min, Drop of oil was added to the dried smear and examined under the light microscope by oil lens 100X.

### 3.4.2. Biochemical tests

Selected biochemical tests were done for the identification of the isolates. All tests were done according to Barrow and Feltham (1993).

#### 3.4.2.1. Catalase test

A drop of 3% hydrogen peroxide ($H_2O_2$) was placed on a clean microscopic slide; a small portion of the bacterium under test was mixed with $H_2O_2$. Production of air bubbles indicates releasing of $O_2$ from the destruction of $H_2O_2$ by catalase enzyme which is produced by the bacteria.

#### 3.4.2.2. Urease test

Urease medium slope (Appendix I) were inoculated with each of test strain, incubated aerobically at 37°C for 48 -h and examined daily for seven days. A pink or red color indicates a positive result according to (Cheesbrough *et al.*,2007) (Appendix I)

### 2.4.3 Degradation tests

Details of composition and preparation of each medium were given (Appendix I).

#### 3.4.3.1 Casein degradation

Fresh plates of casein medium (Appendix I) were each streaked with the test strain, incubated aerobically at 37°C for 3to 7days and examined daily up to 2 weeks to detect clear zones around or under bacterial growth which indicate positive result(Appendix) .

#### 3.4.3.2 Tyrosine degradation

Tyrosine medium plate (Appendix I) were each streaked with test strains then incubated at 37°C for 3 to 7 days and examined daily for dissolution of tyrosine crystal under and around bacterial growth.

#### 3.4.3.3 Xanthine degradation

Xanthine agar medium (Appendix I) were streaked with the test strains then incubated at 37°C for 3 to 7days. A positive result is indicated by a clear zone around and under the growth which means utilization of xanthine.

#### 3.4.3.4 Starch degradation

The test strains were inoculated on starch agar medium(Appendix). The plates were incubated at 37°C for 3 to 7days . After 3-5 days when good growth was noted, the plates were then flooded with lugal`s iodine solution. A clear zone around and under the growth indicates positive starch degradation (Appendix).

### 3.4.4 Sugars fermentation

#### 3.4.4.1 Mannitol

The test strains were cultured in to mannitol sugar medium, the tube test were incubated at 37°C for 24 to 48 hours (Appendix I) .Positive result gave a pink colour ,indicating the fermentation .

#### 3.4.4.2 Rhaminose

The test strains were cultured in to rhaminose sugar medium ,the tube test were incubated at 37°C for 24 to 48 hours, (Appendix I) ,Positive result gave a pink colour , indicating acid production .

#### 3.4.4.3 Sorbitol

The test strains were cultured in to sorbitol sugar medium the tube test were incubated at 37°C for 24 to 72 hours (Appendix I) .Positive result gave a pink color, indicating acid production.

#### 3.4.4.4 Arabinose

The test strains were cultured in to arabinose sugar medium the tube test were incubated at 37°C for 24 to 72 hours (Appendix I). If the bacteria used the sugar and produced acid the color of the medium changed to pink color**.**

### 3.4.5. Growth at 45°C

All isolates were cultured and subjected to incubation at temperature of 45°C for 48 hours . The inoculated plates were examined daily for growth .Ability of the tested strains to grow at 45°C was taken as a positive.

### 3.4.6 Difco and Oxoid discs Anti –microbial Susceptibility Test

Oxoid and Difco anti –microbial susceptibility test discs were used. TSA plates were streaked with the each of test strains .Anti-microbial discs were then applied to the surface of the media and pressed gently using sterile forceps .The plates were incubated at 37 C for 36 to 48 hours , zones of inhibition 25 mm a round each growth of strains that determine the organism was sensitive to anti –microbial agent.

### 3.4.7. Analysis of mycolic acids

Extraction of Mycolic Acid was conducted according to Minnikin (1988) as follows: Cell biomass (5 mg) from the test strains was placed in a bijoux bottle.One ml of methanol/ toluene/ concentrated sulphuric acid (30:15:1, v/v) was added and the mixture and then incubated overnight at 80°C. After cooling, the preparation was shaken with 1ml petroleum ether for 30 minutes. After centerifugation the supernatant, which contains mycolic acids, was transferred to a capped corning tube and evaporated to dryness at 37°C.The extract was redissolved in 0.1 ml petroleum ether. 5-10µl was spotted into Thin Layer Chromatographic (TLC) aluminum sheet Merk (20X20 cm silica gel; 60 F 254).The sheet was run twice in a solvent containing petroleum ether/ diethyl ether (85:15, v/v). After drying the TLC plates were stained with 5% ethanolic molybdosphosphoric acid and heated at 100-150° C for 5-10 minutes. Mycolic acid appears as dark spots on green background (Minnink, 1988). The test was controlled by including a reference *Nocardia* strain (positive control).And *streptomycetes spp* (negative control).

### 3.4.8 Molecular Approach

### 3.4.8.1 DNA Extraction

#### 3.4.8.1.1. DNA Extraction by boiling

DNA was prepared by whole-cell procedure using heat shock method (Konkel *et al.*,1999).In this study ,the 24-h growth of bacteria isolated on Nutrient agar culture medium were suspended in 2ml of sterile distilled water with a sterile loop .One ml aliquot of the cell suspension was transferred to 1.5ml microcentrfuge tube and add 5 drop of proteinase K ,and the cell mixture was boiled in a water bath 10 min followed by sudden freezing for 10min .The freezing for 10 min and boiled was repeated three times .Cell debris was pelleted by centrifugation at high speed(13000 rpm at 4 °C for 10 min by Sigma 1-14Germany Microcentrifuge Device) (Appendix IV-Figure 24).The supernatant was transferred to new 1.5 ml tube and subsequently used immediately as a new as a template for PCR amplification or kept at 4 °C for up to 1 month. The purity of the extracted DNA was determined by running the DNA sample on 2% gel agarose (Sambrook *et al*., 1989).

#### 3.4.8.1.2 DNA Extraction by STET Buffer

In this presented study we used simple protocol for DNA extraction from the genus *Nocardia* using STET buffer. Pure colonies were picked from nutrient agar plated inoculated in 5 mL on 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 × 108 bacterial cells). Bacterial suspension was pelleted via centrifugation at 13000 rpm for 5 min by Sigma 1-14 Germany Microcentrifuge (Appendix IV-Figure 24). The pellet was washed with sterile distilled water and re-suspended in 200 of μL STET buffer (10 mM Tris-HCl, 0.1M

NaCl, 1mM 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 50 µL sterile distilled water and stored at −20°C until the PCR amplification. Purity and quality of the nucleic acid were determined by measuring A260/A280 ratio using UV spectrophotometry (Figure 28) and electrophoresis in 2% agarose gel (10 µL of DNA sample running by Gel electrophoresis device (Figure 27)

**3.4.8.2 Polymerase Chain Reaction:**

**3.4.8.2.1 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 *et al.*, and Reddy *et al.*, 2009).

### 3.4.8.2.2 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) .

### 3.4.8.2.3 Gel Preparation

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 1.5 μl of Ethidium bromides stock (10 mg/ml) per 100ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed according to (Sambrook *et al*., 2001).

### 3.4.8.2.4 Visualization of the DNA

The gel casting tray was put into the electrophoresis, tank flooded with 10x TBE buffer just to cover the gel surface, 5µl of PCR products from each samples was added to wells of electrophoreses, 5µl of DNA ladder (100-bp DNAladder, iNtRON, Korea), was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 100V, 500 mA, UK) (Appendix IV- Figure 27). The electrophoresis was carried out at 75 Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec – UK) (Appendix IV- Figure 28) according to (Sambrook *et al*., 2001).

# CHAPTER FOUR

# RESULTS

## 4.1. Isolation of *Nocardia* and related *actinomyces*

Three hundred milk samples were collected from cow, goats and sheep and twenty soil samples in the same farms were collected from different Sites at Khartoum state (Table1).

As show in table (3) 7 isolates (35%), 13(13%), 11(11%) and 0(0%) isolates were detected from soil, goats milk, cow and sheep as pectively.

**Table 3. Frequency of the isolates according to the type of Samples**

| **Sample** | **No of Sample** | **Growt h** | ***Nocardia* spp** | **% of *Nocardia*** | **Others isolates** | **%of other isolates** |
|---|---|---|---|---|---|---|
| Soil | 20 | 20 | 7 | 35% | 13 | 65% |
| Milk Goats | 100 | 87 | 13 | 13% | 87 | 87% |
| Milk Cows | 100 | 77 | 11 | 11% | 89 | 89% |
| Milk Sheep | 100 | 60 | 0 | 0% | 0 | 0% |

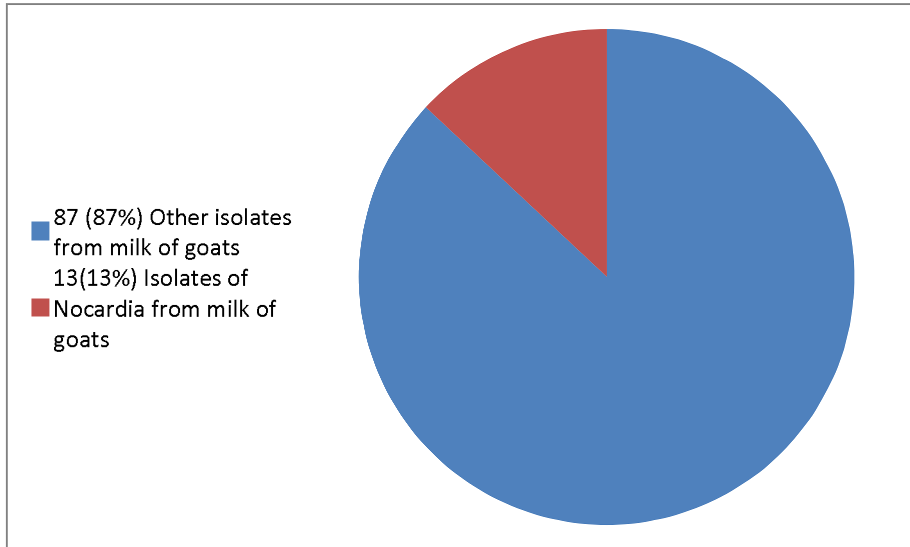



**Fig1: Frequency of *Nocardia* and *Nocardia* –like organism in milk sample of Goats.**

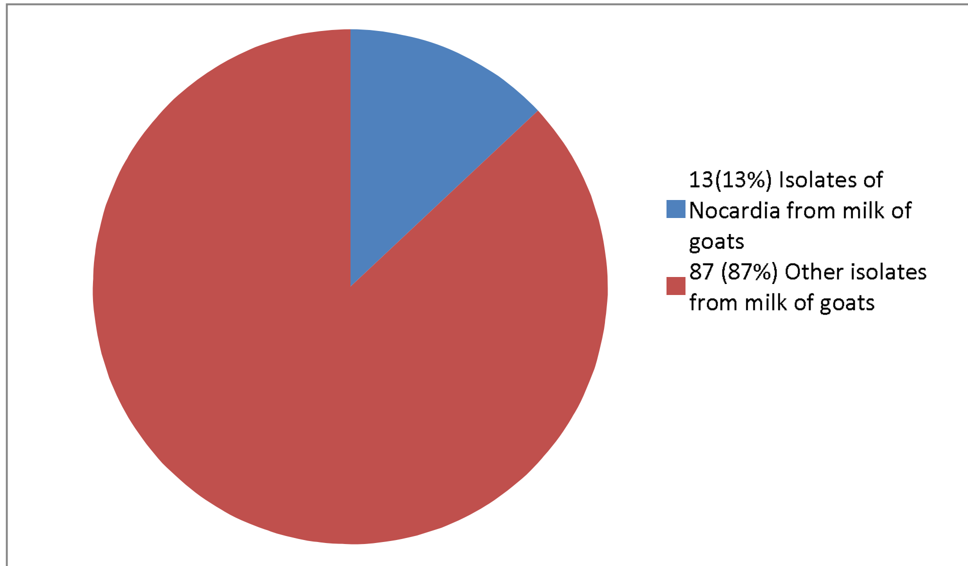



**Fig 2: Frequency of *Nocardia* and others organism from milk samples of cows .**

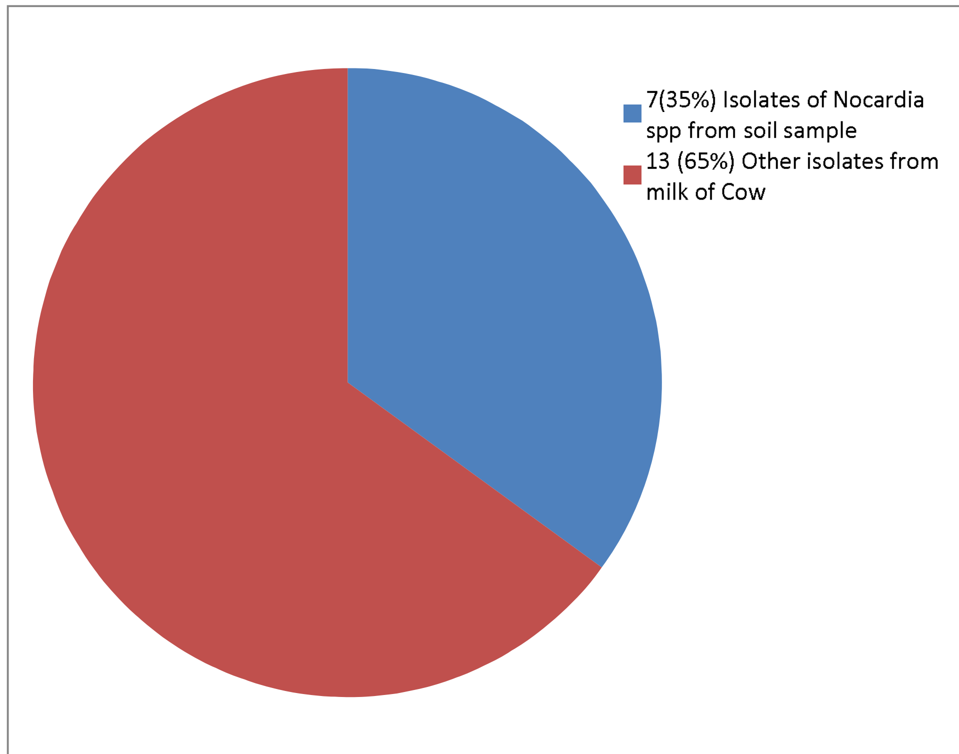



**Figure 3: Percentage of *Nocardia* and others organism from soil samples**

The most visible sign of mastitis is change the milk such as clots, looking watery or blood, the udder alos be warm swollen and painful to the touch and change in appearance it like big mass (Figure 4,5,6,7)

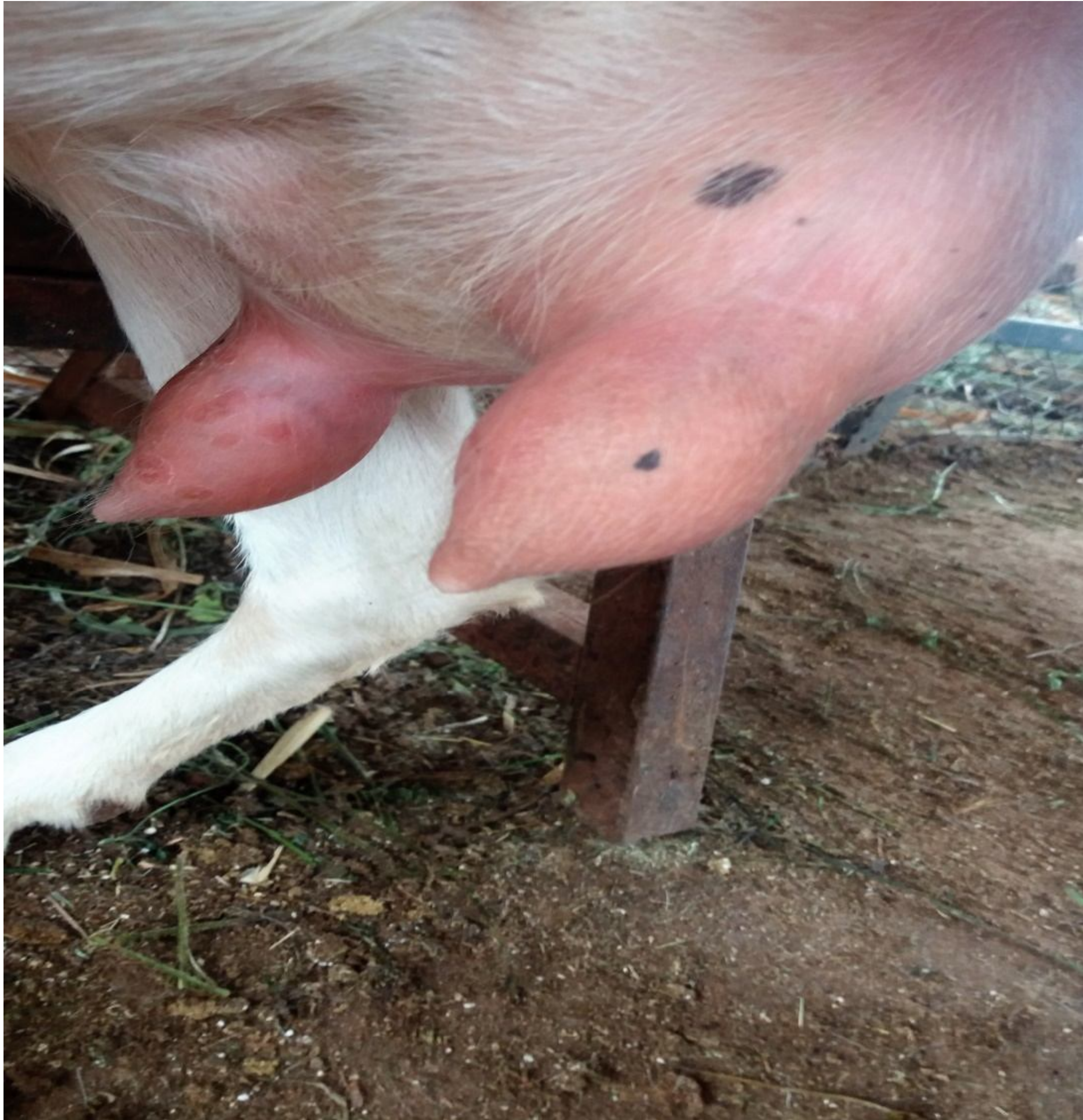


**Fig 4: Note one udder of the goat is small the milk from this udder was their mixed with blood .**

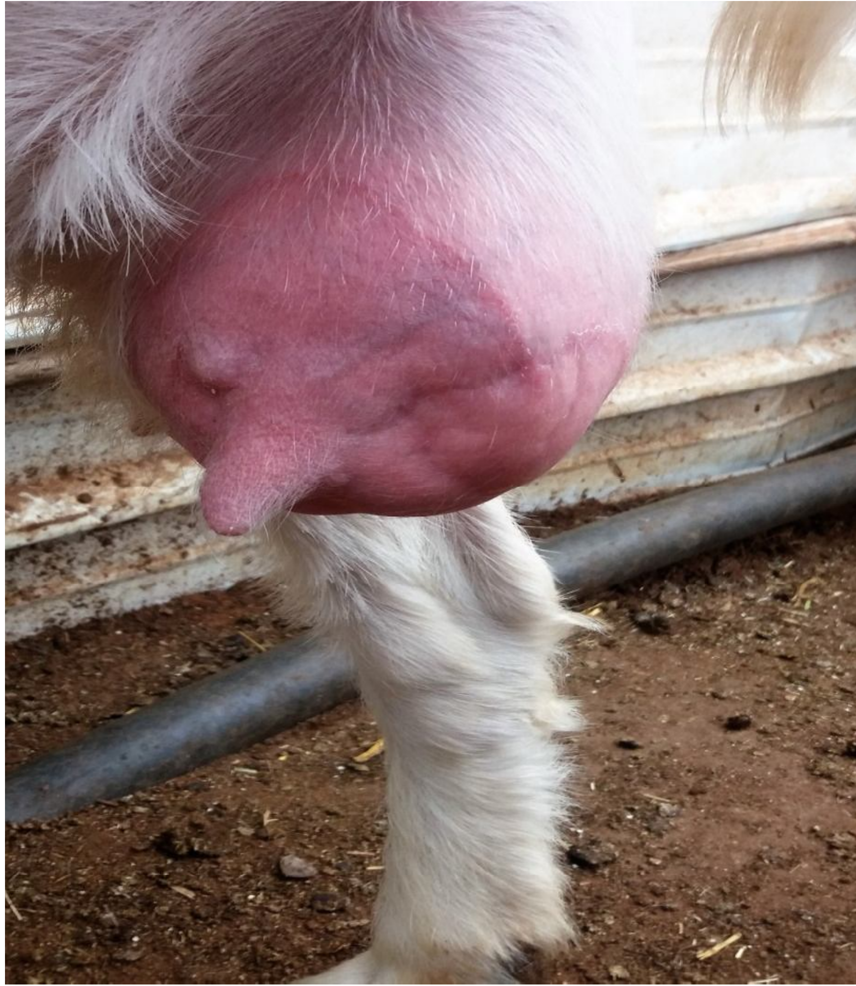


**Fig 5: Note The udder of the goat is big tumour – like mass , The milk from this udder was clotted and watery .**

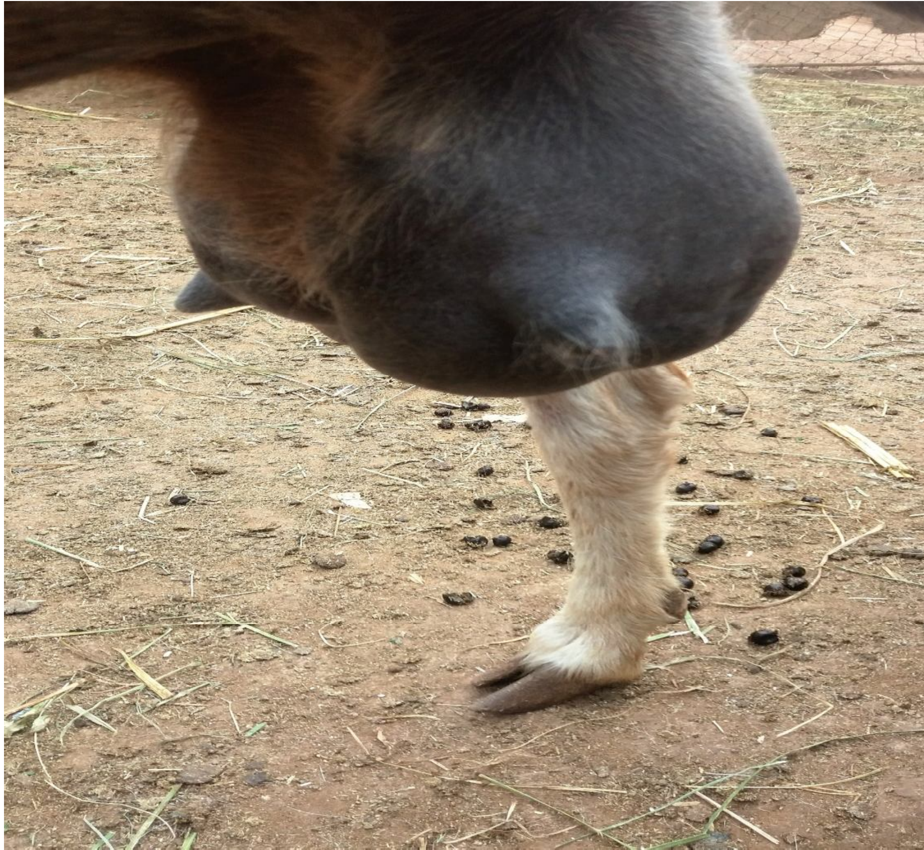


**Fig 6: Note The udder of this goat was in swollen with hard nodules . The milk was thick with blood .**

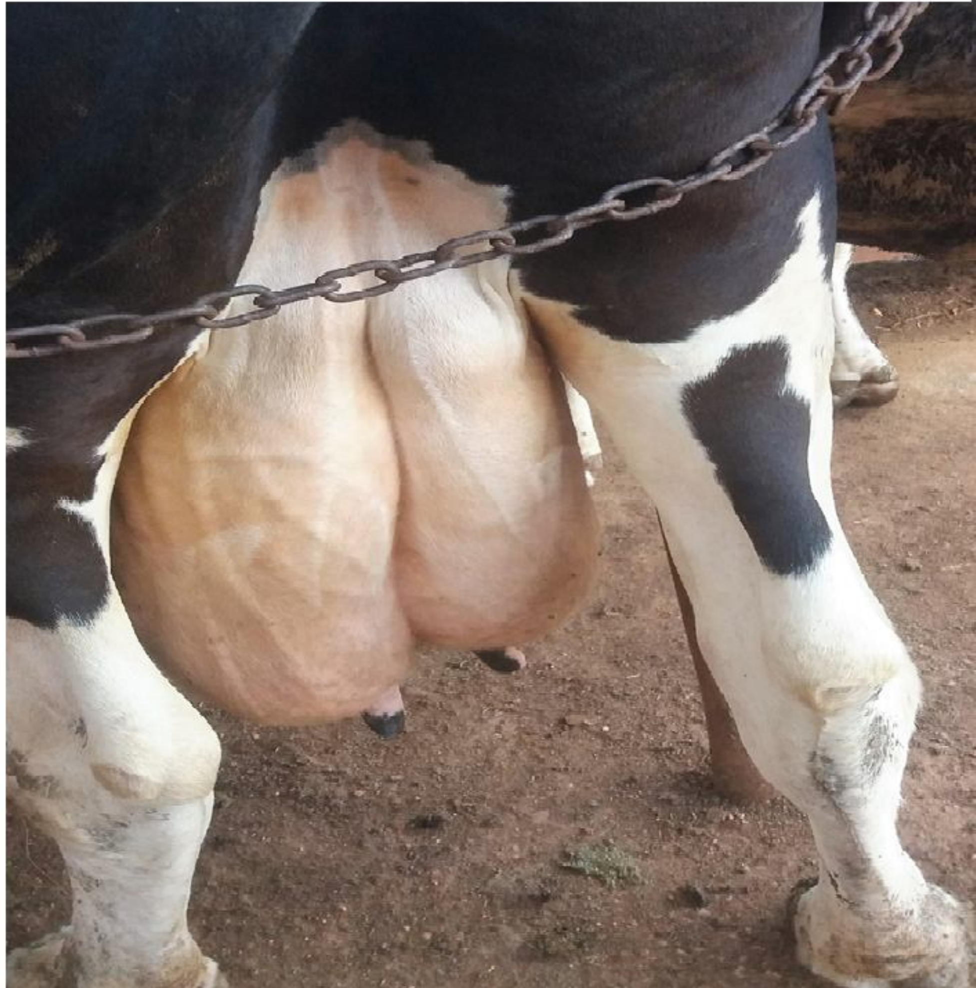


**Fig 7: Note one udder of the cow was in small and there is tumour-like swelling .**

**The milk was thick and with clot.**

## 4-2 Colonies Morphology

The primary culture plates of each of the sample were examined for presences of characteristic *Nocardia* colonies. Morphological and culture pictures of isolated strain are shown in (Table 3). The colour of the colonies which was detected included yellow (Fig- 8), orange to dark (Fig- 9) . Some colonies were dry embedded but some were relatively smooth and easy detected. The aerial hyphae in some strain were heavy.

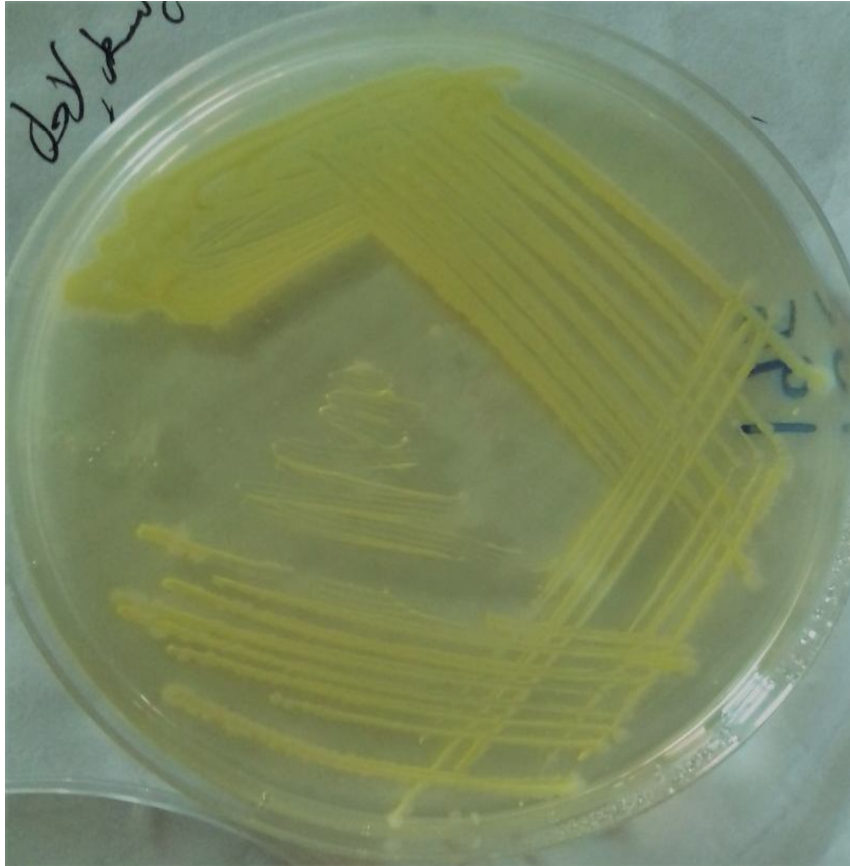


**Fig 8: Growth of *Nocardia* spp strain on GYEA after three days aerobic incubation at 37˚C .The colonies were yellowish smooth and extensive aerial•**

**Fig 9: Growth of *Nocardia* spp on TSA medium, after aerobic incubation at 37 $^{o}$C 96 hours .Note the colonies colours and shapes variation ( orange ,and rough smooth and extensive aerial.**

## 4.3**Microscopic characteristic**

In this study the isolates were Gram positive coccobacilli (figure10). Some isolates were showed coccobacilli and rod partially acid-fast when isolates were stained with modified Ziehl Neelsen method (Figure11).

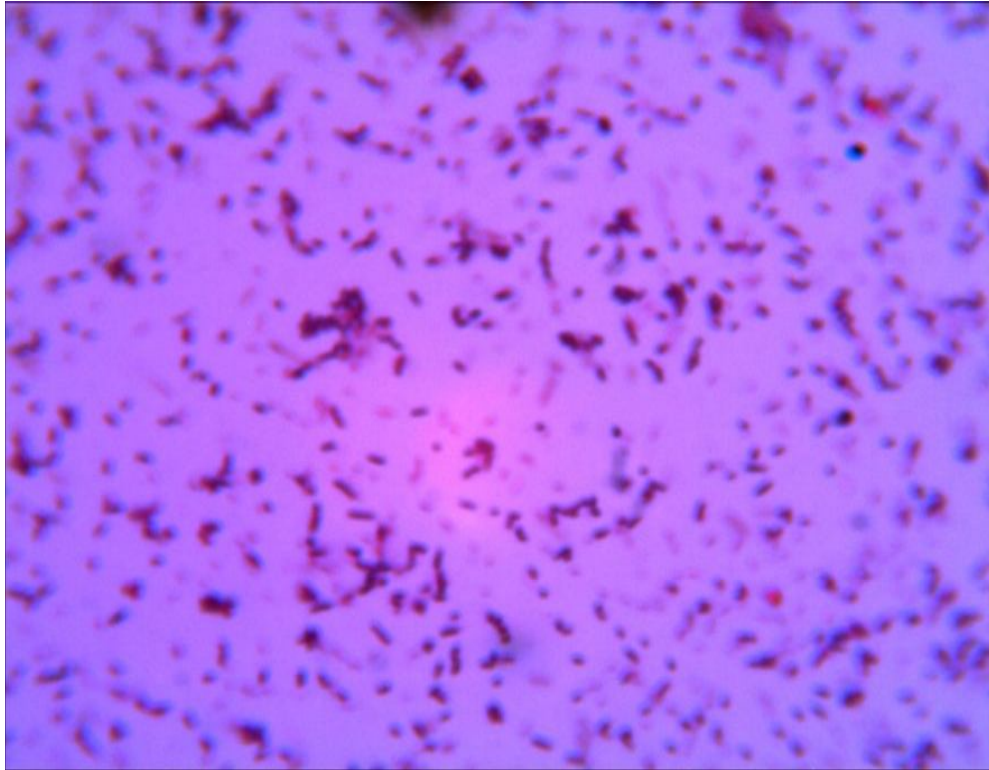


**Fig 10: Gram stained smear of *Nocardia* spp from a goat milk isolates**

**Notice: The colonies were short rods and coccobacilli .**

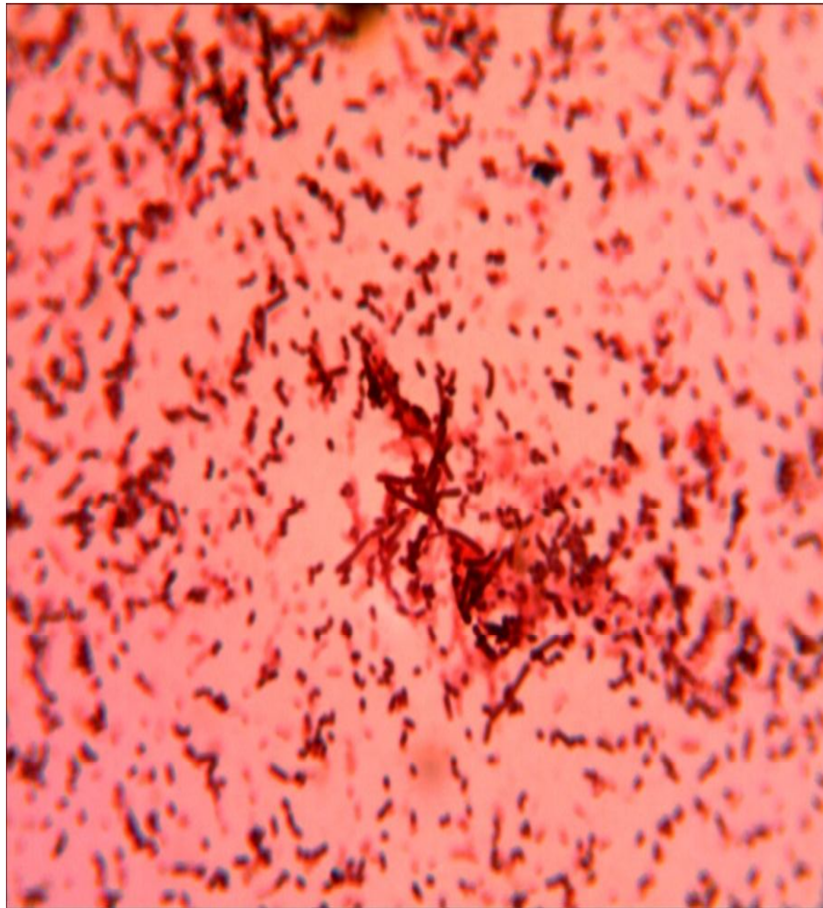


**Fig 11: Modified Ziehl Neelsen stained smear from a cow milk isolates from mastitis**

**Notice the presence of acid-fast short rods and coccobacilli .**

### 4.5 Biochemical test

The isolates were further identified by morphological ,biochemical reactions (urease test, catalase test and degradation of xanthine, tyrosine, casein, and starch), sugar fermentations (Manitol, Sorbitol, Rhaminose and Arbinose sugar),the result  of each isolate are shown in (Table4). In this study the isolated were gave urease and catalase positive (Table 4, Appendix II).

**Table 4  Biochemical Test**

### 4.6 Degradation Tests

In this study all N.*asteroids* they were negative for Tyrosine , xanthine, casein and arabinose medium plate details in(Table 4). They were positive for urea and grew well at 45 ºC .The isolates of N.*facinica* they were positive for Tyrosine, casein medium, they were gave clear zone around colonies (Table 4,Fig 12 and13). All of this strains grew well at 45 ºC.

**Fig 12: Growth of *Nocardia* spp strain on Tyrosine medium. Notes :rough orange colonies and clearance crystal around the colonies indicating degradation of Tyrosine.**

**Fig 13 : Growth of *Nocardia* spp strain on Casein medium after five days aerobic incubation in 37° .Notes clearance zone around the colonies indicating degradation of casein.**

## 4-6 Growth at 45˚C

In this study the isolated of *Nocardia* showed good to growth at 45˚C.

## 4-7 Antibiotic Sensitivity Tests

In this studies the various isolates of *N.asteroides* were resistant to sulphamethaxozole (30µ/ml) and cefotaxime(25µ/ml) but sensitive to ciprofloxacin (25µ/ml), ampicillin (30µ/ml)erythromycin(30µ/ml), ciprofloxacin and tobramycin(25 µ/ml ). The isolates of *N. farcinica* were sensitive to Tobramycin (25µ/ml), Amikacin (25µ/ml) sulphamethoazole (30µ/ml) Trimethoprim (25 µ/ml) but resistant to tobramycin (25µ/ml), ampicillin (25 µ/ml) and ceftazidime (30 µ/ml) (Figure14 ).

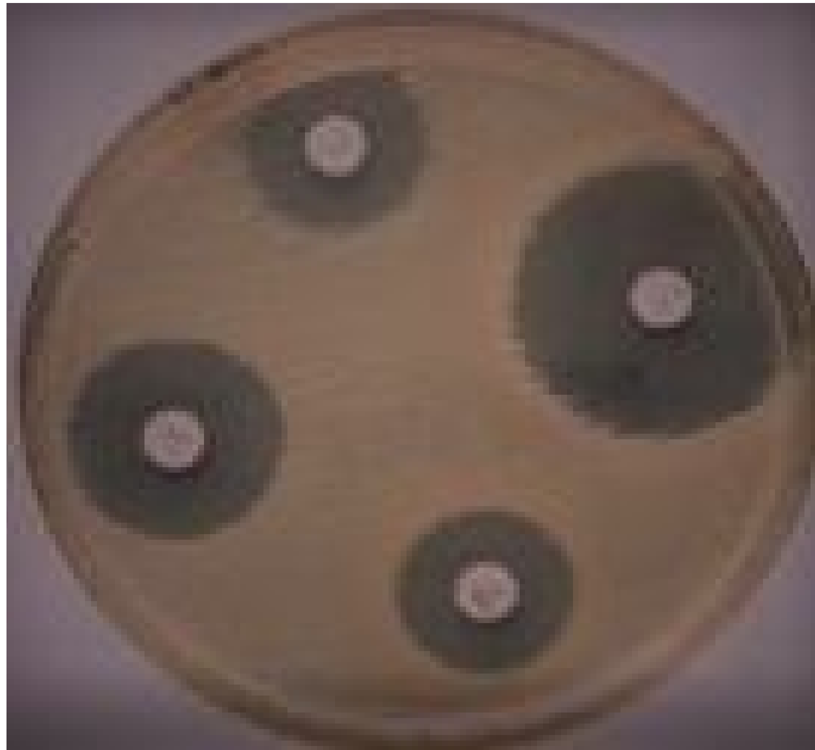


**Fig14 : Susbtibilty test of *Nocardia* . *isolate* on ISO- SENSITEST AGAR Medium plate**

## 4-8 Analysis of Mycolic acids

In the present study *Nocardia spp* positive strains revealed mycolic acid on Thin Layer Chromatography (TLC) plates (Fig .15). The mycolic acid was single spot and it chromatographed with reference *Nocardia* spp (positive control) *and Streptomyces* spp (Negative control).

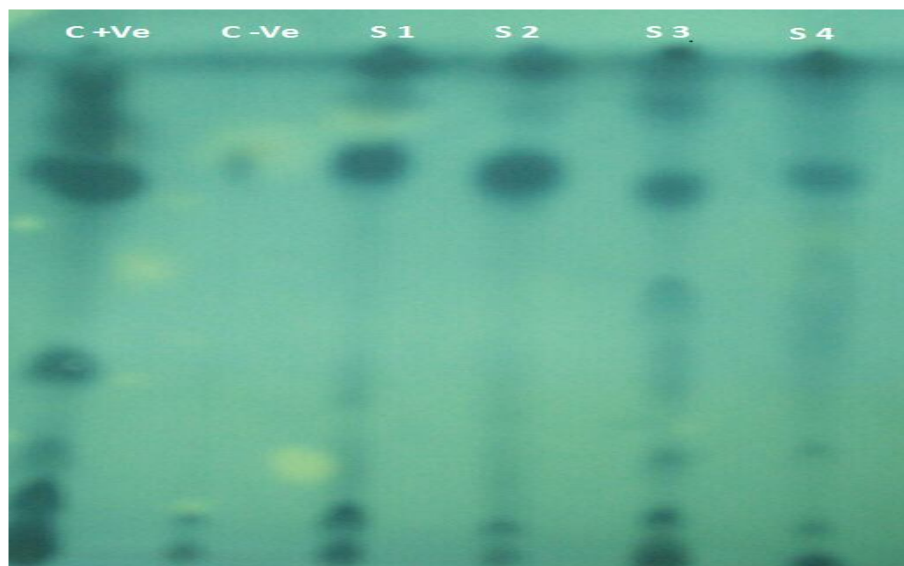



**Fig 15 : Thin layer chromatography analysis of mycolic acids extracted from Nocardia isolate from cow and goats in (S1, S2 .S3 and S4) isolated(C positive control ) (C – ve negative control ).**

## 4.9 Molecular Findings

### 4.9. 1 Purity of the Extracted DNA Chain Reaction

*Nocardia spp* extracted DNA purity was detected by 2% agarose gel, the extracted DNA was clearly seen in pure form and high amounts compared to the DNA marker which contain 40 ng in 5 µL loading (all fragments except typical band DNA). The typical band of DNA fragments is 1000 bp (Figure 16).

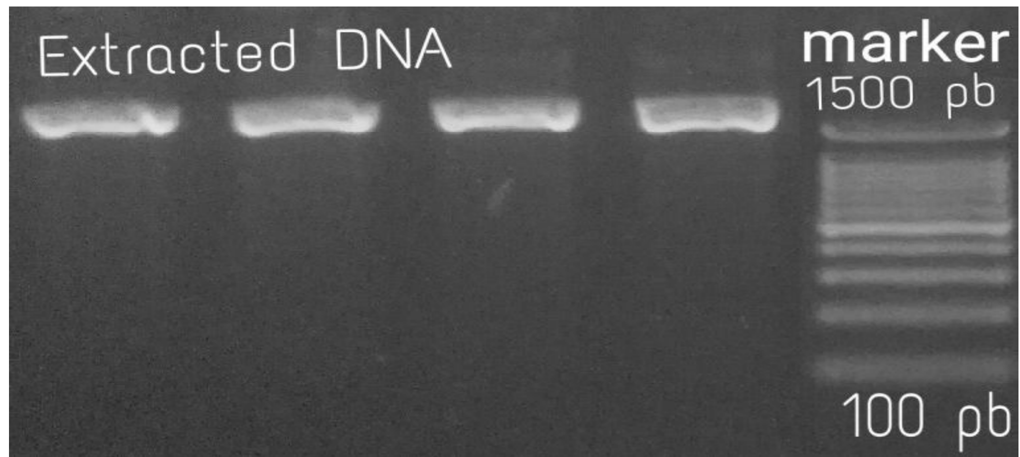



**Figure (16).** ***Nocardia spp*** **extracted DNA separated by 2% agarose gel.**

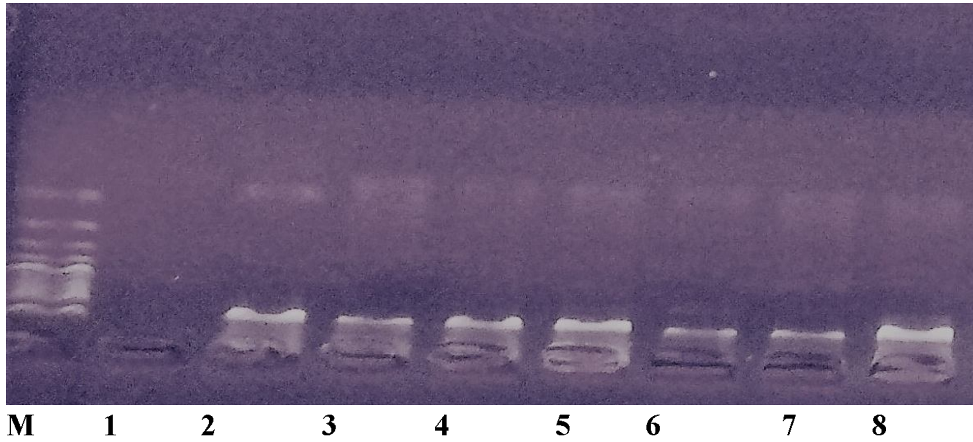



**Fig 17: PCR products separated by 2% agarose gel. M : ladder (100 bp), Lane1:negative control , Lane 2 positive control ( 999bp) and Lane 3 sample ( 1000 bp) , lane ( 4 ,5) samples (999 bp) , samples (6,7,) (1100 bp) and Lane 8 is sample (1000bp).**

### 4.9.2 16S rRNA sequencing and phylogenetic analysis

Isolation of chromosomal DNA and PCR amplification of the 16S rRNA gene were carried out following the methods described by (Kim *et al* 1998). Obtained 16S rRNA nucleotide sequences data were tested on the BLAST electronic system (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to establish a quick phylogenetic position. Following an assignment of the isolate. In the BLAST system, sequences were aligned by computer and corrected manually using PHYDIT for Windows and in comparison to all known sequences of isolates. obtained from Gen Bank database (http://www.ncbi.nlm.nih.gov/nucleotides) (figure18 19,20) Distance estimation and tree topology was done using the neighbor-joining algorithm with the aid of TREECON for windows software (figure 20).

### 4.9.3 Result of 16S rRNA sequencing

In this study out of the 100 milk samples collected from mastitic cow mastitis in Sudan, eleven (11%) revealed *Nocardia* growth which showed gram-positive coccobacilli. eight of eleven samples showed partially acid-fast coccobacilli when were stained with modified Ziehl Neelsen method. The isolates were tentatively identified as members of the genus *Nocardia* on the basis of morphological, biochemical and mycolic acids pattern. Comparative analysis of the 16S rRNA gene sequence confirmed that the isolates fall within the phylogenetic branch which accommodates members of the genus *Dietzia* spp, including *Dietzia maris* (four isolates), and *Dietzia schimae(*one isolates) and other *Dietzia* spp ( Figure 19,23 )

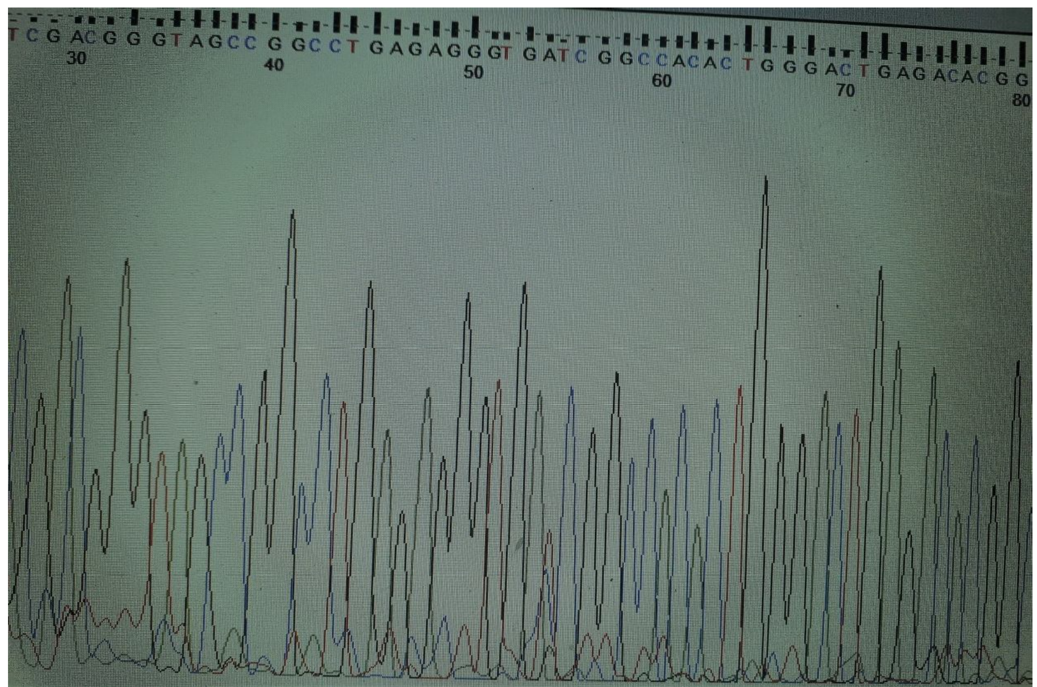
Out of the 100 milk sample collected from Goats in Sudan, 13 (13%) revealed as *Nocardia* and *Nocardia*-Like growth which showed gram- positive coccobacilli, five of thirteen samples showed partially acid-fast cocobacilli when stained with modified Ziehl Neelsen method.The isolates were tentatively identified as member of the genus *Nocardia* on the morphological, biochemical and mycolic acid pattern. Comparative analysis of the 16S rRNA gene sequencing confirm that the isolates fall within the phylogenic branch which accommodates members of the genus of *Rhodococcus* spp as (*Rhodococcus biphenylivorans spp, Rhodococcus pyridine-ivorans*, (figure 20.21.22).

Out of the 20 soil samples collected from the farms in Sudan, seven (7%) revealed as *Nocardia* and *Nocardia* -like growth which showed gram positive coccobacilli, seven sample showed partially acid-fast coccobacilli when stained with modified Ziehl Neelsen method. The isolates were tentatively identified as member of the genus *Nocardia* on the morphological, biochemical and mycolic acid pattern. Comparative analysis of the 16S RNA gene sequencing confirm that the isolates fall within the phylogenic branch which accommodates member genus Mycobacteria including *Mycobacteria flavescence*(two isolate)(Figure 18) and four isolates were gave *Dietzia spp* including *Dietzia papillomatosis* (two isolates) and *Dietzia cinnamea* (two isolates)(figure 23).

The result of 16S RNA and specific of all isolated were analysized by submitted to finch program and analyzed by gene Bank of NCBI database and were recorded there (Figure 18,19,20 ,21,22) . Afterwards, relevant phylogeny or evolutionary tree was depicted

using Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (figure 23).

Fig 18 :




Chromatograms DNA sequencing (sample 18) by Finch TV program . (*Mycobacteria* )

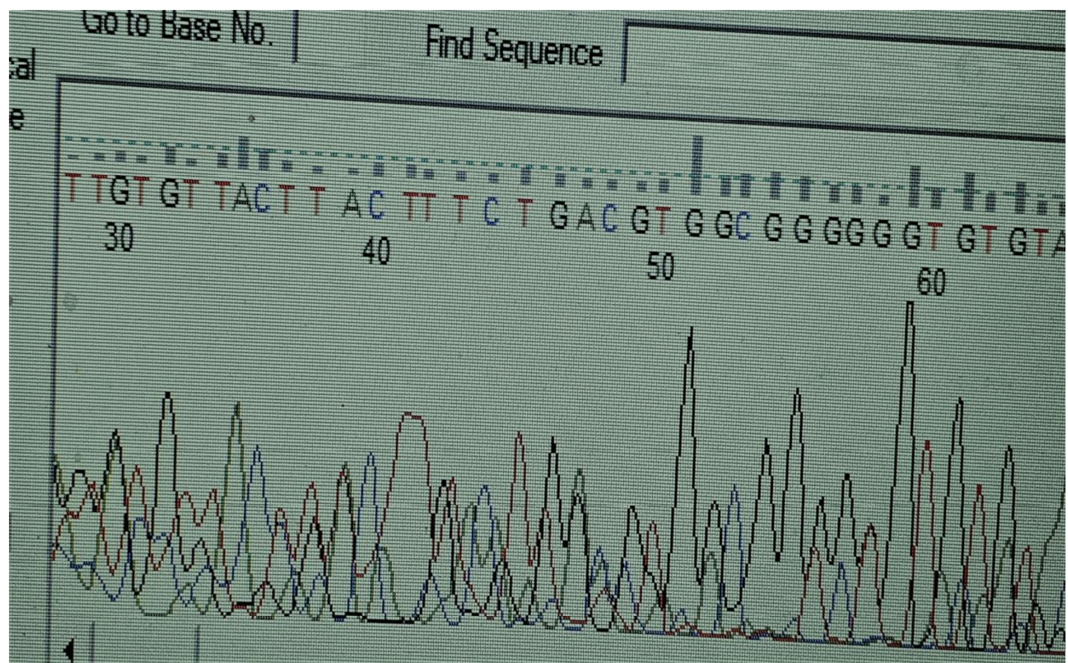



Fig 19: Chromatograms DNA sequencing sample 13 by Finch TV program .(*Dietzia* isolate)

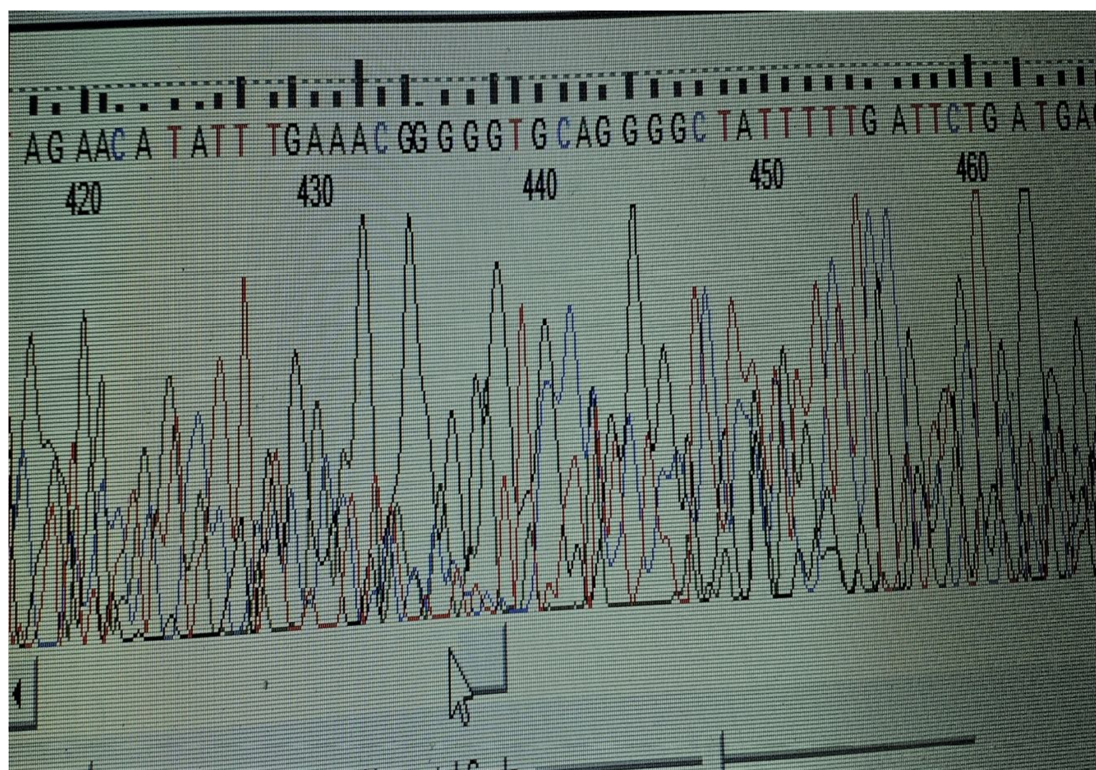



Fig 20 : Chromatograms DNA sequencing sample 13 by Finch TV program .(Rhodoccocus isolate)

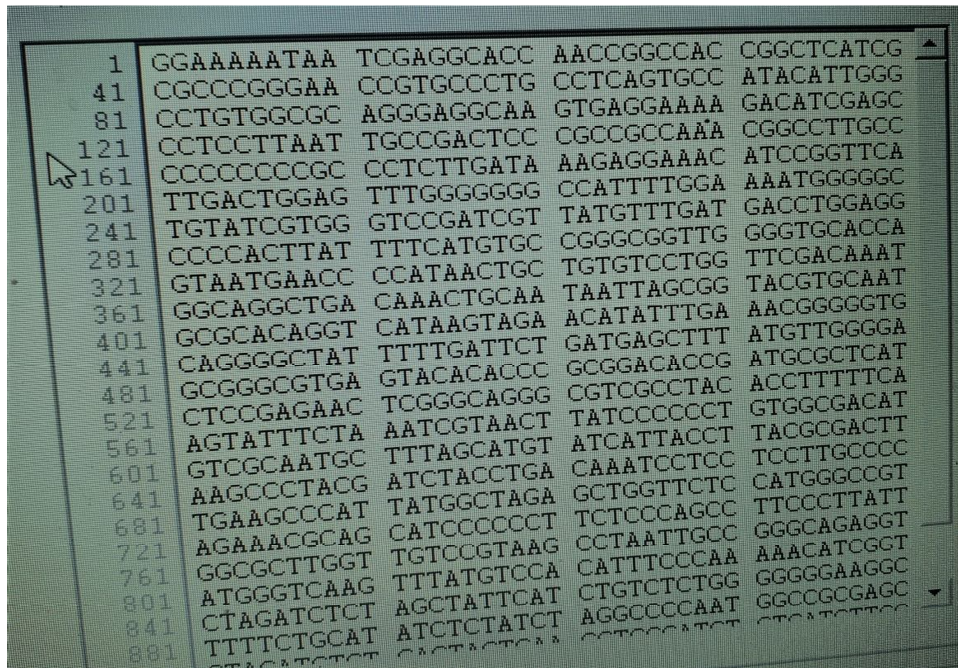



Fig 21 :Chromatograms information of Nucleotide sequences data tests on Finch TV program( sample 20 :*Rhodococcus* ).

Rhodococcus biphenylivorans strain TG9 chromosom

Sequence ID: CP022208.1 Length: 5034221 Number of Match

Range 1: 5003425 to 5003453 GenBank Graphics

| Score | Expect | Identities |
|---|---|---|
| 44.6 bits(48) | 6.6 | 27/29(93%) |

Features: hypothetical protein
molybdopterin dehydrogenase

```
Query  525      GAGAACTCGGGCAGGGCGTCGCCTACACC  553
                ||||||||||||||| ||||| |||||||
Sbjct  5003453  GAGAACTCGGGCAGGTCGTCGACTACACC  5003
```

Fig 22 : (*Rhodococcus* spp) Result of sample 20 after Nucleotide sequences data were tested on the BLAST electronic system (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

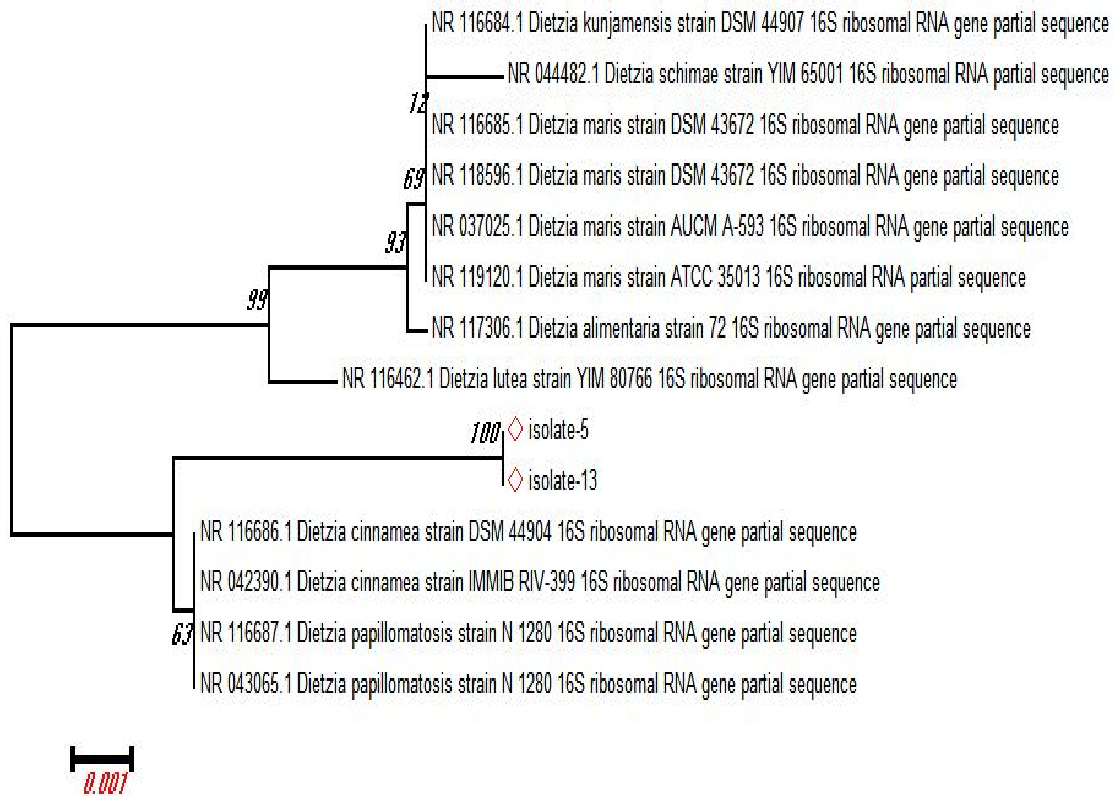



**Fig 23: Phylogenetic tree based on sequences derived from 16S rDNA gene showing the relationship of two milk isolates . The isolates fall within the phylogenetic branch which accommodates members of the genus *Dietzia* but are distinct from *D. maris* and from other *Dietzia* spp.**

# CHAPTER FIVE

# DISCUSSION

The present study was performed to study etiology and prevalence of mastitis in cows, sheep and goats. Mastitis is one of the major disease affecting dairy goats and cows. Bacterial contamination of milk of the affected quarter may pass for human consumption and provide a mechanism of spread of disease to human such as tuberculosis and brucellosis (Blood *et al*., 1983).

In this study the best isolation medium for the soil sample was found to be Tryptic Soya Agar (TSA) supplemented with (5µg/ml) tetracycline, nystatine (5µg/ml) were similar findings reported by (Awatif *et al*., 2001) from soil in Sudan .

Tryptic Soya Agar (TSA) and (GYEA) were found to be successful in the isolation of *Nocardia* and *Nocardia* –like from mastitic milk sample. Other media of *Nocardia and Nocardia*-like such as Tryptic Soya Agar (TSA) Brain Heat Infusion (BHA), Sabouroud Dextrose Agar (SDA), Nutrient Agar and Glucose Yeast Extract Agar (GYEA) had been used for the isolation of *Nocardia* and *Nocardia*-like from clinical sample this result was similar to be found by (Good Fellow *et al*.,1998). All *Nocardia* and *Nocardia*-like grew aerobically on Tryptic Soya Agar Medium (TSAM) slope at 37°C, colonies were visible after 3 – 5 days as yellowish rough, orange, creamy, with aerial hyphae. The edge were with irregular shape, the characteristics which enable making the genus identification of the isolates this results were finding agreed by ( Beaman *et al*.,1995 ).

In this study the frequency 7(35%) similar isolated from *Nocardia* and *Nocardia*-like based on conventional method were

findings agreed by (Awatif et al., 2001; 35% ) from Soil in Khartoum state.

On the other hand among twenty soil samples 7 (35%) of them showed typical occurance of *Nocardia and Nocardia –like* . similar study was directed towards isolation and identification of *Nocardia and Nocardia –like* based on conventional methods, this clearly indicates that the soil can be considered as one of the reservoir for *Nocardia* and *Nocardia* –like this result was a greed by (Awatif et *al* ., 2001 , Al Said et al., 2001,and Good fellow *et*., *al* 1998) isolation of *Nocardia* spp from Sudanese soil.

Genera from the aerobic actinomycetes that closely related to each other were *Dietzia*, *Mycobacterium* and *Rhodococcus* and (Good Fellow *et al*.,1998 and Mohamed *et al*.,2013).

In this study there were indistinguishable between *Dietzia* spp and *Rhodococcus* spp in phonotypic properties, chemical tests and analyzed of mycolic acid confirmed by (Lilian *et.,al* 2010 and Mohamed *et.,al*,2013).

The study also confirmed that *Nocardia and Nocardia-like* was isolated from 11(11%) and 13(13%) of mastitic milk sample from cow and goats revealed the presence of *Nocardia* and *Nocardia*-like based on conventional method were indistinguishable confirmed by (Hamid *et.,al*,1993).

In this study based on conventional method and sensitivity test *N.asteroides* was found sensitive to neomycin, gentamycin streptomycin and resistant to ampicillin, chloroaphinicol ,

On the other hand *N. farcinica* were found sensitive to Tobramycin, Amikacin, sulphamethoazole, Trimethoprim and resistant to tobramycin ampicillin and ceftazidime this result were finding agreed by (Awatif *et al*.,;2001and Savalia *et al* ;1990).

In the present study we were isolated 7(35%) as *N.asteriodes* (5 isolates) and *N.farcinica* (2isolates) from soil samples based on conventional methods, this were finding similar result by (Awatif *et.al* ;2001) from soil in Khartoum State and different findings; in Sudan. Different studies appeared with different findings; in Sudan (six of 15%) as N.*farcinica* by (Maldonado *et al*.,2004).

The results obtained from this study suggested a frequency of (35%) of *Nocardia and Nocardia*-like in soil sample based *on* conventional method which is nearly by (Masoumeh *et al*., 2017; (32% ) and higher than (Hossein *et al*.,2017;( 27.7%) from Iran soil hospital and (Samane *et.al*.,2016; 8.5%) from Iran.

In this study based on 16S rRNA the five *Dietzia* of seven isolated from soil and milk samples was fining similar agreed by(Mohamed *et*., *al* 2013 ;5% ) from cattle in Sudan, and less than (Lilian *et*.,*al* 2010 ;8% ) from human patient in Spain.

In this study mastitis in goats has little attention in many countries including the Sudan, although the disease is known to cause significant economic loss this result was finding agreed by (Radostitis *et al*., 1996; and Omyama et *al*., 2007).

In the present study the isolated 11(11%) from goats which was almost similar the results by (Maldonado *et al*., 2004; 15%) from Goats in Sudan, this result was high percent compared by (Mogahid *et al* 2010; 3.3%) among patients in Sudan.

In our own data suggest that *Dietziae* have the potential to act as opportunistic pathogens the result was agreed by (Lilian *et al* 2010 8%) from human patient in Spain.

In this study the DNA extraction method was used STET buffer showed acceptable and satisfactory results for molecular epidemiology techniques such as PCR and PCR-RFLP.The described method is simple, fast, cost-effective, sensitive, and highly reproducible for DNA extraction from *Nocardia* and *Nocardia* –like, and there is no need for a skillful. This result finding was agreed by (Mehdi *et at*., 2017) in Iran.

In this study the Sequencing of the 16S rRNA gene 11(11%) isolates from milk sample of cow indicated that the isolates fall within the phylogentic branch that accommodates members of the genus *Dietzia* spp. *Rhodococcus* equi and *Dietzia* spp, are closely related actinomycetes that show similar phenotypic properties these were findings agreed by (Pilares *et al*., 2010) In humans pathogen.

In this study we found *Nocardia spp*, *Rhodococcus* ,*Mycobacteria* and *Dietzia spp* were similar phenotypic properties this result was a greed by (Mohamed *et*., *al* 2013) from cattle in Sudan.

In this study the isolation of *Dietzia* spp, Could represent a new significant cause of clinical mastitis in cattle, these findings are agreed with previous study conducted in Sudan by (Mohamed *et.,al* 2013).

In this study from *Nocardia* and *Nocardia*-like in phenotypic it were indistinguishable this result finding agreed by (Awatif *et al*., 2001; and Mohamed *et. al* 2013) in Sudan

In this study were found the result 16S RNA rapid and specific to distinguishable for *Nocardia* and *Nocardia* –like except between

*Nocardia* ,*Mycobacteria*, *Rhodococcus*-like and *Dietzia* these result findings were agreed with previous study conducted by (Mohamed *et al*., 2013) from cattle in Khartoum state.

*Dietzia* bacteria appear to be widely distributed in the environment, and reports of isolates from clinical material are increasing (Koerner *et al*., 2009).

Therefore molecular identification is necessary for definitive identification of *Nocardia* and *Nocardia* –like this result finding agreed by (Minero *et al.,* 2009, Almeida *et al*., 2013 and Muñoz *et al*., 2014).

Finally we consider the recognition of *Nocardia* and *Nocardia*-like based on phenotypic and mycolic acids test were strenuous, but definitive identification was attainable by molecular methods, this result was finding agreed by (Mohamed *et al*., 2013) from human patient in Saudi.

Most of the surveyed farms were small in size , so problem of disposal, ventilation and draining have been clearly observed .Most of the studied farms building material were traditional made of mud ,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 .Also according to my own observations during this study these traditional building materials causes injury to udder and teat of the cow 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 this finding agreed with (William *et al*., 1995).

According to my own observations during this study hygienic procedures in general are very important in controlling mastitis this were findings agreed by(Bushnell *et al*., 1984 ). Among this study the presence of large numbers of the potential pathogens in the immediate environment of the animal might have induced mastitis ,milking management and milking technique have been shown to be an important risk factors with machine milking being more risky than hand milking and calf suckling was findings agreed (Hamman *et al*., 1991 ) .

In the study of the causes emphasis was put on determine role of *actinomycetes* notably Nocardiae in causing bovine mastitis .little work was done in the Sudan regarding bovine mastitis due to actinomycetes infections (Shigidi and Mamoum, 1981; Hamid *et al*., 1998).

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATION

## 6.1. Conclusions

The present study concluded that:

**1**-Soil can be considered as main source of *Nocardia andNocardia* -like .

**2**-Mastitis due to *Nocardia* in dairy Ruminant is a real problem .

3-Mixed infection with more than one type of organism was more common among goats showing clinical evidence of mastitis.

**4**-The milk samples examined confirm the present of *Nocardia* as part of the causative agent of mastitis in goats.

**5**-Nocardia and Nocardia-like dairy Cows and sheep is real problems.

**6**-To prevent goats , Cows and sheep against *Nocardia* and *Nocardia* like are need more efforts and advice to farmers.

**7**-Much efforts are needed to forward technical advice to farmers on proper hygienic procedures, and consistent extension services are needed to address farmers on dairy farms .

**8**-Mastitis as disease that has received little attention in Sudan, so far especially the subclinical cases .

**9**-They was a needed for more systemic survey to investigate the extent and the clinical significance of *Nocardia* and *Nocardia*-like infection associated with mastitis in dairy cows and goats in the Khartoum state .

**10**- The PCR is very fast to detected microorganism and more sensitive and useful.

**11**-The farmers are not helpful for researchers to detect microorganism that cause mastitis .

**12**- The DNA extraction method using STET buffer showed acceptable and satisfactory results for molecular epidemiology techniques such as PCR ,the described method is simple, fast, cost-effective, sensitive, and highly reproducible for DNA extraction from *Nocardia*, and *Nocardia*- like there is no need for a skill full specialist to perform this method.

13-The recovery of isolates belonging to the genus *Dietzia* in the present study represents a new significant cause of clinical mastitis in cattle..

**6.2. Recommendation**

From the result of the present study the following can be recommended:

**1**-The common observation in many in many of the farms studied were poor sanitary conditions and practices , problems of nutritional management ,confinement of animals in over –crowed and poorly ventilated and constructed housing. All of the factors increase mastitis prevalence and incident. So advice must be forwarded to the farmers on proper hygiene measurement, and these advices should be turned into practicality by large farms owners .

**2**-Farmers used drugs for treatment of mastitis without selection, the disk sensitivity test did provide guidance to the choice of effective antibiotic ,therapy .

**3**- Many mastitis pathogens in field showed a wide spectrum resistance to antibiotics treatment . So this need more investigation

,especially in cases of actinomycetes for mastitis and to determine antibiotic sensitivity for these microorganism .

**4-**Daily examination of the udder strip cup and reporting and the milk using the suspected cases to veterinarian or his assistant for necessary care.

**5-**The standards of milking hygiene equally was very low and the preventive measures like the use of udder disinfectants ,strip cup, post milking teat dipping, dry cow therapy and treatment of clinical cases as they occur was a rare sight on these farms. Adoption of conventional milking hygiene practices is suggestion for mastitis control.

**6-**Vaccination of the dairy cows has so far proved to be a very limited value in the control of mastitis through out world .

**7-**Little efforts and works are done in farms investigated to detected subclinical mastitis of the dairy herd .No clear awareness of the farmes regarding this subject .

**8-**Early diagnosis of *Nocardia* mastitis is needed for treatment in order to avoid serious chronic consequences.

**9-**Further research is needed to detect the environmental sources accurate of *Nocardia.*

**10-**The Veterinary Medicine Centers should be provided with tools for microbiological investigation to prevent milk from beeing source of infection.

11-Application of molecular techniques to it more useful and facilitate the identification of *Nocardia* and *Nocardia*-like than conventional methods.

12-More Efforts are needed to validate the detection methods and to conclude on their true prevalence in comparison to other pathogens associated with mastitis.

13- We need more effort to detect *Dietzia* as causative agent for mastitis in cows in Sudan .

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# APPENDEX I

# MEDIA

**Tryptic Soya Agar**

Tryptic soya agar, 30 g; distilled water, 1 litre; pH 7.2 autoclaved at 121°C for 15 minutes.

**Brain heart infusion agar**

Brain heart infusion agar, 30 g, distilled water, 1litre; pH 7.2 autaclaved at 12A1 ˚C for 15 minutes.

**Glucose Yeast Extract Agar GYEA**

Glucose 10g, yeast 10g, agar 14g, distilled water, 1litre; pH 6 to 8 autoclaved at 121 ˚C for 15 min.

**Nutrient agar (Oxoid, $CM_3$)**

Nutrient agar, 28g, distilled water 1 litre, pH 6.8; sterilized by autoclaving at 121°C.

**Starch medium agar**

Starch ten gram, nutrient agar (oxoid) twenty eight gram, one liter distilled water sterilized by autoclaving at 121°C for 15 minutes.

**Tyrosine medium**

L- Tyrosine, 4 gm, nutrient agar (oxoid) twenty eight gram litre distilled water sterilized by autoclaved at 121°C for 15 minutes.

**Xanthine medium**

Xanthine, 4 gm, nutrient agar (oxoid) $CM_3$, twenty eight gram one liter distilled water sterilized by autoclaved at 121°C for 15 minutes.

**Casein medium**

Hundred ml of skimmed milk powder (10% W/V, oxoid) was autoclaved at 121°C for 15 minutes, and then was added to sterile molten GYEA to give a final concentration of 10% W/V.

**Urea medium:**

Four gram urea agar base, 95 ml of distilled water was sterilized by autoclaving at 121°C for 15 minutes, and then 2gms of urea crystals were added to 5ml distilled water. The preparation was dispersed in a bottle and allowed to set in slope position.

**Peptone water sugars:**

The reaction of 900 ml peptone water was adjusted to pH 7.1-7.3 so that the addition of 10 ml of Androde's indicator will bring it to pH 7.5 the preparation was then sterilized by autoclaving at 121°C for 20 minutes. This medium is pink when it is hot but the color fades out by cooling. Then 5-10gms of appropriate sugar was dissolved in 90ml of distilled water and steamed for 30 minutes. The sterilized peptone water with Androde's indicator has a pink color in acid medium (Salicin, Sorbitol, Mannitol, Rhaminose ).

**Rhamnose Sugar**

Rhamnose Sugar was prepared according to Barrow and Feltam, (1993) by dissolving 0.5 gram of Rhamnose in 90ml water and steamed for 30 minutes. Aseptically this was added to 90 ml of

sterile peptone water with indicator then distributed in to sterile test tubes (5mleach ) and steamed for 30 minutes the positive result change the color of medium to pink.

**Mannitol Sugar**

Mannitol Sugar was prepared according to Barrow and Feltam, (1993) by dissolving 0.5 gram of Mannitol in 90ml water and steamed for 30 minutes. Aseptically this was added to 90 ml of sterile peptone water with indicator then distributed in to sterile test tubes (5mleach ) and steamed for 30 minutes the positive result change the color of medium to pink.

**Arabinose Sugar**

Arabinose Sugar was prepared according to Barrow and Feltam, (1993) by dissolving 0.5 gram of Arabinose in 90ml water and steamed for 30 minutes. Aseptically this was added to 90 ml of sterile peptone water with indicator then distributed in to sterile test tubes (5mleach ) and steamed for 30 minutes the positive result change the color of medium to pink.

**Sorbitol Sugar**

Sorbitol Sugar was prepared according to Barrow and Feltam, (1993) by dissolving 0.5 gram of Sorbitoin 90ml water and steamed for 30 minutes. Aseptically this was added to 90 ml of sterile peptone water with indicator then distributed in to sterile test tubes (5mleach ) and steamed for 30 minutes the positive result change the color of medium to pink.

## ISO –SENSITEST AGAR

| | gm/litre |
|---|---|
| Hydrolysed casein | 11.0 |
| Peptones | 3.0 |
| Glucose | 2.0 |
| Sodiumchloride | 3.0 |
| Soluble starch | 1.0 |
| Disodium hydrogen phosphate | 2.0 |
| Sodium acetate | 1 |
| Magnesium glycerophosphate | 0.2 |
| Calcium gluconate | 0.1 |
| Cobaltous sulphate | 0.0001 |
| Cupric sulphate | 0.0001 |
| Zinc sulphate | 0.0001 |
| Ferrous sulphate | 0.0001 |
| Manganous chloride | 0.0002 |
| Menadione | 0.0001 |
| Cyanocobalamin | 0.0001 |
| L-Cysteine hydrochloride | 0.2 |
| L-Tryptophan | 0.1 |
| Pyridoxine | 0.003 |

| | |
|---|---|
| Pantothenate | 0.003 |
| Nicotinamide | 0.003 |
| Biotin | 0.0003 |
| Thiamine | 0.0004 |
| Adenine | 0.01 |
| Guanine | 0.01 |
| Xanthine | 0.01 |
| Uracil | 0.01 |
| Agar | 8.0 |
| pH | 7.4 |

# APPENDIX II

# STAINS

## Modified Ziehl –Neelsen Stain ( ZN stain)

### Solutions:

**(A)ZN carbol fuchsin**

| | |
|---|---|
| Basic fuchsin (powder) | 10 g |
| Phenol (crystalline) | 50 g |
| Alcohol (95% or100% ethanol) | |
| Distilled water | 1000mL |

Dissolve the fuchsin in phenol by placing them in 1L flask over boiling water. Bass for about 1mint, shaking the content from time to time when solution complete add the Alcohol and mix thoroughly.

Then add the distilled water. Filter the mixture before use.

**(B)Acid Alcohol decolorizer**

| | |
|---|---|
| Concentrated hydrochloric acid (HCL) | 75ml |
| Industrial methylated spirit | 2425ml |

Pour the methylated spirit in toa large flask. Place the flask 5-8 cm of cold water in the snik, add the HCL and cover the top of the flask to top fumed escaping. Leave for 10 minutes. Decant into a labeled bottle for use. The final concentration of HCL is 3%.

**(C)Methylene counter stain**

| | |
|---|---|
| 1% stock solution | 40 ml |
| Distilled water | 360 ml |

Add the dye diluted to make the working solution to the distilled water in a bottle and shake to dissolve.

## Procedure:

**Results:**

- Acid fast organisms are red.
- Non acid fast are blue.

1. **Gram 's stain**

## Solutions:

**(A) Crystal violet stain**

To prepare 1 liter

| | |
|---|---|
| Crystal violet | 29g |
| Ammonium oxalate | 9g |
| Ethanol or methanol, absolute | 95 ml |
| Distilled water | to liter |

Weight the crystal violate on a piece of clean filter paper. Transfer to a brown bottle Premark to hold 1 liter.

Add the absolute alcohol and mix until the dye is completely dissolved.

Weight the ammonium oxalate and dissolve in about 200 ml of distilled water, add to the stain. Make up to 1 liter mark with distilled water, and mix well.

Label the bottle and store it at room temperature. The stain is stable for several months.

**(B) lugol's iodine**

To prepare 1 liter:

Potassium iodide 20g

Iodine 10g

Distilled water to 1 liter

Weight the potassium iodide and transfer to a brown bottle premarket to hold 1 liter.

Add about a quarter of the volume of water and mix until the potassium iodide is completely dissolved.

Weight the iodine, and add to potassium iodide. Mix until iodine is dissolved.

Make up to 1 liter mark with distilled water, and mix well. Label the bottle and mark it toxic. Store in a dark place at room temperature.

**(C) Acetone-alcohol decolorize**

To prepare 1liter:

| | |
|---|---|
| Acetone | 500 ml |
| Ethanol or methanol, absolute | 475 ml |
| Distilled water | 25 ml |

Mix the distilled water with the absolute alcohol. Transfer the solution to screw-cap bottle of 1 liter capacity.

Measure the acetone and add immediately to the alcohol solution, mix well.

Label the bottle, and mark it highly flammable. Store at room temperature.

**( D) Neutral red, 1g/L**

To make 1liter:

| | |
|---|---|
| Neutral red | 1g |
| Distilled water | 1liter |

Weight the neutral red on a piece of filter paper and transfer it to a bottle of 1liter capacity.

Add about quarter of the volume of water, and mix until the dye is completely dissolved.

Add the remainder of the water, and mix well.

Label the bottle and store at room temperature. The stain is stable for several months.

**Procedure**

- Athin film smear is prepared , dried and fixed

  Crystal violet is applied for 30 second s and

  Replaced lugol's iodine for 30 second s.

  - Decolorized with absolute alcohol acetic or acetone until on more violet emanates from the smear (1-2 second s )
- Then washed with water.

Counter stain 1:10 carbol fuchion for 30 second s.

- Washed in water, blot dry examined under oil immersion lens
- **Results:**
- Gram positive bacteria strains violet.
- Gram negative bacteria strains red.

# APPENDIX III

## Molecular reagents

**10 X TBE buffer**

Formula in grams per liter

Tris base……………………………………108 gm

Boric acid……………………………..……..…..55gm

EDTA………………………..…………...40 ml of 0.5M

Deionized water………………..…………..…1 liter

**Preparation**

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

**1X TBE buffer**

Formula in ml per liter

10 X TBE……………………………………….10 ml

Deionized water………………………………….90 ml

**Preparation**

Ten ml of 10 X TBE buffer was added to 90 ml deionized water were heated until completely dissolved.

**Ethidium bromide solution**

**Formula in grams per 1ml**

Ethidium bromide……………………………10 mg

Deionized water…………………………….…I ml

**Preparation**

Ten milligrams of ethidium bromide powder were dissolved into 1000 µl deionized water, and kept into brown bottle.

# APPENDIX IV

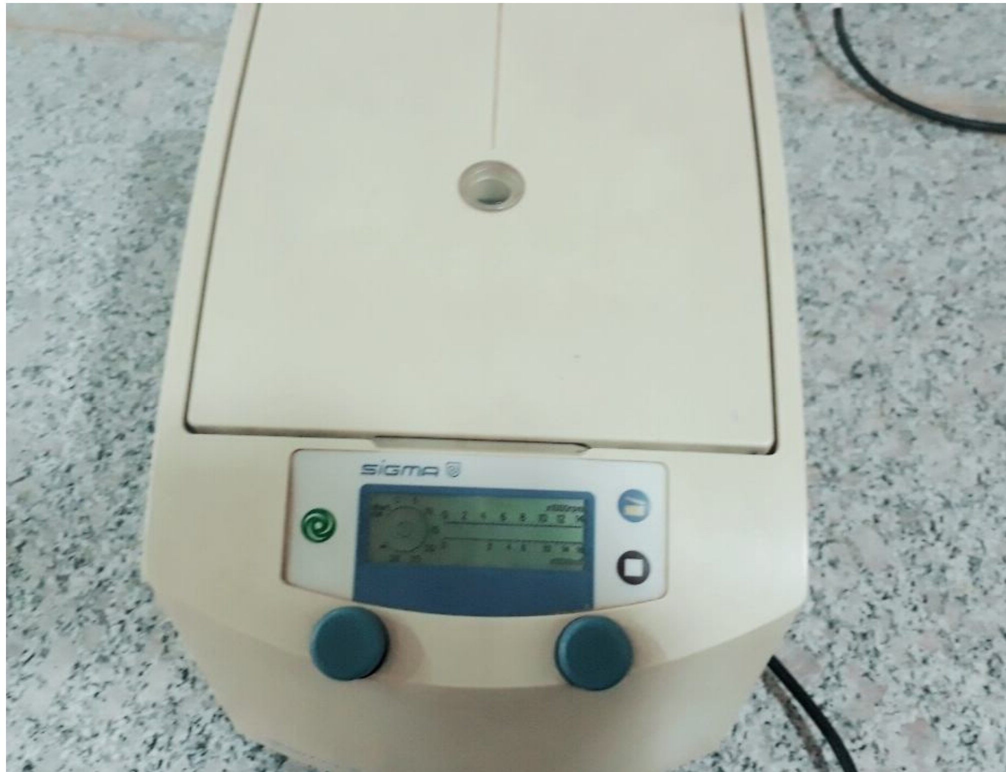


**Fig24 : Sigma 1-14 Germany Microcentrifuge Device (In Medical Research lab at Sudan University of Sciences and Technology).**

**Fig 25 :CLASSIC K960 China Thermocycle Device. (In Medical Research laboratory at Sudan University of Sciences and Technology).**

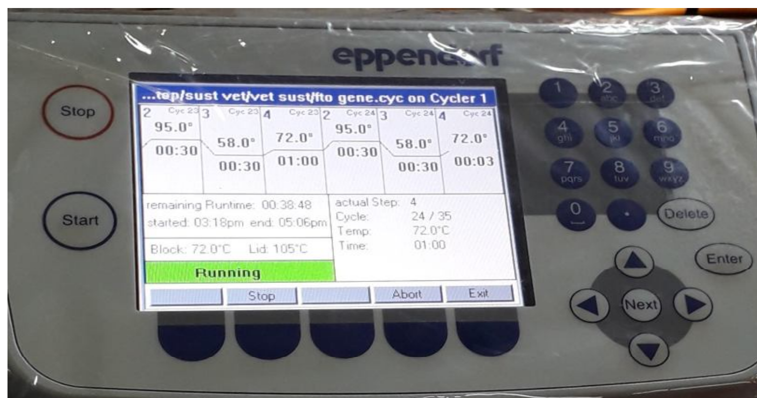


**Fig 26: Eppendrof Thermocycl Device (In Research Laboratory College of Veterinary Medicine Sudan University of Science and Technology) .**

**Fig27: Gel Electrophoresis and Power Supply Device(In Medical Research lab at Sudan University of Sciences and Technology).**

**Fig 28: UV Light Transilluminater Device (In Research laboratory College of Veterinary Medicine Sudan University of Science and Technology) .**

**Fig 29: Block Heater bathroom (In Research laboratory College of Veterinary Medicine Sudan University of Science and Technology) .**

# APPENDIX V

*Maxime* **PCR PreMix Series** *Research Use Only*

**ISO 9001/14001 Certified Company**

# *Maxime* PCR PreMix Kit ( *i*-Taq )

**for 20µl rxn / 50µl rxn**

**Cat. No. 25025**(for 20µl rxn, 96 tubes) **Cat. No. 25026**(for 20µl rxn, 480 tubes)
**Cat. No. 25035**(for 50µl rxn, 96 tubes)

## DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
*Maxime* PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

## STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

## CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

## CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20µl rxn) — 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50µl rxn) — 96 tubes

| Component in | 20 µl reaction | 50 µl reaction |
|---|---|---|
| *i*-Taq™ DNA Polymerase(5U/µl) | 2.5U | 5U |
| dNTPs | 2.5mM each | 2.5mM each |
| Reaction Buffer(10x) | 1x | 1x |
| Gel Loading buffer | 1x | 1x |

**Note :** The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

## PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   **Note 1 :** Recommended volume of template and primer : 3µl~9µl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   **Note 2 :** Appropriate amounts of primers
   - Primer : 5-20pmol/µl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20µl or 50µl.
   Do not calculate the dried components.

**Example** **Total 20µl or 50µl reaction volume**

| PCR reaction mixture | Add | Add |
|---|---|---|
| Template DNA | 1 ~ 2µl | 2 ~ 4µl |
| Primer (F : 10pmol/µl) | 1µl | 2 ~ 2.5µl |
| Primer (R : 10pmol/µl) | 1µl | 2 ~ 2.5µl |
| Distilled Water | 16 ~ 17µl | 44 ~ 41µl |
| Total reaction volume | 20 µl | 50 µl |

**Note :** This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   **Note :** If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   **Note :** This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

## SUGGESTED CYCLING PARAMETERS

| PCR cycle | | Temp. | PCR product size: 100-500bp | 500-1000bp | 1Kb-5Kb |
|---|---|---|---|---|---|
| Initial denaturation | | 94℃ | 2min | 2min | 2min |
| 30-40 Cycles | Denaturation | 94℃ | 20sec | 20sec | 20sec |
| | Annealing | 50-65℃ | 10sec | 10sec | 20sec |
| | Extension | 65-72℃ | 20-30sec | 40-50sec | 1min/Kb |
| Final extension | | 72℃ | Optional. Normally, 2-5min | | |

## EXPERIMENTAL INFORMATION

- *Comparison with different company kit*

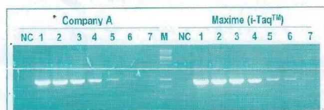


**Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.**
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
**Lane M,** SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC,** Negative control

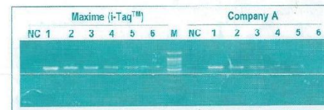


**Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).**
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
**lane M,** SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC,** Negative control

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*For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.*

*ISO 9001/14001 Certified Company*

# SiZer™ DNA Markers

## DESCRIPTION

iNtRON supplies a wide range of products for accurate size and mass estimations (quantitation) of nucleic acid fragments. Nucleic Acid Markers are available for sizing linear, or supercoiled DNA and single-stranded RNA fragments. A variety of these markers are available in the ready-to-use SiZer™ formats.

SiZer™ DNA Markers are ideal for determining the size of double-stranded DNA from 60~10,000bp base pairs. The SiZer™ DNA Markers consist of 7 ~ 15linear double-stranded DNA fragments. Several fragments are present at increased intensity to allow easy identification. All fragments are precisely quantified and mixed during the production.

For 5 µl loading, all fragments except typical band DNA fragments are 40 ng. The typical band of DNA fragments is 100 ng. These ladders are pre-mixed with loading dye and are ready to use.

All DNA Markers can be stained with RedSafe™ Nucleic Acid Staining Solution, ethidium bromide (EtBr) or other DNA stains.

## CHARACTERISTICS

- Ideal for determining the size of DNA
- Stable for more than 12 months at - 20 ℃
- Ready to use without any handlings.

## KIT CONTENTS

| Product | Contents | Cat. No. |
|---|---|---|
| SiZer™-20 DNA Marker | 0.3 ml | 24071 |
| SiZer™-50 plus DNA Marker | 0.5 ml | 24072 |
| SiZer™-100 DNA Marker | 0.5 ml | 24073 |
| SiZer™-1000 DNA Marker | 0.5 ml | 24074 |
| SiZer™-1000 plus DNA Marker | 0.5 ml | 24075 |
| SiZer™-15K DNA Marker | 0.5 ml | 24076 |
| SiZer™- λDNA/HindIII DNA Marker | 0.5 ml | 24077 |

## STORAGE

- Store at 4℃ and stable for more than 6 months. For more stable use, should be eliquoted and then stored at -20℃. (stable for more than 12 months)

## GENERAL USE

- No DNase and RNase detected.
- Load 5 µl per each well of Agarose gel.

## QUALITY CONTROL

Well-defined bands are formed during agarose gel electrophoresis. The DNA concentration is determined spectrophotometrically.

The absence of nucleases is confirmed by a direct nuclease activity assay.

## PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

## NOTICE BEFORE USE

- Do not heat before loading
- For quantification, adjust the concentration of the sample to equalize, it approximately with the amount of DNA in the nearest band of the ladder.
- Visualize DNA by staining RedSafe™, ethidium bromide (EtBr) or other DNA stains.

## DETAIL INFORMATION

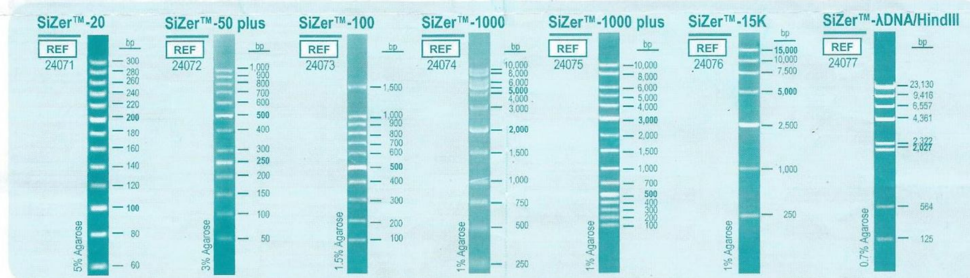
| | Size range (bp) | Conc. (ng/µl) | Typical bands | Other bands | Loading Vol. | Band number | Contents |
|---|---|---|---|---|---|---|---|
| SiZer™ -20 | 60-300 | 128 | 100ng/5µl | 40ng/5µl | 5µl | 13 | 60,80,**100**, 120,140, 160, 180,**200**,220, 240,260, 280,300 |
| SiZer™ -50plus | 50-500 | 128 | 100ng/5µl | 40ng/5µl | 5µl | 13 | 50,100,150, 200, **250**, 300, 400,**500**, 600, 700,800,900,1000 |
| SiZer™ -100 | 100-1500 | 100 | 100ng/5µl | 40ng/5µl | 5µl | 11 | 100,200,300, 400, **500**, 600, 700,800, 900, 1000,1500 |
| SiZer™ -1000 | 250-10000 | 120 | 100ng/5µl | 40ng/5µl | 5µl | 12 | 250, 500, 750, 1000,1500, **2000**, 3000,4000, **5000**, 6000,8000, 10000 |
| SiZer™ -1000 plus | 100-10000 | 144 | 100ng/5µl | 40ng/5µl | 5µl | 15 | 100,200,300, 400,**500**, 700, 1000,1500,2000, **3000**,4000,5000, 6000,8000, 10000 |
| SiZer™ -15K | 250-15000 | 85 | 125ng/5µl | 50ng/5µl | 5µl | 7 | 250, 1000, **2500**, 5000, 7500, 10000, 15000 |
| SiZer™ - λDNA/HindIII | 125-23130 | 100 | 350ng/5µl | - | 5µl | 8 | 125, 564, 2027, 2322, 4361, 6557, 9416, 23130 |

## RELATED PRODUCTS

| Product Name | Cat.No. |
|---|---|
| RedSafe™ Nucleic Acid Staining Solution (20,000x) | 21411 |
| DNA-spin™ Plasmid DNA Extraction Kit | 17096/17097/17098 |
| MEGAquick-spin™ Total Fragment DNA Purification Kit | 17286 / 17287/17288 |
| Maxime™ PCR PreMix (i-StarTaq) | 25165 |
| Maxime™ PCR PreMix (i-pfu) | 25185 |

## ELECTROPHORESIS

- The 5 µl of ladder DNA was loaded, and then electrophoresed for 1hr at appropriate concentration of gel





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iNtRON Biotechnology, Inc.

# Questionnaire of Cow, Sheep and Goats  Mastitis

**Area data:**

Area ..... Name of farm ......... Farm system ...............

**Cow data:**

Breed ......... Age ....... Number of calving ............

Date of delivery ........... Average production ............

History of mastitis ........ Previous treatment ..............

Condition ........ Depression ..... Anorexia .........................

**Udder data:**

Quarter envolved ........ Fibrosis .........................

Heat ......................... Induration ........................

Pain ......................... Abrasion .........................

Swelling ................. Size .............................

Hypermia ............... Supramammary L.N .........

Symmetry ...............

**Milk data:**

Colour ..................... Odour

Milk yield ............... Abnormal contents

**Milk tests:**

MWST ...................

RMT .....................

**Culture:**

Identification