Clinical and Bacteriological Aspects of Bovine Mastitis Caused by Actinomycetes

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Preface

This work had been carried out in the Department of Preventive Medicine and Public Health, Faculty of Veterinary Medicine, University of Khartoum, under the supervision of Dr. Mohamed El Amin Hamid.
To

My beloved family

My lovely husband

Vet 96

those whom believes that knowledge is power.

All my dearest colleges and friends who rely on themselves.

With my best wishes for all of you; believing that the one who makes no mistakes, makes nothing.

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ABSTRACT

In the present study 170 milk samples were collected from dairy cows from around Khartoum state between October, 2001 and October 2002. 150 were from mastitic milk and 50 were from apparently normal milk samples. The aim was to assess the role of nocardiae and related actinomycetes in the etiology of bovine mastitis and whether they exist in udder without causing clinical mastitis.

A pilot isolation procedure which aimed to improve the selective isolation of actinomycetes from milk was conducted. It showed that concentration of milk samples with centrifugation then heating of milk deposits at 60°C to 10 minutes, followed by culturing onto Tryptic Say Agar medium supplemented with sodium chloride (5%) was found to be suitable combination for the selective isolation of actinomycetes from milk.

Out of the 170 milk samples, 50 actinomycete strains were isolated. The isolated strains were identified using a phenotypic identification scheme comprised of cultural, morphological, biochemical, physiological and chemotaxonomic (mycolic acids) tests. The identification scheme of the 50 actinomycetes revealed the following clusters: *N. farcinica* 18%, *N. braseilensis* 14 %, *N. carnea* 10 %, *N. asteroides* 8 %, *N. otitidiscavarium* 6 % and 24 % un-identified Nocardia-like organisms, *Streptomyces* sp. 10 %, *Dietzia* sp. and *Williamsia* sp. as 6 % and 4 % respectively.

One strain from the cluster that denoted *Dietzia* sp. was confirmed by the gene sequence analysis of 16S rDNA as a member of the genus
Dietzia. Whereas the identification of the remaining clusters had been based on phenotypic criteria only. Confirmation of Dietzia as a cause of bovine mastitis represents, to our knowledge a first report worldwide.

Descriptive questionnaires were collected for the positive 50 mastitis cases which were caused by nocardiae and related actinomycetes in order to correlate clinical signs of mastitis to the identified phenotypic clusters. Nocardial mastitis appeared in one quarter (67.9%), chronic type (75%) with watery (14.3%) or watery with clotted (32.1%) secretions. Mastitis due to Streptomyces sp. appeared as acute (60%), affecting one or two (40%) swelled quarter with high percentage of watery and clotted secretions (40%). Dietzia sp. caused acute mastitis (66.7%), hypertrophied and swollen quarters (66.7%) with bloody (66.7%) or watery (33.3%) secretions. Williamsia sp. caused acute (50%) or chronic (50%) mastatic cases that appeared in one (50%) and two (50%) quarters with (50%) watery and bloody, or (50%) yellowish and clotted secretions.

The second part of the study was the testing of 50 apparently normal milk samples with California Mastitis Test; this was followed by culturing the samples onto blood agar plates. 34% of the samples were CMT positive and the results of the culture revealed the followings: 24% cocci, 10% Staphylococcus spp. 6% bacilli, 6% coccobacilli, 4% rods, 4% Streptococcus spp., 2% branching filaments organisms and 2% revealed no growth.

66% out of the 50 samples were found CMT negative, revealing no growth in 32%, 18% bacilli, 16 % Staphylococcus spp., 8 % rods, 8 % cocci, 4 % branching filaments organisms and 2% Streptococcus spp.
The text contains a detailed study of a disease in 170 men during the period from August to June in the state of Health and concerning 120 animals. The study was conducted in October 2001. The study found that the incidence of disease was 50 per cent in the sample. The study also investigated the role of the pharynx and the role of the tonsils in the disease. The study was conducted by a team using various methods and techniques. The study concluded that the incidence of disease was higher in males than in females. The study also found that the disease was more common in older individuals. The study was conducted by a team using various methods and techniques. The study was conducted by a team using various methods and techniques.
60% (or 1/3) of the total number of infected

40% (or 2/5) of the number of the infected

40% (or 2/5) of the infected

66.7% (or 2/3) of the infected

33.3% (or 1/3) of the infected

66% of the infected

50% (or 1/2) of the infected

50% (or 1/2) of the infected

50% (or 1/2) of the infected

50% (or 1/2) of the infected

50% (or 1/2) of the infected

40% (or 2/5) of the infected

66% of the infected

33.3% of the infected
INTRODUCTION AND OBJECTIVES

Introduction

Mastitis likes most livestock diseases; it is a result of between the host (cows), pathogens and environment (Heeschen, 1984). Mastitis is a complex disease, in view of the complexity of causes, pathogeneses, intensity, duration and therapy (Jain, 1979). Mastitis can be defined as an inflammation of the mammary glands caused by physical or chemical agents, but the majority of the infections are usually caused by bacteria (Quinn et al. 1994).

Bovine mastitis is of great economic importance to dairy industry worldwide (Miller et al., 1984). Growth and multiplication of microorganisms in the udder result in tissue damage and hence decreasing ability of the udder cells to synthesize the major constituents of the milk (Kitchen et al., 1984), and also leads to injury of the secretary tissue resulting in decreased milk yield (Shuster, 1989). Moreover, mastitic quarters milk yield will never retain to it is perfection values (Macfaedeen et al., 1988) even in the following lactation (Lucery and Rowlands, 1984). Similarly, the cost of treating the diseased animals and culling of the therapy-resistant cows (Shommein et al., 1988) are the major concern to dairymen.

The major causes of bovine mastitis are Stahp. aureus, Strep. agalactia and E. coli (Radositis et al., 2000), but considerable reports of bovine mastitis caused by nocardiae and related actinomycetes have been published, that is because of the worldwide distribution of actinomycetes
e.g. nocardiae and streptomycyes in soil (Goodfellow, 1998), environment and dust (Hagan and Bruner, 1988).

Nocardiae are known to infect bovine mammary glands causing enzootic (Wendt et al., 1969) and endemic mastitis (Nicolet et al., 1968), characterized by acute or chronic mastitis types (Ditchfield et al., 1959; Shigidi and Mamoun, 1981; Hamid et al., 1998). Nocardial mastitis does not usually respond to treatment with common antibiotics, immediate culling is the most appropriate approach (Quinn et al., 1994).

Actinomyces viscosus occurs in soil and dust where animals are kept and on animal's body (Bakhiet et al., 1992). It had been recently isolated from seven haemorrhagic mastitis cases in Sudan (Bakhiet et al., 1998). Actinomyces pyogenes was also isolated from 17 (9.77%) out of 173 mastitic dairy herds (Ibrahim et al., 1997). No much data is available on mastitis caused by other actinomycetes.

The objectives of this study were:

- To determine the role of nocardiae and related actinomycetes in mastitis among dairy cattle in Khartoum State.
- To correlate the clinical pictures to the isolated nocardiae and related actinomycetes mastitis.
- To improve the methods of isolation of nocardiae and related actinomycetes from milk.
- To identify the isolated actinomycetes using phenotypic and genotypic tests.
- To determine actinomycetes and other bacteria in apparently normal milk samples and to evaluate California Mastitis Test by bacteriological examination for the tested samples.
CHAPTER ONE
LITERATURE REVIEW

1.1 Bovine Mastitis

1.1.1 Definition
Mastitis is the most common disease that affects adult dairy cows (Mendonca et al., 2003). Mastitis is an inflammation of the mammary glands which is characterized by physical, chemical and usually bacteriological changes in the milk and by pathological changes in the glandular tissues (Blood et al., 1983). Mastitis can be caused by physical or chemical agents but the majority of cases are usually caused by bacteria (Quinn et al., 1994).

1.1.2 Types of mastitis
Mastitis has many forms which are usually classified according to their severity (Aiello, 1998):

1.1.2.1 Peracute mastitis: Peracute mastitis is a severe inflammation with swelling, heat, pain and abnormal secretions in the glands, accompanied by systemic disturbances such as fever, depression, rapid or weak pulse, sunken eyes, weakness and complete anorexia (Blood et al., 1983). Gangrenous mastitis is included in this category (Quinn et al., 1994).

1.1.2.2 Acute mastitis: Acute mastitis occurs with similar changes as in peracute mastitis but fever; anorexia and depression are slight to moderate (Blood et al., 1983).

1.1.2.3 Subacute mastitis: Subacute mastitis is often not characterized by systemic reaction and marked changes in glands and secretions (Quinn et al., 1994).
1.1.2.4 **Chronic mastitis**: chronic mastitis is characterized by the absence of systemic signs and very few external signs of changes in the udder, but abnormal secretions in the glands occur intermittently (Quinn *et al.*, 1994).

1.1.2.5 **Subclinical mastitis**: In this type the infection in the mammary glands is detectable only by bacterial culture or by tests such as California Mastitis Test to demonstrate a high leukocyte count in the milk (Quinn *et al.*, 1994).

1.1.3 **Milk tests**

Various tests were used to determine the type of the causative organisms and the infection level of the herd or individual cow (Bodman and Rise, 2003).

Appropriate tests include California Mastitis Test (CMT), Somatic Cell Count (SCC) and electric cell count (Boadman and Rise, 2003) which are used at intervals to determine somatic cell counts of the milk (Blood *et al.*, 1983).

Mastitic milk can be tested by the indirect chemical tests, which are based on the increase of sodium and chloride ions (Quinn *et al.*, 1994). These tests depend on measurement of the actual damage in the udder, not the cow response to the damage as the cell count does (Blood *et al.*, 1983).

Radial immunodiffusion test is used to assess the integrity of the mammary mucosa. This test accurately determines the increases of serum albumin concentration in the milk (Quinn *et al.*, 1994).

The CMT has the advantage that it can be used in bulk milk (Blood *et al.*, 1983) and in quarter samples under field conditions to determine the extent of positive reaction and identify the physiological and the management factors most closely correlated with a high proportion of
positive reaction. The physiological factors have great effects on CMT such as parity, stage of lactation, dry period (Braud and Schultz, 1963), end of milking in the chronic mastitis and the presence of a non-pathogenic *Corynobaeterium bovis* in the teat duct which causes rise in the cell count (Quinn et al., 1994).

Buss and Möller (1934) concluded that the rapid test for the diagnosis of mastitis was of great value in early detection of mastitis, but Schalm (1960) did not recommended CMT to be used in early and late stage of lactation because at these periods there was physiological increase in the leukocytes number in milk.

According to Jansen et al. (1997) a combined use of CMT information and the result of bacteriological analysis and culture lead to development of better selective treatment.

1.1.4 Milk abnormalities

Abnormalities in the milk occur as discolorations which vary from white with few flakes to watery and brownish with fine mealy flakes (Aiello, 1998). Clots or flacks usually indicate sever degree of inflammations. Flakes at the end of milking may be indicative of mammary tuberculosis in cattle (Blood et al., 1983).

Wateriness in a first ten streams is considered to be normal, and also blood clots and small plugs of wax in the first few days after calving, especially in heifers.

During the dry period in normal cows, the secretion changes from normal milk to clear watery fluid, then honey and finally to cholesterol in the last few days before parturition (Blood et al., 1983).

1.1.5 Udder abnormalities

Abnormalities of the udder varied according to the severity of the inflammation (Aiello, 1998). In severe cases there may be early gangrene,
abscess may develop in the glandular tissues, diffused swelling accompanied by heat and pain is marked to acute inflammations. Local areas of fibrosis may occur in a quarter, varying in size from peak-like lesion to masses as large as a fist (Blood et al., 1983). In sever chronic mastitis the affected glands gradually become less productive and may atrophy and slowly become firm (Aiello, 1998).

1.1.6 Etiology

Over 130 microorganisms have been isolated from bovine mastitic milk samples (Quinn et al., 1999); examples are Streptococcus agalatia, Streptococcus uberis, Corynobaeterium pyogenes, Corynobaeterium ulcerans, Klebseilla sp., Mycobacterium bovis, Mycobacterium lacticola, Bacillus cereus, Nocardia asteroides, N. braziliensis and N. farcinica.

Fungal infections including Tricosporum sp. Aspergillus fumigatos and Conidia sp. (Blood et al., 1983).

Lerondlle and Poutrel (1984) reported that, coagulase negative staphylococci comprised 24.1% of the isolates while the major pathogens constituted 7.5% and of these Staphylococcus aureus was the most prevalent (75.2%) from farm animals.

In Saudi Arabia, Barbourrek et al. (1985) found that the most predominant bacteria isolates were Micrococcus sp., Staphylococcus aureus, Streptococcus sp. and Corynobaeterium sp. Most of the microorganisms, which were isolated by Kopur in 1984 were Gram positive mainly streptococci and staphylococci. Hafez and El-Amarosui (1987) reported the isolation of Staphylococcus aureus, Streptococcus spp. and Pasteurella spp.

Hassanein et al. (1984) isolated Corynobaeterium pyogenes in Egypt.
Karmy (1990) isolated *Streptococcus agalactia* from 5 cases, *E. coli* from 6 cases and *Corynebacterium pyogenes* from 4 cases out of 32 camels suffering from mastitis.

*E. coli* was isolated as the causative agent in 32% out of 311 clinical mastitis cases (Longo *et al.*, 2001).

Anderson (1983) reported that many subclinical infections were caused by *Staphylococcus epidermis* and also clinical mastitis could be caused by it. Some authors regarded *Staphylococcus epidermis* as mere part of the normal udder flora (Brumely, 1978).

Out of 567 herds samples which were analyzed separately from 7-100 and from 101-305 days post partum was 21.2% for lactating period 7-10 days and 34.5% for 101-305 days post partum. The prevalence of subclinical mastitis was *Staph. aureus* in 16.5% and 7.4%, coagulase negative staphylococci in 51.5% and 50.6%, *Corynobacterium bovis* 25.7 and 45.1%, respectively (Busato *et al.*, 2000).

In a study of 187 specimens of udder tissue and milk from mastitic goats, Bozhilove *et al.* (1970) isolated 80-85% staphylococci and 40% *Staphylococcus aureus*. El Nasri (1960) reported the isolation of staphylococci, Streptococci and coliform, but no isolation of anaerobic organisms from goats.

### 1.1.7 Etiology of bovine mastitis in Sudan

Bovine mastitis was first reported in Sudan in 1953 (Annual Report of the Sudan Veterinary Service, 1953). The first survey of causal organisms of chronic mastitis in herds was done on 57 cows, 33 showed indurations of one or more quarters, 10 showed slighter degrees of indurations, and 14 cases had normal udders. The isolated causative agents were Staphylococci and Streptococci (Wakeem and El-Tayeb, 1962).
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 which was isolated from clinical (92.2 %) and sub clinical cases (44.2 %).

Obeid (1983) tested a total of 763 composite milk samples, the most commonly isolated organisms in descending order were: *Strep. agalactia* (29.5%), *Staph. aureus* (16.9%), *Staph. albus* (6%) *Strepcoccus* sp. (1.7%), *Micrococcus* sp. (1.6%) and coliforms (0.7%).

The predominant pathogens encountered were *Staphylococcus* sp., *Corynobacterium* sp. and *E. coli* out of three hundred twenty-two lactating Friesian cows (Abdel Rahim *et al.*, 1990).

*Actinomyces pyogenes* was isolated out of 173 mastitic milk as (9.77%), (Ibrahim *et al.*, 1997). Seven haemorrhagic mastitics cases caused by *Actinomyces pyogenes* were observed among 108 dairy cows surveyed for the prevalence of mastitis.

Nocardiae were isolated from bovine mastitis by Shigidi and Mamoun (1981) who isolated *N. asteroides*, but Hamid *et al.* (1998) isolated *N. farcinica* from Zebu cattle in Elobaid.

1.1.8 Epidemiology

Pathogens causing mastitis often enter via the teat canal. The infection often occurs when the teat sphincter is slack, for a period of 20 minutes to 2 hours after milking (Quinn *et al.*, 1994). On the other hand, both the beginning and the end of the dry period represent times of increased risk of intramammary infections (Eberhart, 1986).

The infections come mainly from the contaminated udder or the environment (Blood *et al.*, 1983). Also the cow and seasonality play an important role in the infection (Leslie, 2003).
Herd mastitis can be caused by both environmental and contagious pathogens which may occur separately or simultaneously (Bodman and Rice, 2003).

Environmental infection with streptococci, klebsiellae and enterobacters occur more frequently early in the dry period. On the other hand *E. coli* infections tend to occur immediately before or after calving (Smith *et al.*, 1985a, b). Mastitis caused by these organisms is much less frequent but when they do, the disease is more resistant to hygienic control measures (Blood *et al.*, 1983). Also the variation in the load of coliforms and environmental streptococci in the environment are important predictors of new infection rates (Smith *et al.*, 1985).

The rate of new intramammary infections is significantly higher in the dry period than during lactation (Eberhart, 1986). The greatest increase in susceptibility is during the first three weeks of the dry period, in which the new infection rate is higher than during the preceding lactation. The second period of heightened susceptibility occurs just prior to calving and in the immediate post partum period (Bush and Oliver, 1987).

The organisms are primarily contracted from contamination of udder by manure, bedding (Eberhart, 1986), and contaminated milking machine cups (Blood *et al.*, 1983).

It is still difficult to assess objectively the impact of milking machine on teat tissue. Panel was done included teat shape, assessment, teat skin, teat apex visual scoring and teat thickness measured before and after milking (Casirani *et al.*, 2002).

Successful results were obtained when Seeleman and Sienoweik (1932) transmitted the infection in bovine by milking with hands, which had been wetted with mastitic secretion. Poor milking procedures, teat injuries, teat sore and milkers behavior play a secondary role as a
predisposing factors in transmitting and distributing the disease among herds (Aiello, 1998).

There are also important herd – level effects, such as the prevalence of existing infection at drying off and the method of drying off (Bush and Oliver, 1987). The average rate of new infections in untreated dry cows is expected to be between 8 and 12% of quarters (Eberhart, 1986).

The ability and severity of the infection depend on the characteristics of the organism and its ability to adhere to mammary epithelium, to colonize the teat duct and its resistance to antibiotic therapy.

The transmission mechanisms which depend on the bulk of infections in the environment including infected quarter, efficiency of milking personnel and high milking speed also play their role in the severity of the infection (Blood et al., 1983).

Susceptibility of cows to mastitis is determined by its lactation stage as mentioned above, age, and level of inherited resistance which include teat shape and the anatomy of the teat canal (Blood et al., 1983; Leslie, 2003). Cousins et al. (1980) suggested that the teat canal is more easily penetrated by bacteria during the early dry period. Similarly, swelling of the mammary gland and the increasing volume of secretions, contribute to high risk of new infection during the prepartum period.

Schmahlstei (1960) studied 100 infected udders with mastitis and had classified their teat shape, teat canal, orifice and sphincter, the author found that there was no single feature that was associated with mastitis, and also thickness and deposition of teat sphincter muscle play no role in incidence of bovine mastitis.

Sometimes mastitis arises as a sequel of toxin production in the alimentary tract following traumatic reticulitis and reticulo-pericarditis or as a result of infection of the reproductive organs, or may follow as
complication of heart, kidney and other diseases (Illin and Pospetov, 1968). The environmental mastitis increases during the wet weather, which was observed by Costa et al. (1998), who suggested a seasonal influence.

### 1.1.9 Economical impact

Economically mastitis plays a very important role because of its variable effects through many factors in animal health, production, human health, treatment expenses and the most important effects is the fatalitis largest economic losses in dairy farms and the extended usage of antibiotics (Kromber and Grabowski, 2002).

The effects of mastitis on animal health depend on the severity of the inflammation. Systemic signs which affect animal production mostly occur in per acute mastitis as fever, anorexia, depression and weakness, these signs varies according to the type of mastitis and the causative agent (Aiello, 1998).

Estimates showed that an affected quarter suffers 30% reduction in productivity and is estimated to lose 15% of it is production (Blood et al., 1983). Infection in late lactation had 48% reduction in yield, compared with 11% depression on dry period after calving (Blood et al., 1983). Even more the loss is supplemented by loss of about 1% of total solids by changes of the composition of casein, fat, lactose and glycogen which are reduced, whereas whey proteins, pH and chlorides are increased (Blood et al., 1983).

The additional danger of the contaminating bacteria in the milk for human is its unsuitability for human consumption and in transmission of tuberculosis, streptococcal sore throat and brucellosis (Aiello, 1998).

The cost of treating the diseased animals and culling of the therapy- resistant cows are of major concern to dairymen (Shommein et
al., 1988). In 2001, Longo et al., found that the total of economic losses due to clinical mastitis were 1307 FF (218US) for *E. coli* mastitis and 891 FF (148US) for Gram-positive mastitis.

Increasing culling and loss of life caused by *Staph. aureus*, *E. coli* and nocardial mastitis are much less occasioned (Blood et al., 1983).

1.2 Historical background of actinomycetes infections in Sudan

Most of actinomycetes members were saprophytic, distributed in soil, water but can cause diseases in man and animals (McNeil and Brown, 1994). Notable example of actinomycetes infections is Bovine farcy, which was first reported by Annual Reports of Veterinary Services (1924) as a cause of bovine lymphangitis that came under notice in a bull in Nuba mountains. Laboratory examination revealed the presence of *Actinomyces farcinicus*. The disease has received more attention in second half of the 20th century (Awad and Karib, 1958; Awad, 1958). In 1960 Awad reported nocardiosis of the udder and testes; El Nasri (1960) observed the spread of the disease among the Arab cattle in Nuba Mountains. Awad (1961) and El Nasri (1961) contradicted each other concerning the distribution of the disease. More recent studies were subsequently done (El Sanousi et al., 1979; Hamid, 1988; Hamid et al., 1991).

*Nocardia asteroides* was isolated from canine nocardiosis from five cases since 1942, then two were added (Awad, 1958). Four cases were added in relatively short time for four months, suggested that the disease and the causal agents were more common than suspected (Fawi et al., 1963).

Nocardiosis is considerable in the Sudan (Awad and Karib, 1958). In Omdurman 7.0% causeous lymphadenitis were found to be due to
nocardiosis (Awad El Kareem and Mustafa, 1974). Mustafa (1966) recorded 36.6% nocardiosis.

*Actinomyces viscosus* occurs in soil and dust where animals are kept and on animal body (Bakhit *et al.*, 1992). It was isolated from seven haemorrhagic mastitic cows (Bakhit *et al.*, 1998).

Bovine dermatophilosis is described by local name “Abu-Giglies” particularly among the Western Sudanese nomads. The first report of the diseases was presented by Soltys (1965), who observed prevalence role of 15-20% among cattle in Kordofan State. The etiological agents of the disease were studied extensively by Abu-Samra (1974, 1978, and Abu-Samra *et al.*, 1976).

The south Darfur State has the highest cattle population in the Sudan so, many reports were done in this State. Abu-Samra (1974, 1976 and 1978), diagnosed the disease in 0.2% and 0.9% among Kordofan and Darfur cattle, respectively. The occurrence of the disease was recorded during rainy and dry season as 25% and 5%, respectively. Abu-Samra and Ibrahim (1989) conducted a survey on the economic losses due to bovine dermatophilosis. Musa (2000) surveyed the prevalence rate of the disease among cattle in South Darfur.
1.3 Nocardial mastitis

1.3.1 Source

Pathogenic nocardiae are saprophytes which are found in many climates in soil, water either as indigenous flora or contamination (Duright and Yuan, 1999), grass and udder skin are thought to be the primary sources of nocardiae (Anonymous, 2001).

Nocardiae have a worldwide distribution in soil (Orchard and Goodfellow, 1974; Orchard et al., 1977; Orchard, 1981) and implicated in the biodegradation of vulcanized natural rubber (Huchinson et al., 1977), including N. asteroids (Snijders, 1924), N. carnea (Castellani, 1913) and other species.

N. brasiliensis was isolated from a cow with chronic granulomatous mastitis with abscess formation (Ditchfield et al., 1959). Two nocardiae strains were isolated from mastatic bovine udders (Lindner, 1968).

One hundred and forty-nine nocardiae strains, freshly isolated from soil samples were isolated from a number of countries with either tropical or temperate climates (Valere and Goodfellow, 1979). Pathogenic nocardiae had been isolated from soil samples from around Khartoum State (Sid Ahmid, 2001).

Abdel Fattah (1996) isolated 21.4 % and 23.33 % Nocardia sp. from soil and mastatic cow milk samples, respectively.

1.3.2 Spread and transmission

Nocardiae enter through wounds, ingestion, inhalation, by contaminated infusion equipment used in treatment of other forms of mastitis (Hagan and Bruner, 1988), and by trauma (Duright and Yuan, 1999). Acute nocardial mastitis is triggered when onset of lactation flushes the organism from limited foci through the lactiferous duct system.
(Duright and Yuan, 1999). Organisms enter the udder when udder washing is ineffective or udder infusion is not carried out aseptically (Blood et al., 1983).

Contaminated treatment or treatment devices and improper teat sanitation prior to treatment are common means of spread (Anonymous, 2001), also the contaminated mastitis treatment preparations and infusion needles are suspected as the vector of nocardial mastitis. The organism can survive in the preparation and infused into the gland during subsequent use of the antibiotics (Anonymous, 2003). *Nocardia asteroides* is capable of surviving in mixtures used for intramammary infusion for up to 7 weeks (Blood et al., 1983).

1.3.3 Etiology

Numerous reports of bovine mastitis caused by nocardiae have been published. *N. brasiliensis* was reported as a cause of chronic mastitis in one case (Ditchfield, 1959), *N. farcinica* (Awad, 1970) from two cases and also *N. asteroides* (Pier et al., 1958, 1961), *N. caviae* (Hagan and Bruner, 1988) were reported.

*Nocardia asteroides* was mentioned as a causative agent of an enzootic nocardial mastitis (Wendt et al., 1969) and as a causative of endemic mastitis by Nicolet et al. (1968), it had been also isolated with increasing frequency as an opportunistic pathogen from cases of bovine mastitis (Hagan and Bruner, 1988)

*N. asteroides* as a cause of bovine mastitis was first reported in Peru from a milk of mastitic cows on the third day after their second calving (Moreya et al., 1980) it was also recorded as a relatively common causes of chronic mastitis in Cuba (Merino et al., 1973).

From a questionnaire on the occurrence of bovine mastitis due to nocardiae between January 1981 and August 1985 the result showed that
only three herds were infected in 1981, 55 in 1982, a peak at 103 in 1983, followed by fall to 67, in 1984 and 14 in the first 8 months in 1985. Suggested factors contributing to the 1983 peak were the hot, wet weather at 1982, favoring multiplication of nocardiae in soil and the use of silage with high lactic acid content in 1983 (Pellerin et al., 1988).

In a study made to examine the effects of repeated episodes of clinical mastitis in chronically infected quarters Nocardia sp. was isolated as 11.9% (Wilson et al., 1991).

Dacosta et al. (1996) isolated Nocardia sp. from 107 milk samples and 186 samples of mammary secretion from dry cows as 4.55%, 2.15% respectively.

1.3.4 Nocardial mastitis in Sudan

In Sudan there were some reports indicating the presence of nocardial mastitis.

Nocardia asteroides is one of the causal agents of granulomatous mastitis which may affect half of the goats udder (Dafalla and Garib, 1958). N. farcinica was isolated from udder and testis (Awad, 1960). Nocardia was isolated from secretions of chronic mastitis affecting one half of goat's udders (Ibrahim, 1962).

Nocardia asteroides was isolated from bovine mastitis in Sudan by Shigidi and Mamoun in 1981 and also N. farcinica was isolated from zebu cattle in Elobied (Westren Sudan) by Hamid et al., 1998.

1.3.5 Pathogenesis

The inflammation of the teat sinus and lower parts of the gland suggest invasion of nocardiae via the teat canal. The inflammation spread from lobule to lobule (Blood et al., 1983). The organism multiplies in the devitalized tissue producing a purulent granulomatous inflammation
which may develop draining sinus tracts (Anonymous, 2001). Hard nodules or extensive fibrosis may be found during palpation of affected quarters (wood consistency) (Anonymous, 2001). Systemic signs may or may not be present (Hagan and Bruner, 1988), but badly affect may affected gland by rupture of develop sinus tracts (Radostitis et al., 2000).

The severe forms of the disease formed by high body temperature and rapidly progressive fibrosis of the udder. Pire et al. (1958) described enzootic nocardial mastitis in dairy herds in California from outbreak with acute, high fever developed in herds soon after calving.

In the chronic form of the disease the udder become in the form of big trauma-like mass with multiple chronic abscesses (Ibrahim, 1962).

An investigation of seven nocardial bovine mastitis by Battig et al. (1990) revealed that all investigated cows had been treated from mastitis during previous lactating periods. After parturition six cows had a severe acute mastitis, the infected quarter became indurated and strongly enlarged which can not be influenced by therapy. The seventh case had a chronic *Nocardia asteroides* mastitis with involution of the affected quarter. The histopathological examination revealed an acute chronic, necrotic to granulomatous mastitis.

The nocardial mastitis caused by *N. asteroides* is characterized by granulomatous inflammations which may lead to extensive fibrosis and formation of palpable nodules in the udder tissue (Quinn, 1994; Blood et al., 1983).

### 1.3.6 Clinical signs

Affected animals may show a systemic reaction with pyrexia (42°C) loss of apetite (Moreyra et al., 1980) and depression. The affected gland is swollen, hot, painful, (Duright and Yuan, 1999), with fibroses
(Blood et al., 1983) and abscesses in the subramammary nodes (Moreyra et al., 1980). The affected gland usually becomes non-functional. Fatalities (5-10%) may occur during the acute stage (Duright and Yuan, 1999).

Milk secretions varied from watery with floccules, to yellowish with clots and pieces of necrotic tissue (Moreyra et al., 1980), then clots in grayish (Blood et al., 1983). Lastly the udder secretions may be purulent (Anonymous, 2001), gray clumps, abscess content were viscous, yellowish, gray and mixed yellowish-green solid materials and white mycelial clumps maybe observed (Hillermark, 1960).

1.3.7 Diagnosis

Direct microscopy of stained smear made from deposits of centrifuged milk samples is sometimes used as a routine simple diagnostic tool (Quinn et al., 1994).

Culturing the deposit of milk sample up to 4 days, show the characteristic picture of Nocardia sp. (Quinn et al., 1994). Specimens of milk should not be refrigerated before culture because storage of samples can jeopardize the isolation of the organism (Anonymous, 2001). According to Hagan and Bruner (1988), it reduces the chances of successful culture of N. asteroides.

1.3.8 Treatment

In vitro drug sensitivy tests are not accurate guide to treat nocardiosis (Hagan and Bruner, 1988). Nocardia sp. was considered to be difficult to treat (Dacosta et al., 1996). Nocardial mastitis had most responded to treatment with common antibiotics, but novobiocin (500 mg) combined with 25- 40 ml of 0.2% nitrofurazone solution showed some positive results (Pier et al., 1958).
Antimicrobial therapy of nocardial mastitis may produce temporary clinical relief and cessation of shedding but no permanent cures. Trimethoprim/sulphanomide therapy combined with surgery produced impressive results. Alternative drugs included minocycline and doxycycline (Duright and Yuan, 1999).

According to Quinn et al., (1994) mastitis due to *Nocardia* sp. is unresponsive to treatment. Immediate culling is the most appropriate measure. Quinn et al. (1994) agreed with Pellerin et al. (1988) because the infected animal represented a hazard for other animals.

A study in 13 nocardial strains isolated from mastitic milk to 12 selective antibiotics revealed that chloramphenicol was the most active antibiotic (Pellerin et al., 1988). Nassal (1967) reported that *N. asteroides* was sensitive only to erythromycin and kanamycin but penicillin and tetracycline had no effect. According to Lerner and Baum (1973) *N. asteroides* was sensitive to doxycycline, minocycline, erythromycin and streptomycin. It was also sensitive to gentamycin, neomycin and streptomycin, but resistant to ampicillin, chloramphenicol and oxytetracyclin (Savalia et al., 1990).

### 1.3.9 Control

The probable invasion of the causal agents of mastitis may be from soil-borne, or as proper hygiene problem at milking (Blood et al., 1983), also mastitis may appear spontaneously in one animal and spreads in the course of milking operations (Duright and Yuan, 1999).

Good infusion technique, proper sanitation of hands and teat ends, wearing gloves and using single treatment devices are highly effective prevention measures (NYSCHAP, 2003). Keeping stalls and loafing areas clean and dry when treating, either during lactation or dry period, use an aseptically clean prepared antibiotic and thoroughly and disinfect the teat
with alcohol prior to infusion lead to good control (Anonymous, 2003). The organism appears to be sensitive to sodium hydrochloride (Blood et al., 1983).

The use of neomycin as a dry cow therapy increased the risk of nocardial mastitis (Ollis et al., 1989). Also Ferns et al. (1991) agreed with the use of neomycin including specific dry cow product. The author mentioned other factors which increase the risk such as higher level of production on a large herd size.

Larocque et al. (1992) recorded that teat dips containing dodecylbenzene sulfuric acid and nonylphenoxy poly (ethylenoxy) ethanol – iodine complex were effective against Nocardia spp.

1.4 The genus Nocardia

The genus Nocardia was proposed by Trevisam in 1889. The genus now is well defined and belongs to the mycolic acids group of actinomycetes, that is, to the suborder Corynbactericeae (Stackebrand et al., 1997) which forms a distinct monophyletic line that also encompasses the genera Corynebacterium, Dietzia, Gorodonia, Mycobacterium, Nocardia, Rhodococcus, Skermania, Tsukamurella and Williamsia (Chun et al., 1996; Goodfellow et al., 1998). These taxa can be separated from one another using a combination of biochemical, chemical and morphological features (Goodfellow et al., 1999; John et al., 2000).

Nocardiae are aerobic, catalase-positive, nonmotile, Gram variable to Gram positive and typically acid-alcohol fast at some stage of the growth cycle (Goodfellow, 1998). This is the most distinguish characteristic of Nocardia sp., some times called partial acid fastness (Baron et al., 1994).

Nocardiae have extensive branched vegetative hyphae that grow on the surface and penetrate agar media, these hyphae often fragment in situ.
or on mechanical disruption into rods shape or coccoid elements. Arial hyphae at times are visible only microscopically, formed short to long chains. Well to poorly formed conidia may occasionally be found on the aerial hyphae and more rarely on both aerial and vegetative hyphae (Goodfellow and lechevalier, 1989).

Nocadiae colonies are visible from 3-7 days after inoculation, appears as waxy, bumpy, or velvety rougose forms often with yellow to orange pigment, also may appear chalky white (Baron et al., 1994).

Most nocardiae produce carotenoid-like pigment, which result in colonies with various shades of blue, violet or green (Hagan and Bruner, 1988), orange, pink or yellow. Soluble brown, yellowish (Goodfellow, 1998), Nocardiae colonies may be rough or smooth of a soft to dough-like consistency, or compact and leathery (Hagan and Bruner, 1988; John et al., 2000).

1.5 The genus Streptomyces

The genus *Streptomyces* is considered as a sole member of the family *Streptomycetaceae* at present. Streptomyces strains are widespread in natural environment land, sea, river, and atmosphere and as a causative agents of human, animal and plant diseases but soil is the most popular habitat of streptomyces (Chun, 1995).

Streptomyces are Gram positive but not acid-alcohol fast and do not fragment into bacillary forms (Jametz et al., 1984) catalase, escullin, casein, glatin, hypoxanthine, starch and L-tyrosine positive (John et al., 2000). Streptomyces produce well-developed branching and rarely fragmented substrate mycelia. Arial mycelia usually bear long or short chains of spores (Chun, 1995). Spores are non motile (John et al., 2000) with smooth, warty, spiny, hairy or knobby surface which is used for identification of species in the genus (Chun, 1995).
1.6 The genus *Dietzia*

The new genus *Dietzia* (Rainey *et al*., 1995) was proposed with a single species. *Dietzia maris*, formerly *Rhodococcus maris* (Nesternko *et al*., 1982). It was associated with a variety of environmental habitats especially with soil and animal farms (Goodfellow, 1986). This bacteria unfortunately sometimes considered as contaminants and are underdiagnosed (Bezit *et al*., 1997) but it was frequently isolated as opportunities pathogens (McNeil *et al*., 1994).

*Dietzia* are aerobic, catalase positive, gram positive, nonmotil and oxidase-negative (Yumoto *et al*., 2002).

1.7 The genus *Rhodococcus*

The epithet rhodochrous (Zopf, 1891) was reintroduced by Gordon and Mihm (1957). Recent taxonomic revisions that reassigned certain members of the genus to 3 new genera, namely *Dietzia*, *Gordona*, *Tsukamurella*, that have left *Rhodococcus* (Goodfellow, 1998). At present the genus contains 12 validly described species (Stoecker *et al*., 1994), which are quite variable in their morphology, biochemical characteristics, growth patterns and capacity to cause diseases (Baron *et al*., 2003).

Rhodococci are widely distributed but particularly abundant in soil and herbivore dungs. Some species are pathogenic for animals and human (Chun, 1995).

*Rhodococcus* are aerobic, gram positive, nonmotile, catalase positive actinomycetes that form rods to extensively branched and usually partial acid-alcohol-fast. Colonies may be rough, smooth or mucoid and pigmented puff, cream, yellow, orange or red although colorless variants do occur (John *et al*., 2000).
1.8 The genus *Gordonia*

the genus *Gordonia* (Tsukamura and Mizuno, 1971) was proposed for some slightly acid-alcohol-fast actinomycete isolated from soil, sputa of patients with pulmonary disease (Goodfellow, 1998) and some strains have been associated with foams in an activated sludge of sewage treatment plants (Chun, 1995).

Stackebrandt *et al.* (1997) redefined the genus to include 13 validly described species, only 5 of these species are of medical importance.

*Gordonae* are aerobic, gram positive to gram variable, nonmotile, catalase positive actinomycete that forms short rods and cocci. They are usually acid-alcohol-fast. Colonies are formed on glucose yeast extract agar as rough brownish, pink or orange to red (Goodfellow, 1998; John *et al.*, 2000).

1.9 The genus *Tsukamurella*

This genus *Tsukamurella* was proposed by Collins *et al.* (1988) for actinomycetes previously known as "*Corynebacterium paurometabolum*". *Tsukamurella paurometabola* was first described as *Gordoni aurantiaca* in human in 1971 by Tsukamura and Mizuno. Recently 5 additional species have been assigned to this genus (Baron *et al.*, 2003).

*Tsukamurellae* are obligately aerobic, Gram positive, nonmotile, catalase positive actinomycete that forms straight to slightly curved rods and weakly to strongly acid-alcohol-fast and contain mycolic acids. Colonies are formed on lowenstein-jensen medium and Brain-Heart-infusion agar as rough, dry, cream to orange colonies (Goodfellow, 1998; John *et al.*, 2000).
1.10 The genus *Actinomadura*

*Actinomadura madurae* was first recognized in 1894 by Vicent, who named the organism “*Streptothrix madura*” and described it as the causative agent of madura foot (Baron *et al.*, 2003). The genus is well defined now and composed of 27 validly described species (Kampfer *et al.*, 1990).

*Actenomadurae* are aerobic, Gram positive, non-motile, non-acid-alcohol-fast actinomycete that forms a non-fragmenting, extensively branching sub state mycelium. The aerial mycelium may be blue, brown, cream, grey, green, pink, red, white or yellow. In the absence of aerial mycelium colonies have a leathery or cartilaginous appearance (Goodfellow, 1998).
CHAPTER TWO

MATERIALS AND METHODS

2.1 Samples and area of investigation

One hundred and seventy (170) milk samples were collected from dairy cattle in Khartoum State between October 2001 and October 2002, 120 were mastitic milk samples and 50 were apparently normal milk samples. Investigated dairy farms were mainly cross-breed (Local × Friesian) were located in Khartoum North and Omdurman. They were kept under poor level of hygiene and management.

2.2 Clinical examination and samples collection

Data about herds, infected cows including cow data, udder data and milk data were collected (Appendix D).

Milk samples were collected from mastitic and normal cows under aseptic conditions. Before the collection of quarter milk samples, the teats were disinfected with cotton moistened with 70 % ethyl alcohol. The first few drops of milk were discarded and about 2-10 ml were collected in sterile universal bottles and transported on ice to the laboratory in not more than three hours.

2.3 California Mastitis Test (C.M.T)

CMT test was carried out using the method described by Scham et al. (1971) and Quinn et al. (1994). Briefly, equal volumes of commercial CMT reagent and milk were mixed. Interpretation of the result was done as described by Quinn et al. (1994). Negative (0) and trace (±) were considered as negative and different intensities of positives (1, 2 and 3) were considered as positive as shown in Table 1.
2.4 Isolation and preservation of Actinomycetes

2.4.1 Culture media

Tryptic soy agar (T.S.A. Difco™) medium, T.S.A. supplemented with 5% NaCl and 5% sheep blood agar were used for the primary isolation of actinomycetes. T.S.A supplemented with 5% NaCl was used for subsequent subculturing of actinomycetes. (Appendix A).

2.4.2 Culture methods

2.4.2.1 Primary culture

One ml mastatic milk was centrifugated in microcentrifug tubes for 5 min. The cultures were made from the milk deposit by spreading a loopfull over dried T.S.A plates containing 5% NaCl. The plates were incubated aerobically at 37°C and examined after 5, 7, and 10 days for the growth of Nocardia spp., Streptomyces sp. and related actinomycetes. The present of these was known from characteristic described previously by Goodfellow (1998) and in the ATLAS OF ACTINOMYCETES (JSM, 1996).

2.4.2.2 Purification of culture

Typical and well isolated nocardiae and streptomyces-like colonies from the primary culture were picked with a wireloop and streaked on the surface of fresh plates of T.S.A. Pure cultures were obtained by repeating the subcultures on T.S.A., which were identified macroscopically by the presence of identical discrete colonies along the streak lines.
**Table 1: Interpretation of CMT results**

<table>
<thead>
<tr>
<th>CMT Score</th>
<th>Interpretation</th>
<th>Visible reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negative</td>
<td>Milk fluid and normal</td>
</tr>
<tr>
<td>±</td>
<td>Trace</td>
<td>Slight precipitation</td>
</tr>
<tr>
<td>1</td>
<td>Weak positive</td>
<td>Distinct precipitation but no gel formation</td>
</tr>
<tr>
<td>2</td>
<td>Distinct positive</td>
<td>Mixture thickness with a gel formation</td>
</tr>
<tr>
<td>3</td>
<td><strong>Strong positive</strong></td>
<td>Viscosity greatly increased strong gel that is cohesive with convex surface</td>
</tr>
</tbody>
</table>

According to Quinn *et al.*, 1994
Nocardia colonies were known by the characteristics described by Goodfellow (1998); JSM (1996) and John et al. (2000) as follows: most nocardiae produce carotene-like pigment that result in colonies with various shades of orange, pink, red or yellow. Colonies may be smooth or granular and irregular wrinkled or heaped. Aerial hyphae may be lacking, sparse or abundant and visible to the naked eye and they appear as white powder on top of the colonies.

Microscopically, using Modified Ziehl-Neelsen stain (MZN) Nocardia spp. smears show partial acid fast, often branching filaments which fragment into rods and cocci.

Streptomyces colonies were known by the characteristics described by Williams et al. (1983) and John et al. (2000) as follows: Streptomyces colonies form discrete and lichenoid, leathery or butyrous colonies. Initially colonies are relatively smooth surfaced, but later they develop a weft of aerial mycelia that they appear floccose, granular, powdery or velvety. It produces a wide variety of pigments responsible for the color of the vegetative and aerial mycelia. Colored diffusible pigment may also be seen. The aerial mycelium at maturity forms chains of three to many spores. A few species bear short chains of spores on the substrate mycelium. Sclerotia-, pycnidial-, sporangia-, and synnemata-like structures may be formed by some species. Spores are non-motile (Williams et al., 1983; John et al., 2000).

2.4.3 Preservation of culture

2.4.3.1 Slants

Growth on T.S.A slants (Bijoux, Universal or McCartney bottles) remains viable at room temperature or at 4°C for several months. But subculturing every three months ensures no loss of organisms. Culture can be kept at room temperature for up to eight months.
2.4.3.2 Frozen glycerol suspensions

Colonies from grown culture were kept in sterile 20% glycerol as described by Willington and Williams (1978). Heavy cell biomass from young culture was transferred onto sterile tube containing 1 to 5 ml 20% glycerol. The cell suspension was kept in deep-freezing at -20°C. This method provides long preservation for up to 10 years.

2.5 Identification

Identification of the 50 isolates was done using microscopical, cultural, biochemical, physiological and mycolic acid analysis. One isolate that was tentatively identified as *Dietzia* sp. was subjected to genotypic identification.

2.5.1 Microscopic examination

Gram’s and Modified Ziehl-Neelsen (MZN) staining methods (Appendix C) were used to stain smears from all samples in this study. Smears were spread on microscopic slides, fixed by gentle flaming, stained with Gram’s and MZN methods. Gram-positive organism appears purple, violet color whereas MZN posture organism appears red in a blue background (Quinn et al., 1994).

2.5.2 Biochemical and degradation tests

All the biochemical and degradation tests were done according to the methods detailed in Cowan and Steel's Manual of Medical Microbiology (Barrow and Feltham, 1993, Appendix A). Tests performed included: catalase production, degradation of casein, tyrosine, xanthine, starch hydrolysis, urease production, nitrate reduction, and fermentation of rhamnose, manitol, sorbitol and salicin.
2.5.2.1 Catalase test

One ml of 3-6% H$_2$O$_2$ was placed on a clean slide. A loopfull of each culture was mixed with the H$_2$O$_2$ and examined within 5 minutes for evaluation of gas, which indicates a positive catalase activity.

2.5.2.2 Casein degradation

The composition and preparation of casein medium is described in Appendix A.

Actinomycetes-like colonies (nocardiae, streptomycetes) were picked with a wireloop and streaked on a dry surface of a fresh plate of casein agar. Cultured plates were incubated at 37°C and examined daily for up to 14 days. Positive result gave a clear zone around the growth indicating the utilization of casein by the grown organism.

2.5.2.3 Tyrosine degradation

Ingredients and preparation of tyrosine agar is shown in Appendix A. A wireloop of each isolated colonies was streaked on a dry plate of tyrosine agar and incubated at 37°C, examined daily. A positive result showed a clear zone under and around the grown organism.

2.5.2.4 Xanthine degradation

The tested strains were streaked on the surface of a dry, fresh plate of xanthine agar (Appendix A). A clear zone around and under the growth indicates the utilization of xanthine by the tested strain.

2.5.2.5 Starch hydrolysis
Starch hydrolysis result was read after adding Lugols iodine to grown culture in starch medium. Positive organism showed a clear colorless zone around the grown bacteria.

2.5.2.6 Urea degradation

Christens urea medium was prepared as described in Appendix A. A wireloop with suspected colonies were streaked on a dry, fresh slope of Christens urea medium, incubated at 37°C and examined daily for up to 5 days. Positive organism changes the color of the medium into red.

2.5.2.7 Nitrite reductase

Preparation, materials and reagents of nitrate test are shown in the Appendix A and B. The test strains were incubated in nitrate broth for 7-14 days at 37°C. One ml of reagent solution A was added to the culture tube, followed with 1ml reagent solution B. Deep red color indicates reduced nitrate to nitrite, which mean positive result. Powdered zink was added to the tubes that did not show red color within 5 min. Change of color into red means nitrate was still present in the medium, this considered as a negative result.

2.5.2.8 Sugar fermentation

2.5.2.8.1 Rhamnose

The test strains were cultured onto rhamnose sugar, which was prepared as described in Appendix A. A pink color is an indication of positive result due to acid production from fermentation of rhamnose.

2.5.2.8.2 Salicin

The test strains were each cultured onto salicin sugar (Appendix A). Positive Salicin fermentation gave pink color.
2.5.2.8.3 Sorbitol

Materials and preparation are shown in Appendix A. Test strains were cultured onto sorbitol sugar. Pink color is an indication of positive result due to acid production from sorbitol fermentation.

2.5.2.8.4 Mannitol

The test strains were cultured onto mannitol sugar (Appendix A). Positive results give pink color, which indicates manitol fermentation.

2.5.3 Physiological tests

2.5.3.1 Growth at 45°C

The test strains were cultured on TSA medium containing 5% NaCl incubated at 45°C and examined daily for up to 7 days. Growth indicates ability of the test strains to grow at this incubation temperature.

The following tests are not identified tests but used as experimental treatment to the samples.

2.5.3.2 Tolerance to 60°C

Six strains were subjected to 60°C in a water bath for 10, 20, 30 and 360 minutes, they were cultured between every limit of time in T.S.A media, then incubated at 37°C and the growth was recorded which indicate tolerance of strains to 60°C at the time limit.

2.5.3.3 Tolerance to Sodium Chloride

The test strains were cultured on different concentrations of NaCl: 3, 5 and 7% to test their tolerance to various degrees of salty.

2.5.3.4 Tolerance to NaOH
Eight positive mastatic milk samples to *Nocardia* sp. and *Streptomyces* sp. were tested for growth after treatment with 1% NaOH. One ml from each milk sample was added to sterile tubes containing sterile 1ml 2% NaOH and cultured after 10, 20, 30, 360 minutes in fresh plates of T.S.A medium, incubated at 37°C and the growth was recorded. Seven of the previously grown test strains were subjected to the same test and the result was recorded.

### 2.5.4 Extraction and analysis of mycolic acids

The test strains were degraded by acid methanolysis (Minnikin, 1988; Hamid *et al.*, 1994). Three to five colonies were scraped from a well grown culture and placed in bijoux bottle contained 1ml methanol\toluene\concentrated sulfuric acid (30:15:1; V\V). The mixture was then heated overnight at 80°C. After cooling, one ml petroleum ether (b.p.60-80) was added to each bottle. The preparation was shaken for 15 min (by hand), then centrifugated. The supernatant which contained mycolic acids was transferred to cap Corning tubes. Supernatant was evaporated at room temperature; redisolved in 100µL petroleum ether and 5-10µL were spotted onto thin layer chromatographic (TLC) aluminum sheets (20×20 cm silica gel 60 F254, Merk, Germany). The sheet was run twice in solvent contained petroleum ether\diethyl ether (85:15 V\V), dried then stained with 5% ethanolic molybdophosphoric acid. Stained sheet was heated at 150°C for up to 10 min; Mycolic acid appears as dark spots on green background (Minnikin, 1982). Nocardia mycolic acids are single spot, whereas mycobacteria have multiple spots on the other hand other genera such as Streptomyces contained no mycolic acids (Minnikin, 1982, 1988).

### 2.5.5 Genotypic identification

One strain (SD 1716) was submitted for genotypic analysis using the 16S rDNA sequences. DNA extraction, purification and PCR amplification of 16S rDNA was done as described by Chun and Goodfellow (1995).

Nucleotides sequences were obtained from Newcastle University in (ACGT) format. Revised final data in the form of (ACGT) were the subject of an initial BLAST online program (www.ncbi.nlm.nih.gov/BLAST).
The resultant BLAST clustering was subjected to further analysis using PC
installed phylogenetic programs, namely PHYDIT (Moleculer Sequence Editor,
Jongsikchun, version 3.1).

Sequence data from tests strain and from related actinomycetes obtained from
were fed into PHYDIT following programs www.ncbi.nlm.nih.gov/nucleotide
guidelines. The programs use the join- neighbor method (Saito and Nei, 1987) to
establish similarity between tested organisms and to identify unknown.

The program was set to construct the tree and to estimate the
similarity between tested strains and related species included in the
analysis.

2.6 Antibiotic sensitivity

Eight test representative strains were examined for their in vitro drug
sensitivity. The isolates were first cultured in nutrient broth for 48 hrs.
One ml of the suspension was spreaded on T.S.A plate using a sterile
swab. The sensitivity discs (DIFCO, OXIDO) were placed on the
inoculated media, inhibition zone were measured after 48-72 hrs aerobic
incubation at 37°C. 0.5 to 1 cm inhibition diameter was scored +; 1.0-1.5
cm scored ++; 1.5-2.0 scored +++; 2.0 cm and above scored ++++. 
CHAPTER THREE

RESULTES

3.1 Microscopic examination

All samples were tested firstly by direct microscopy on smears from deposits of the centrifuged milk samples. 50 actinomycetes were isolated from 170 collected milk samples. Direct smears were done from the deposits of the positive milk samples for the 50 isolated actinomycetes. Some samples showed (29.4%) Gram positive, partial acid-fast cocci, coccobacilli, rods or branching filamentous forms (Fig. 1 and 2), but 2.9% showed non-acid fast branching filamentous forms.

Pure cultures of the 50 isolated actinomycetes were examined microscopically using MZN and Gram stains. 56% were *Nocardia* spp. which revealed Gram positive, partial acid-fast often branching filaments some times fragments into cocci, coccobacilli or rods forms under microscope (Table 2).

Microscopically, *Streptomyces* sp. 10% appears as non-fragmented branching filamentous with Gram positive and non acid fast stains. *Dietzia* sp. 6% appears as coccobacilli, gram positive and partial acid-fast (Table 2).

3.2 Morphological and cultural characterization of the isolates

Fifty (50) Nocardia spp. and related actinomycetes were isolated from the 170 milk samples.
Figure 1. Microscopic appearance of partial acid-fast cocci forms of *N. asteroides* strain SD 1733 from direct smear of centrifuged milk sample (Modified Ziehl-Neelsen stain X100).

Figure 2. Partial-acid fast rods of *N. farcinica* strain SD 1701. Direct smear from deposit of centrifuged mastatic milk sample (Modified Ziehl-Neelsen) X100
Figure 3. Dominant growth of Nocardia sp. in primary culture from milk on T.S.A. medium after five days under aerobic incubation at 37°C. Note the dry cream and the white powdery colonies.

Figure 3. Primary culture of *Streptomyces* sp. on Tryptic Say Agar medium after five days under aerobic incubation at 37°C. Note the predominant white powdery colonies the indicating heavy aerial hyphae.
Table 2. Phylogenetic clusters of Actinomyces spp. Isolated from bovine milk and their morphologic, biochemical, physiological properties.

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Cluster One: N. loccinea

Cluster Two: N. brasiliensis

Cluster Three: N. carnea

Cluster Four: N. asteriodes

Cluster Five: N. otitidiscavarum

Cluster Six: Nocardia-like organisms

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Cluster Seven: Nocardia-like organisms

T.S.A. + 5% NaCl | T.S.A. + 10% NaCl | TD + 5% NaCl | TD + 10% NaCl | TD + 15% NaCl | TD + 20% NaCl

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<td></td>
<td>Cluster Nine: Actinomycetes sp</td>
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</tr>
<tr>
<td>1716</td>
<td>P.A.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>N/D</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1718</td>
<td>P.A.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>N/D</td>
<td>+</td>
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<tr>
<td>1720</td>
<td>P.A.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>N/D</td>
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<tr>
<td>1722</td>
<td>P.A.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>N/D</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

P/A parcial acid-fast  P Poor growth  + Positive  
N/D Not done  R Resistance  - Negative
The primary culture plates for all samples were examined morphologically for the presence of characteristic *Nocardia* spp. (Fig. 3) and other actinomycetes (Fig. 4), then the observed results were supported with microscopic examination from the direct deposit milk (Fig. 1 and 2) then the isolated strains were authenticated upon subculturing. The isolated strains revealed 8 clusters, abundance and frequency of each type is illustrated in Table 2.

*Streptomyces* sp. colonies had diffusible green pigment (Fig. 5). *Nocardia* sp. colonies ranged from rough cream (Fig. 6), to deeply embedded yellowish and white with heavy aerial hyphae (Fig. 7). Colonies of *Nocardia*-like organisms appeared as smooth colonies embedded or easily detached colonies, with red (Fig. 8 and 9), and slight pink color. These patterns were identified in two clusters as *Dietzia* sp. and *Williamsia* sp. respectively.

### 3.3 Isolation and identification of actinomycetes from mastitic and normal milk samples

#### 3.3.1 Mastatic milk

Forty six (38.3%) *Nocardia* spp. and related actinomycetes were isolated from 120 mastatic milk samples. Actinomycetes were isolated using Tryptic Say Agar medium (T.S.A), under aerobic incubation at 37°C for up to five day's (Fig. 3 and 4). The isolates were identified using phenotypic characters (Table 2). Detailed phenotypic identify of these 46 isolates are given in section 4.4.

#### 3.3.2 Apparently normal milk samples

Fifty (50) apparently normal milk samples were first tested by California Mastitis Test (CMT) which shows 34% prevalence of
Figure 5. Growth of *Streptomyces* sp. strain SD 1728 on T.S.A after 72 hour under aerobic incubation at 37°C. Note the dark green diffusible pigment and extensive aerial hyphae.

Figure 6. Growth of *N. farcinica* SD 1701 on yeast extract agar medium after 72 hour aerobic incubation at 37°C. Note the rough cream yellow colonies.
Figure 7. Growth of *N. asteroids* strain SD 1741 on Yeast Extract Agar medium after 72 hour under aerobic incubation at 37°C. Note the rough wrinkled colonies with heavy aerial hyphae.
Figure 8. Growth of *Dietzia* sp. strain SD 1716 on Tryptic Say Agar medium after five days under aerobic incubation at 37°C. Note the deeply red, abundant and smooth colonies.

Figure 9. Growth of *Dietzia* sp. SD1716 on Yeast Extract Agar medium after 72 hour under aerobic incubation at 37°C. Note red and smooth colonies.
Figure 10. Reaction of 50 apparently normal milk samples from cows to California Mastitis test
Figure 11. Recovery of various bacteria from apparently normal milk samples with positive and negative reactions to California Mastitis Test.
subclinical mastitis and 66% normal milk (Fig. 10). Four (8%) *Nocardia* spp. and related actinomycetes were isolated from the 50 apparently normal milk samples, one of the isolated strains from CMT positive samples was branching filamentous appearance which is characteristic for nocardiae as *N. farcinica*. On the other hand CMT negative cultured samples revealed one isolate of each of *N. asteroides*, un-identified *Nocardia*-like organism and *Streptomyces* sp. (Fig. 11). Details of these and other isolates are given in section 4.4.

### 3.4 Phenotypic identification

The main phenotypic properties studied were the morphological, cultural, biochemical, physiological and genotypic properties. 10 clusters were obtained on the bases of overall similarity (Table 2). Details of these clusters are as follows:

#### 3.4.1 Cluster One: *N. farcinica*

This cluster contained 9 isolates. Results are shown in Table 2. Strains showed variations in the microscopic appearances from rods (SD 1701, 1702, 1720, 1727, 1745) to coccobacilli (SD1704, 1713, 1721, 1749). Most strains were partial-acid fast (Fig. 2) to non acid-fast, but all were Gram positive. Colonies were mainly rough, cream and embedded into agar (Fig. 6). The isolates were positive for starch hydrolysis, nitrate reduction, urea, rhamnose and catalase but revealed negative results to sorbitol, salicin, casein, tyrosine and xanthine degradation (Table 2). All the tested strains grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids (Fig. 12 lane 4). SD 1727 revealed mild sensitivity to amoxicillin (30µg), erythromycin (10µg) and chloramphinicol (30µg), moderate sensitivity to gentamicin (10µg),
Figure 12. Thin layer chromatographic analysis of mycolic acid from representative actinomycetes. Lane 1, positive control N. farcinica; lane 2, N. asteroids SD 1713; lane 3, Nocardia like- organism SD 1703; lane 4, N. farcinica SD 1745; lane 5, Nocardia-like organisms sp. SD 1737; lane 6, treptomyces sp. SD 1744; lane 7, Dietzia sp. SD1718; lane 8, Streptomyces sp. SD 1728 and lane 9, Streptomyces sp. as negative control. Arrow indicates solvent development direction.
sulphamethoxazole/trimethoprim (25µg) and resistance to penicillin G (10 I.U.) (Table 3).

3.4.2 Cluster Two: *N. brasiliensis*

This cluster contains 7 isolates. Results are shown in Table 3. Strains showed variations in the microscopic appearances from coccobacilli (SD 1708, 1724, 1743) to rods (SD 1714, 1715, 1717, 1719). Most strains were partial-acid fast, but all were Gram positive. Colonies were mainly rough, yellowish and embedded into agar. The isolates degraded casein, tyrosine, hydrolyzed starch and revealed positive reaction to nitrate, urea, catalase and manitol, but didn’t degrade xanthine, were negative to rhamnose, salicin and sorbitol. SD1717 was sorbitol positive. All the tested strains in this cluster grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids. SD1715 revealed mild sensitivity to amoxicillin (25µg), erythromycin (10µg), moderate sensitivity to gentamicin (10µg), sulphamethoxazole/trimethoprim (25µg), penicillin G (10 I.U.) and chloramphillin (30µg) (Table 3).

3.4.3 Cluster Three: *N. carneae*

This cluster contained 5 isolates. Results are shown in Table 2. Strains showed microscopic appearance of cocci and coccobacilli (SD 1722, 1725, 1726, 1735, 1736). Most strains were partial-acid fast, but all were Gram positive. Colonies were mainly rough, deeply embedded into agar and cream in color. The isolates were positive to catalase and nitrate, but negative to manitol, rhamnose, sorbitol, salicin and urea and did not degrade tyrosine, xanthine and casein, but hydrolyzed starch (Table 2).

60° C (Table SD 1722 was positive to salicin. SD 1736 was examined to
Table 3 Sensitivity tests to the isolated actinomycetes from milk samples.

<table>
<thead>
<tr>
<th>Isolated strains of actinomycetes</th>
<th>Pencill in 10 I.U.</th>
<th>Erythromycin in 10µg</th>
<th>Gentamicin 10µg</th>
<th>sulphamethoxazole/trimethoprime 25µg</th>
<th>Ampicillin 25µg</th>
<th>Chloramphini col 30µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD 1711</td>
<td>R</td>
<td>++</td>
<td>++</td>
<td>R</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>SD 1712</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SD 1715</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>SD 1727</td>
<td>R</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>SD 1733</td>
<td>R</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SD 1737</td>
<td>R</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SD 1739</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>R</td>
<td>+++</td>
</tr>
<tr>
<td>SD 1744</td>
<td>R</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

R  Resistant  
+  0.5-1 cm  
++  1-1.5 cm  
+++  1.5-2  
++++  Above 2
4) and NaOH 1% (Table 5) tolerance for time limit, which revealed poor and good growth (Table 2). All the tested strains grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids.

### 3.4.4 Cluster Four: *N. asteroides*

This cluster contains four isolates (SD 1731, 1733, 1741, 1750). Results are shown in Table 2. Strains showed variations in the microscopic appearances from branching filaments and fragmented into rods. Most strains were partial-acid fast, but all were Gram positive. Colonies were mainly rough deeply embedded into agar with white surface, but no pigments (Fig. 7). The isolates were positive for catalase, nitrate and urea, but negative to casein, tyrosine and xanthine degradation and didn't hydrolyze starch. All the tested strains grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids (Fig. 12 lane 2). SD1733 revealed mild sensitivity to amoxicillin (25µg), erythromycin (10µg), chloramphinicol (30µg) and sulphamethoxazole/trimethoprim (25µg) but moderate sensitivity to gentamicin (10µg), and resistance to penicillin G (10 I.U.) (Table 3).

### 3.4.5 Cluster Five: *N. otitidiscavarium*

This cluster contains three isolates (SD 1706, SD 1707 and SD 1710). Results are shown in Table 2. Strains showed variations in the microscopic appearances which ranged from branching filaments, except SD 1710, to fragmented rods. Most strains were partial-acid fast, but all were Gram positive. Colonies were mainly rough, cream and deeply embedded into agar. The isolates were positive for catalase, manitol, urea and nitrate; they did not degrade casein and tyrosine but degraded xanthine. They were negative for starch hydrolysis, rhamnose, salicin and
### Table 4. Tolerance of actinomycetes to 60°C

<table>
<thead>
<tr>
<th>Actinomycetes strains</th>
<th>0 minutes</th>
<th>10 minutes</th>
<th>20 minutes</th>
<th>30 minutes</th>
<th>360 minutes</th>
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<tr>
<td>SD 1718</td>
<td>3</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>SD 1723</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>2</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>SD 1736</td>
<td>3</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SD 1925</td>
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<td>3</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>SD 573</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 5. Tolerance of actinomycetes to 1% NaOH

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<th>Actinomycetes strains</th>
<th>0 minutes</th>
<th>10 minutes</th>
<th>20 minutes</th>
<th>30 minutes</th>
<th>360 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD 1718</td>
<td>3</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>SD 1723</td>
<td>3</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>SD 1733</td>
<td>3</td>
<td>2.5</td>
<td>2.5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>SD 1736</td>
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<td>2.5</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>SD 1925</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2.5</td>
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<tr>
<td>SD 573</td>
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<td>3</td>
<td>2.5</td>
<td>2</td>
<td>1.5</td>
</tr>
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</table>
sorbitol. All the tested strains grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids.

3.4.6 Cluster Six: *Nocardia*-like organisms

This cluster contained 8 isolates. Results are shown in Table 2. Strains showed rods and branching filamentous forms in the microscopic appearances. Most strains were partial-acid fast to non acid fast (SD 1737, 1738, 742, 1746), but all were Gram positive. Colonies were mainly smooth deeply, embedded into agar with powdery and white surface and showed brown pigment (Fig. 13 and 14). The isolates were positive for catalase and nitrate, negative reaction to urea, manitol, sorbitol, rhamnose and salicin SD 1737 and revealed positive results to rhamnose and salicin, respectively. But degraded casein and tyrosine, hydrolyzed starch but did not degrade xanthine. All the tested strains grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids (Fig. 12 lane 3 and 5). Strains in this cluster were similar to one another but could not be assigned to specific known species within the actinomycetes. SD1739 revealed mild sensitivity to erythromycin (10µg), chloramphinicol (30µg) and gentamicin (10µg) but moderate sensitivity to penicillin G (10 I.U.), sulphamethoxazole/trimethoprim (25µg) and resistance to amoxicillin (25µg) (Table 3).

3.4.7 Cluster Seven: *Nocardia*-like organisms

This cluster contains four strains (SD 1705, 1709, 1712, 1740). Results are shown in Table 2. Isolated strains revealed partial-acid fast and Gram positive rods. Colonies were rough, cream to yellowish and deeply embedded into the media. The isolates were positive to catalase
Figure 13. Growth of *Nocardia*-like organisms on Tryptic Say Agar medium after 72 hour under aerobic incubation at 37°C. Note the rough colonies with brown pigments and white powdery surface indicating aerial hyphae.

Figure 14. Growth of *Nocardia*-like organism strain SD 1703 on Yeast Extract Agar after 72 hour under aerobic incubation at 37°C. Note the rough colonies with white powdery appearance indicating heavy aerial hyphae.
and hydrolyzed starch, but were negative to manitol, rhamnose, sorbitol, salicin, nitrate and urea, but didn't degrade casein, tyrosine and xanthine. SD1712 was positive to manitol. All the tested strains grew at 45°C under aerobic incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids. Strains in this cluster were similar to one another but could not be assigned to specific known species within the actinomycetes. SD 17120 revealed moderate sensitivity to erythromycin (10µg), chloramphinicol (30µg), gentamicin (10µg), sulphamethoxazole/trimethoprim (25µg), amoxicillin (25µg) and penicillin G 10 (I.U.), (Table 3).

### 3.4.8 Cluster eight: *Streptomyces* sp.

This cluster contains five isolates (SD 1728, 1730, 1732, 1744, 1747). Results are shown in Table 3. Strains showed non-acid fast branching filamentous, but all strains were Gram positive. Colonies were rough deeply embedded into agar with white surface and green diffusible pigment (Fig. 5). The isolates were positive to catalase and nitrate, but negative to urea, manitol, sorbitol, salicin and rhamnose; degrading casein, tyrosine, hydrolyses starch but did not degrade xanthine. All the tested strains in this cluster grew at 45°C under aerobic incubation, tolerated 3% and 5% NaCl concentrations, but they did not contained mycolic acids (Fig. 12 lane 6 and 8). SD1744 revealed mailed sensitivity to erythromycin (10µg), chloramphinicol (30µg), and amoxicillin (25µg), moderate sensitivity to gentamicin (10µg) and sulphamethoxazole/trimethoprim (25µg), but resistance to penicillin G (10 I.U.) (Table 3)

### 3.4.9 Cluster nine: *Dietzia* sp.
This cluster contains three strains (SD 1716, 1718, 1729). Results are shown in Table 3. Strains showed partial-acid fast and Gram positive coccobacilli forms. Colonies were smooth, red, abundant and deeply embedded into agar (Fig. 8 and 9). The isolates were positive to catalase, urea, nitrate, and hydrolyses starch but didn't degraded casein, tyrosine or xanthine, negative to manitol, sorbitol, salicin and rhamnose, but SD1718 was positive to salicin. All the tested strains grew at 45°C incubation and tolerated 3% and 5% NaCl concentrations and contained mycolic acids (Fig. 12 lane 7).

3.4.10 Cluster Ten: Williamsia sp.

This cluster contains two strains (SD1711, 1723). Results are shown in Table 3. Strains appeared predominantly as cocci. Colonies were smooth and slight pink color. The isolates were positive to catalase, urea and nitrate, hydrolyse starch, degraded casein, tyrosine but did not degrading xanthine, negative to manitol, sorbitol, salicin and rhamnose, except SD1711 which was positive to salicin. All the tested strains grew at 45°C incubation, tolerated 3% and 5% NaCl concentrations and contained mycolic acids. SD1723 revealed mild sensitivity to erythromycin 10µg, chloramphenicol (30µg), and gentamicin (10µg), moderate sensitivity to amoxicillin (25µg), but resistance to penicillin G (10 I.U.) and sulphamethoxazole/trimethoprim (25µg) (Table 3).

3.5 Genotypic analysis

Complete sequencing of the gene 16S rDNA (1450 nucleotides) of strain sp. SD 1716 was obtained. When compared with corresponding nucleotide sequences of all known bacteria it resulted in a high similarity to members of genus Dietzia especially to Dietzia maris. The result came
Figure 15. Phylogenetic tree showing the position of *Dietzia* sp. SD 1716 in relation to validly described species of the genus *Dietzia*. 

 oppose
<table>
<thead>
<tr>
<th>Species</th>
<th>SD1716</th>
<th>90.8</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>C. renale</strong></td>
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<tr>
<td>D. maris</td>
<td>100</td>
<td>90.9</td>
<td>---</td>
</tr>
<tr>
<td>D. natrolim</td>
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<td>97.9</td>
<td>97.9</td>
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<td>D. psychral</td>
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<td>97.1</td>
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<td>G. bronchia</td>
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<td>93.3</td>
<td>93.4</td>
</tr>
<tr>
<td>M. avium</td>
<td>90 92.3</td>
<td>92.3</td>
<td>90.9</td>
</tr>
<tr>
<td>M. bovis</td>
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<tr>
<td>N. otitidis</td>
<td>89 92.6</td>
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<td>92.9</td>
<td>93</td>
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<td>91.4</td>
<td>93.2</td>
<td>93.3</td>
</tr>
</tbody>
</table>

**SD1716**
Table of similarity showing the position of *Dietzia* sp. SD 1716 in relation to validly described species.
with high bootstrap value indicating confidence in the analysis. The 16S rDNA sequence similarity is shown in Table 6, and the phylogenetic tree is shown in Fig. 15.

3.6 Survey

Data and milk samples from cows with mastitis and from cows with no clinical mastitis (normal) were considered for the descriptive questionnaires. The results of the descriptive questionnaires collected from 50 clinical mastitis cases due to actinomycetes are shown in Tables , 7, 8 and 9; Figures 16, 17 and 18. The results of the descriptive questionnaires for the isolated actinomycetes and other bacteria grown from the 50 normal cases are shown in Fig. 11.

3.7 Clinical pictures of mastitis caused by actinomycetes

3.7.1 Isolated actinomycetes

Twenty eight (28) Nocardiae strains as (56%) were isolated out of the 50 isolates which were identified as: 18% *N. farcinica*, 14% *N. brasiliensis*, 10% *N. carnea*, 8% *N. asteroids* and 4% *N. otitidiscavarium*, *Nocardia*-like organisms isolated as 24% Isolation of related actinomycetes from the collected samples revealed 10% *Streptomyces* sp., *Dietzia* sp. and *Williamsia* sp. 6% and 4% respectively.

3.7.2 Types of mastitis

Among the 50 isolated actinomycetes the clinical picture for each species is shown in Table 7 and Fig. 16. Nocardial mastitis appears in high percentage as chronic mastitis in comparison with other types, *Streptomyces* mastitis was found mainly as acute mastitis, but mastitis due to *Dietzia* sp. and *Williamsia* sp. appears as acute and chronic or acute respectively.
Table 6 Types of mastitis among the 50 isolated actinomycetes

<table>
<thead>
<tr>
<th>Actinomycetes spp.</th>
<th>Acute</th>
<th>Chronic</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocardia spp.</td>
<td>17.9%</td>
<td>75%</td>
<td>7.1%</td>
</tr>
<tr>
<td>Nocardia-like organisms spp.</td>
<td>41.7%</td>
<td>50%</td>
<td>8.3%</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>60%</td>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>Dietzia sp.</td>
<td>66.7%</td>
<td>33.3%</td>
<td>00.0%</td>
</tr>
<tr>
<td>Williamsia sp.</td>
<td>50%</td>
<td>50%</td>
<td>00.0%</td>
</tr>
</tbody>
</table>
Figure 16. Mastitis types in relation to the 50 isolated actinomycetes species
Table 7 Distribution of infected quarters and lesions type among the 50 isolated actinomycetes

<table>
<thead>
<tr>
<th></th>
<th>Number of infected quarters</th>
<th>Changes in Udders size</th>
<th>Changes in udders palpation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hypertrrophy</td>
<td>Normal</td>
</tr>
<tr>
<td>Actinomycetes spp.</td>
<td>0  1  2  3</td>
<td>7.1% 67.9% 17.9% 7.1%</td>
<td>14.3%</td>
</tr>
<tr>
<td>Nocardia spp.</td>
<td>8.3% 75% 8.3% 8.3%</td>
<td>14.7%</td>
<td>50%</td>
</tr>
<tr>
<td>Nocardia-like organisms spp.</td>
<td>20% 40% 40% 0.0%</td>
<td>40%</td>
<td>40%</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>0.0% 0.0% 100% 0.0%</td>
<td>66.7%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Dietzia sp.</td>
<td>0.0% 50% 50% 0.0%</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Williamia sp.</td>
<td>0.0% 50% 50% 0.0%</td>
<td>50%</td>
<td>50%</td>
</tr>
</tbody>
</table>
Figure 17. Distributions of infected quarters and lesions types among the 50 isolated actinomycetes
3.7.3 Infected quarters and lesion types

The number of infected quarters and types of lesion of the 50 isolated actinomycetes are shown in Table 8 and Figure 17.

*Nocardia* spp. affected mainly one hypertrophied quarter. However three strains, *N. farcinica* (SD 1749), *N. asteroids* (SD 1750) and *Nocardia*-like organism (SD 1748), were isolated from apparently normal milk samples with no udder lesions. *Streptomyces* sp. were isolated from one and two swelled quarters although one strain (SD 1747) was isolated from apparently normal milk sample. *Dietzia* sp. were isolated from two hypertrophy and swelled quartets, and *Williamsia* sp. were isolated from one and two quarters.

3.7.4 Milk secretions

The 50 isolated actinomycetes were isolated from varied milk samples between apparently normal milk and mastatic milk with various milk secretions (Table 9 and Figure 18).

Most of the isolated nocardiae were from milk secretions varied from watery, watery with clots to clotted yellowish. Although three isolates: SD 1748, SD1749 and SD 1750 were from apparently normal milk. *Streptomyces* sp. were isolated from mastitis characterized by watery and clotted secretions. Nevertheless, *Streptomyces* sp. SD 1747 was isolated from apparently normal milk. *Dietzia* sp. were isolated from bloody milk, but *Williamsia* sp. were isolated from milk secretion which varied from watery with clots and clotted yellowish.

3.8 Recovery of bacteria from apparently normal milk samples

Fifty apparently normal milk samples were examined by CMT and blood agar culture. The result showed that 34% were CMT positive (Fig. 10). The 34% samples revealed 24% cocci, 10% *Staphylococcus* spp., 6%
Table 9 Variation among milk secretions in the 50 isolated actinomycetes

<table>
<thead>
<tr>
<th>Actinomycetes spp.</th>
<th>Normal</th>
<th>Watery</th>
<th>Yellowish</th>
<th>Clots</th>
<th>Bloody</th>
<th>Watery &amp; clots</th>
<th>Yellow &amp; Clots</th>
<th>Water Bloody</th>
<th>Clots &amp; Bloody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocardia spp.</td>
<td>7.1%</td>
<td>14.3%</td>
<td>3.6%</td>
<td>10.7%</td>
<td>3.8%</td>
<td>32.1%</td>
<td>14.3%</td>
<td>3.6%</td>
<td>3.6%</td>
</tr>
<tr>
<td>Nocardia-like organisms spp.</td>
<td>8.3%</td>
<td>8.3%</td>
<td>0.0%</td>
<td>41.7%</td>
<td>0.0%</td>
<td>16.7%</td>
<td>8.3%</td>
<td>0.0%</td>
<td>8.3%</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>20%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>20%</td>
<td>0.0%</td>
<td>40%</td>
<td>20%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Dietzia sp.</td>
<td>00.0%</td>
<td>33.3%</td>
<td>00.0%</td>
<td>00.0%</td>
<td>66.7%</td>
<td>00.0%</td>
<td>00.0%</td>
<td>00.0%</td>
<td>00.0%</td>
</tr>
<tr>
<td>Williamsia sp.</td>
<td>00.0%</td>
<td>00.0%</td>
<td>00.0%</td>
<td>00.0%</td>
<td>00.0%</td>
<td>50%</td>
<td>50%</td>
<td>00.0%</td>
<td>00.0%</td>
</tr>
</tbody>
</table>
Figure 18. Distributions of the milk secretion abnormalities among actinomycetes clusters
bacilli, 6% coccobacilli, 4% rods, *Streptococcus* spp., 4%, branching filaments 2% and no growth in 2% of the samples. (Fig. 11).

66% out of the 50 samples were found CMT negative (Fig. 10), revealing no growth in 32%, 18% bacilli, 16% *Staphylococcus* spp., 8% rods, 8% cocci, 4% branching filaments, 2% *Streptococcus* spp. (Fig. 11).

### 3.9 Improvement of actinomycetes selective isolation procedure

#### 3.9.1 Media

The 120 mastatic milk samples were primary cultured onto Tryptic Say Agar medium. T.S.A. was then supplemented then NaCl 3% and 5% for the subculturing. Tolerance to various degree of salt had greatly enhanced the isolation and subsequent subculturing, apparently by suppressing contaminations (Table 3).

#### 3.9.2 Centrifugation of milk

Seven actinomycetes strains were cultured twice, from whole milk and from deposits of centrifuged mastatic milk samples. Three were *Nocardia*-like organisms (SD 1739, 1740, 1742), 2 strains were *Streptomyces* sp. (SD1730, SD 1744), *N. carnea* SD 1736 and *N. farcinica* SD 1745. Detailed results are shown in Table 10 and Figure 19. The results revealed that the deposit of the milk samples is suitable for isolating actinomycetes than from whole milk.

#### 3.9.3 Tolerance to 60°C

Six actinomycetes were treated experimentally to tolerate 60°C to time limit (0, 10, 20, 30 and 360 minutes). Four species were from mastatic milk samples SD1718, SD1723, SD 1733, SD 1736. Two were *Streptomyces* sp. SD 573 and SD 1925 which were isolated from donkey
Table 10. Comparison between the recoveries of actinomycetes isolated from whole milk and from deposit of centrifuged milk samples

<table>
<thead>
<tr>
<th>Actinomycetes strains</th>
<th>Whole milk</th>
<th>Deposit</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD 1730</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SD 1736</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SD 1739</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SD 1740</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>SD 1742</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SD 1744</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SD 1745</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>
Figure 19. Comparison between actinomycetes inoculated from whole and deposit milk
Figure 20 Tolerance of actinomycetes strains to 60° C

Figure 21. Tolerance of actinomycetes strains to 1% NaOH
fistulous withers and a goat suffered from mastitis, respectively. Details of the results are mentioned in Table 4 and Figure 20. The results revealed that 60°C for 10 minutes is tolerable for actinomycetes, and this can be used to inhibit contaminations.

3.9.4 Tolerance to 1% NaOH

The six actinomycetes were also tested to tolerate 1% NaOH to time limit (0, 10, 20, 30 and 360 minutes). Details of the results are shown in Table 5 and Figure 21. The results indicated that 1% NaOH for 10 minutes is tolerable by actinomycetes, without significantly affecting the quality of the growth.

3.9.5 The final selective isolation strategies

From the above results a combination of milk treatment and the selective media used is concluded as a best strategy for the selective isolation of actinomycetes from milk samples. This strategy is as follows: heating of milk to 60°C for 10 minutes or adding 1% NaOH to equal volume of milk for 10 minutes for the two together, followed by centrifugation. Deposit is cultured onto T.S.A medium supplemented with 5% NaCl (Tables 4, 5, 10 and Figures 19, 20, 21).
Mastitis though not often a fatal disease causes great economic losses worldwide. Being so importance in dairy cows but as well it causes significant losses in other domestic animals, particularly sheep, goats, camels and pigs. Difficulties in mastitis treatment and control are the main factors contributing to these losses. It is however that prompt knowledge on the causal agents, their isolation, identification and sensitivity to antimicrobial agents will undoubtedly remain important detrimental factors towards effective control and treatment strategies (Quinn et al., 1994; Radostitis et al., 2000).

The main objective of this study was to examine representative bovine milk samples (mastitic and normal) from Khartoum state for the presence of pathogenic nocardiae and related actinomycetes in association with or without clinical mastitis. Nocardial mastitis is known to be one of the most serious type affecting dairy cows causing enzootic (Wendt et al., 1969) and endemic mastitis (Nicolet et al., 1968). In Sudan nocardial mastitis had been reported but in association with sporadic cases or being found incidentally (Dafalla and Gharib, 1958; Awad, 1960; Ibrahim, 1962; Shigidi and Mamoun et al., 1981; Hamid et al., 1998). Nocardiae and many other clinically significant actinomycetes are widely distributed in the environment notably soil (Hagan and Bruner, 1980; Goodfellow, 1998) and plant materials (Duright and Yuan, 1999).
Nocardiae are opportunistic microorganisms which enter through wounds, inhalation, contaminated infusions equipments (Hagan and Bruner, 1988) and by trauma (Duright and Yuan, 1999), thus causing various types of infection in man and animals.

One of the major outcomes of this work was the isolation and identification of 50 strains which were identified into 10 phenotypic clusters as: 18% *N. farcinica*, 14% *N. brasiliensis*, 10% *N. carnea*, 8% *N. asteroids*, 6% *N. otiitoscavarium*, 10% *Streptomyces* sp., 6% *Dietzia* sp., 4% *Williamsia* sp. and 24% were unidentified *Nocardia*-like organisms. These results agreed to some extent with Pellerin *et al.* (1988) who isolated a numbers of nocardial species from cows with mastitis. Similarly other authors investigated the occurrence of nocardiae bovine mastitis (Wendt *et al.*, 1969; Nicolet *et al.*, 1968) who advocated the role of nocardiae as causative agents of enzootic and endemic mastitis.

Out of these figures (56% *Nocardia* spp., 24% unidentified *Nocardia*-like organisms, 10% *Streptomyces* sp., 6% *Dietzia* sp. and 4% *Williamsia* sp.), it is clear that mastitis due to nocardiae and environmental pathogens in dairy cows could be a real problem with increasing significance either to its emergence as a new health hazard or due to the mis-diagnosis on routine microbiology laboratories.

In the present study *Nocardia* spp. and related actinomycetes and other bacteria were isolated from 50 apparently normal milk samples. These samples were tested by California Mastitis Test (CMT) and bacteria were recovered on blood agar plates. 34% out of the 50 samples were CMT positive and revealed 24% cocci, 10% *Staphylococcus* spp., 6% bacilli, 6% coccobacilli, 4% rods, 4% *Streptococcus* spp., 2% branching filamentous organisms and no growth in 2% of these samples. These findings were considered as subclinical mastitis. These findings agreed with Anderson (1983) who reported causes of many subclinical
and clinical mastitis by \textit{Staphylococcus epidermis}. In the latest edition of the Veterinary Medicine (Radostitis \textit{et al.}, 2000) it is explained that \textit{Streptococcus agalactia} is a high contagious obligate microorganism as a cause of udder infections. Vaz \textit{et al.} (2002) mentioned that \textit{Staphylococcus} spp. is the most frequently isolated microorganism from cases of subclinical mastitis. While, Todhunter \textit{et al.} (1995) reported increased mastitis infections which were caused by environmental \textit{Streptococcus} spp. Moreover, these authors indicated that these pathogens were leading causes of both clinical and subclinical mastitis in dairy cows worldwide. But many factors which contribute towards the production of the clinical mastitis remain to be determined.

Sixty six percent (66\%) of the 50 apparently normal cases were found CMT negative and surprisingly revealed no growth in only 32\% of the samples. 18\% revealed bacilli, 16\% \textit{Staphylococcus} spp., 8\% rods, 8\% cocci, 4\% branching filamentous organisms. These findings agreed with Brumley (1978) who regarded \textit{Staphylococcus} spp. as part of normal udder flora. Also, Radostitis \textit{et al.} (2000) considered coagulase-negative staphylococci as common isolates from normal milk samples, teat canals and teat skins. It is worth mentioning that Gamal El Din (2003) was able to prove experimentally that \textit{N. farcinica} causes serious acute mastitis in Nubian goats. This information contradicts nocardiae as a normal flora whenever it is claimed.

Two percent (2\%) of the positive samples to CMT revealed no growth which may be explained by involvement of physiological, non infectious factors such as stage of lactation. This come in line with Braud and Schultz (1963) and with Schalm (1960), who did not, recommend CMT to be used in early and late stage of lactation because during these periods there is physiological increase in the leukocyte numbers in milk. Organisms such as \textit{Mycoplasma} spp. or \textit{Brucella} spp. which are difficult
to isolate from milk using the current isolation procedures might be the cause in failing to isolate from samples with CMT positive on T.S.A. or Blood Agar media.

Actinomycetes were isolated from the 50 apparently normal milk. One isolate, *N. farcinica*, was isolated from normal milk but with CMT positive. *N. asteroidis*, *Nocardia*–like organisms and *Streptomyces* sp. were also isolated from CMT negative samples. This phenomenon seemed to be normal and it agreed with the common habitat and distribution of these actinomycetes in soil (Roy Cullimore, 2000). Thus it supports their nature as opportunistic pathogens (Leslie, 2003). Such observation represents an important epidemiological information that should be investigated further.

Centrifuged milk samples were found to improve the recovery of *Nocardia* spp. after comparing results with the isolation from whole milk. Such conclusion has been elaborated by Quinn *et al.* (1994). In the present study T.S.A medium was found to be successful in the primary isolation of nocardiae and other actinomycetes from milk. But efforts were done in this study to improve the isolation of actinomycetes from milk. In this regards representative of isolated actinomycetes were subjected to harsh treatment in order to inhibit contaminations and to enhance their recovery in pure forms.

T.S.A supplemented with 3% then 5% NaCl was found to enhance the growth of actinomycetes but inhibit other contaminations. Sid Ahmed (2001) supplemented T.S.A medium with antibiotics, (tetracycline) for the same purposes. The late treatment method was satisfactory for nocardiae, but evaluation for actinomycetes in general was not clear. Descriptive questionnaires obtained in this study revealed clear pictures of the clinical signs and some epidemiological data for actinomycetes as shown in Table 3 and Figure 14. Nocardial mastitis in the present Investigations had been mostly characterized by chronic forms, affected one hypertrophied quarter, and with milk secretions that varied from clotted and watery to clotted yellowish. These results were similar to
descriptions given by other reports (Moeyra et al., 1980; Quinn et al., 1994; Blood et al., 1983; Anonymous, 2001).

Un-identified *Nocardia*-like organisms isolated in the present study also showed a chronic form (50%) and hypertrophied, swollen quarter with watery or watery with clotted secretions. One of the inadequacies of this study was that the phenotypic protocol applied could not identify these *Nocardia*-like strains to species level. Though expensive and need expertise but sequencing 16S rDNA gene will be a good method for identification and classification of unknown organisms (Chun and Goodfellow, 1995).

*Streptomyces* sp. had been isolated from acute mastatic cases as 60% in comparison with 20% chronic and 20% normal cases. The infections were in one or two hypertrophied and swelled quarters. Milk secretions were watery with clots. One isolated *Streptomyces* sp. from a normal milk sample, which may be normal saprophyte come from environmental source (Chun, 1995).

To our knowledge there is no more information about *Streptomyces* sp. as causes of mastitis, but Bakhit et al. (1998) isolated seven *Actinomyces viscosus* strains from bovine haemorrhagic mastitis cases, which occurs during the first week following parturition. The seven isolated *A. viscosus* were gram positive, non-acid fast, non motile, producing catalase, hydrolyzed starch and esculin but did not produce indole, urea and reduced nitrate to nitrite, but they were isolated aerobically. They were not pathogenic to goats when inoculated experimentally. These isolates may be *Streptomyces* sp. and this can be justified by the aerobic growth of the isolates. *Streptomyces* sp. are aerobic, while *Actinomyces* sp. are aerobic or capnophilic actinomycetes (Quinn et al., 1994). Moreover, the failure of the isolated *A. viscosus* to
produce the disease in goats support this opinion when compared with Gamal El Din (2003) findings when inoculated goat experimentally by *Streptomyces* sp. SD 1925, it caused a mild infection with high increase of leucocytes (positive CMT) and the milk return to normal after treatment.

*Dietzia maris* is a saprophytic actinomycetes, emerging as an opportunistic pathogens, which can cause localized infections In human (Pidoux *et al.*, 2001), there is no more information about causing mastitis because this is the first report of *Dietzia* sp. as a cause mastitis in bovine. However, *Dietzia maris* was previously *Rhodococcus maris* (John *et al.*, 2000). are known to be pathogenic and can be confused with *Corynebacterium* species. Notable example being *Corynebacterium equi* which was transferred to *R. equi* the causal agent of foal pneumonia (Radostitis *et al.*, 2000).

Two *Williamsia* sp. strains were isolated. One isolate was from acute mastitis with one swollen quarter, the other was from chronic mastitis case with normal quarters. Milk secretions of this type varied between clotted watery and yellowish. *Williamsia* sp. was isolated for the first time as a cause of mastitis, but to our knowledge, there is no information about *Williamsia* spp. as a cause of animal diseases.

The sensitivity of the isolated actinomycetes in the present study to antibiotics were high to erythromycin (10 μg/ml) ampicillin (25 μg/ml), chloramphenicol (30 μg/ml) and gentamicin (10 μg/ml). Whereas, the isolates were found moderately sensitive to sulphamethoxazole/trimethoprim (25μg/ml), but most of the isolates were resistances to penicillin (10I.U/ml). Our results agreed with Nassal (1967) who reported high sensitivity of nocardiae to erythromycin, Peuerin *et al.*
(1988) who reported the sensitivity of nocardiae to chloramphenicol, Chen (1993) who reported sensitivity of nocardiae to erythromycin, gentamicin and chloramphenicol and Sridhar et al. (2001) who reported species-specific patterns of susceptibility to gentamicin and erythromycin. These patterns were susceptibility to gentamicin and erythromycin typically obtained for \textit{N. nova}, \textit{N. farcinica}. 
CONCLUSIONS AND RECOMMENDATIONS

Conclusions

- In conclusion, the results of the present study indicate high percentage of Nocardiae spp. infections (16.5%), unidentified Nocardia-like organisms (7.1%) and other actinomycetes (5.9%) as causes of bovine mastitis were also found to have a significant role. Clinical pictures of the infected cows by these isolates were characterized mainly by chronic forms with various lesions and secretions, specially hypertrophy and swollen quarters with watery and clotted secretions. Dietzia sp. and Williamsia sp. were isolated as causes of mastitis, this finding represent a first report of this kind.
  – A combination of milk treatment with 1% NaOH for 10 minutes or heating milk to 60°C to 10 minutes and centrifuged milk sample with supplemented Tryptic Say Agar medium with 5% NaCl was a good combination for recovering actinomycetes from milk samples.
  – A combined California Mastitis Test with bacteriology culture to reveal accurate diagnosis especially for subclinical mastitis.

Direct microscopic smear from deposits of centrifuged milk samples was good and easy method to tentative diagnosis of nocardiae and related actinomycetes.

- Phynotypic identification in concluded microscopically, cultural, morphological and biochemical tests tended to very long way which tacks time in diagnosis and identification of the causative agents, especially when compared with the genotypic identification, although it is expensive but more accurate and tacks less time.
**Recommendations:**

The present study recommends for further works the followings:

- Further studies to investigate the role of bacteria found in association with normal milk samples. Their pathogenesis has to be tested experimentally, as well as the determination of host related factors.

- The CMT should be performed to the normal milk and representative positive and negative samples should be at interval by cultured on suitable media.

- Application of molecular biology and 16S rDNA analysis to confirm identification of phenotypic clusters for accurate diagnosis at least such technology could be practiced at selected samples from time to time to be able to determine emerging, re-emerging and commonly mis-diagnosed un-identified organisms.

- As soil and environments are the source of actinomycetes mastitis infections, hygienic measures related to udder health should not be over looked.
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APPENDICES

Appendix A:

Media

Tryptic Soy Agar (TSA) (Difco™)

Composition: Pancreatic diges of casein 15g, enzymatic diges of soybean meal 5g, NaC 15g, agar15g, distilled water 1000 ml.

Preparation: The ingredients were sterilized at 121°C for 15min, then cooled to 50°C. Medium was distributed into the sterile Petri dishes (15ml) under flaming.

T.S.A+NaCl

Composition: T.S.A + 5% NaCl

Preparation: T.S.A + NaCl was prepared as T.S.A above but NaCl (5%) was added before melting and sterilization. Medium was cooled to 50°C before distributing into sterile Petri dishes (15ml medium/Petri dish).

Nutrient agar; Oxoid

Composition: Meat (beef) extracts 10g, peptone 10g, NaCl 5g, Agar 20g, and 1000 ml distilled water.

Preparation: The ingredients were dissolved by heating in water bath, then sterilized at 115°C for 20 minutes, allow to cool to 50°C before distributed into sterile Petri dishes (15 ml).
Blood Agar

**Composition:** Difibrinated blood 50ml, nutrient agar (Oxoid) 950ml.

**Preparation:** Nutrient agar was prepared and sterilized as above, cooled to 50°C before the blood was aseptically added. Sterilized medium was then mixed well and distributed into sterile Petri dishes as 15 ml/Petri dish.

Glucose-Yeast-Extract Agar

**Composition:** Glucose 10g (GPR™), yeast-extract 10g (Merck), agar 14g (idg) and 1000 ml distilled water.

**Preparation:** The ingredients were sterilized at 121°C for 15min after melting, and then cooled to 50°C. Medium was distributed into sterile Petri dishes (15ml) under flaming.

Casein Agar

**Composition:** Skimmed milk 500 ml. double strength nutrient agar 500 ml.

**Preparation:** Skim milk was made by standing fresh whole milk in a refrigerator overnight siphoning the milk below the cream layer, steamed for 30 min on each of 3 successive days. Sterilized by heating at 115°C for 10, min cooled to about 50°C before adding to sterilized double strength Nutrient Agar which had been cooled to 50-55°C. Medium was then mixed and distributed into sterile Petri dishes (15ml/Petri dish).

Tyrosine Agar (Sigma)
Composition: Peptone 5g, meat extracts 3g, agar 20g, and L-tyrosine.

Preparation: Tyrosine agar was prepared on base of Nutrient Agar as mentioned. Preparation was mixed well to produce a uniform suspension of the insoluble Tyrosine, then sterilized at 115°C for 20 minutes and allowed to cool to 60°C before distributed into sterile Petri dishes (15ml).

Xanthine (Gordon & Mihn, 1957; Sigma)
Xanthine agar was prepared as Tyrosine Agar but substituting Xanthine (4g/l) for Tyrosine (5g/l).

Starch Agar
Composition: Potato starch 10g, nutrient agar, 1 liter distilled water.
Preparation: The starch was triturated to smooth cream, then added to the molten Nutrient Agar. Mixed well and sterilized at 115°C for 10min, distributed into sterile Petri dishes.

Urea Agar
Composition: Peptone 1g, NaCl 5g, KH₂PO₄ 2g, agar 20g, glucose 1g, phenol red 0.2% aqu. Soln 6ml dissolved in 950ml distilled water, 20g urea.
Preparation: These ingredients without urea were sterilized at 115°C for 20min. 20g urea was melted in 50ml sterilized distilled water, cooled to 50-55°C before added aseptically to the base. The medium was then mixed well and distributed aseptically into sterilized containers and allowed cooling as slopes.

Nitrate Agar
**Composition:** NaNO₂ 0.01g, nutrient broth 1000 ml.

**Preparation:** Nitrate was dissolved in nutrient broth, distributed into tubes (5ml/tube) and sterilized at 115°C for 20min.

**Peptone water sugar (BDH)**

**Composition:** peptone 19g, NaCl 15g, 1000 ml water.

**Preparation:** Peptone water sugars were prepared according to Barrow and Feltham, (1993), methods of preparation depends on Andrade's indicator. 900 ml peptone water (pH 7.1-7.3) were adjusted to pH 7.5 by adding 10 ml Andrade's indicator. The preparation was sterilized at 115°C for 20 minute. Pink color may appear, but it fades on cooling, 5-10g of sugar was melted in 90 ml steamed water for 30 min or sterilized by filtration. The preparation was added aseptically into sterilized Peptone eater with indicator, distributed into sterilized Test tubes and steamed for 30 minutes.

**Rhamnose Sugar (Sigma)**

Rhamnose Sugar was prepared according to Barrow and Feltham (1993) by melting 0.5g of rhamnose in 9 ml water, steamed for 30 minutes, and then added aseptically to 90 ml sterilized Peptone water with the indicator. Medium was then distributed into sterilized Test tubes (5ml/tube), and steamed for 30 minutes. Color of medium changes into pink in case of positive result.

**Salicin Sugar (Sigma)**

Salicin Sugar was prepared as rhamnose according to Barrow and Feltham (1993) substituting salicin for rhamnose.
Sorbitol Sugar (Sigma)
Salicin Sugar was prepared according to Barrow and Feltham (1993) sorbitol sugar prepared as rhamnose but substituted sorbitol for rhamnose.

Manitol Sugar (Sigma)
Mani3tol Sugar was prepared as rhamnose, according to Barrow and Feltham (1993) substituting manitol for rhamnose.
Appendix B:
Reagents

Hydrogen Peroxide
H2O2 3% aqueous solution
Prepared by this percentage (3%), stored in a refrigerator in a bottle closed with plastic screw cap.

Nitrate Test reagents
Solution A
0.33% Sulphanilic acid dissolved in 5N-acetic acid. Dissolved by gentle heating.

Solution B
0.6% Dimethyl-naphthylamine in N-acetic acid. Dissolved by gentle heating.

Lugols Iodine
Iodine 5g, potassium iodide 10g, distills water100 ml.
Dissolved iodide and iodine into the distilled water and diluted 115 with water for use.
Appendix C:

Stains & staining methods

Gram Stain

Reagents:

Ammonium oxalate-crystal violet:

**Solution A**

- Crystal violet 10g.
- Ethanol (95%) 100 ml.

**Solution B**

- Ammonium oxalate 1% aq. Soln.

For use mix 20 ml. Of soln. A and 80 ml. Of soln. B.

**Grams iodine (mordant):**

- Iodine crystals 1g.
- Potassium iodide 2g.
- Distilled water 200ml.

The iodine crystals and the potassium iodide are ground together in a mortar before adding distilled water slowly.

Decolourizer

- Ethanol 95%
- Dilute carbon fuchsin 10ml.
- Distilled water 90ml.

**Concentrated carbon fuchsin**

- Basic fuchsin 1g.
- Ethanol 95% (V\V) 10m.
### Appendix D

**Questionnaire of Clinical and Bacteriological Aspects of Bovine Mastitis Caused by Actinomycetes**

1. **Sample data**
   - Sample number □ □ □
   - Season □ □ □
   - SD. number □ □ □

2. **Area data**
   - Name □ □ □
   - Place □ □ □
   - Others □ □ □

3. **Type of farming**
   - Intensive □
   - Semi-intensive □
   - Free □

4. **Cow data**
4.1 **Age** □ □ □
4.2 **Breed** □ □ □
4.3 **No. of calving** □ □ □

4.2 **Condition**
   - Healthy □
   - Emaciated □

4.3 **Fever, systemic reaction**
   - Yes □
   - No □

4.4 **Type of mastitis**
   - Acute □
   - Sub-acute □
   - Chronic □
   - Gangrenous □

5. **Udder data**
5.1 **Number of infected quarter** □ □ □

5.2 **Size**
   - Normal □
   - Atrophied □
   - Hypertrophy □

5.3 **Temperature**
   - Normal □
   - Hot □
   - Cold □

5.4 **Supramammary L.N**
   - Normal □
   - Inflamed □

5.5 **Palpation of the udder**
   - Normal □
   - Endulant □
   - Fibrosed □

5.6 **Other’s**
   - Pain:
     - Yes □
     - No □

6. **Milk data**
   - Normal □
   - Watery □
   - Bloody □
   - Cloted □