Some Epidemiological and Zoonotic Aspects of Bovine Tuberculosis in Khartoum State, Sudan

By

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Dedication

To all whose help made this work Possible.....
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ABSTRACT

The present study was conducted in Khartoum State from February 2005 – February 2006 to determine the prevalence of bovine tuberculosis (BTB) and the risk factors associated with the occurrence of the disease on 35 randomly selected dairy herds containing 587 heads of cattle. Moreover, the zoonotic implication of bovine tuberculosis was also investigated.

The methods applied were single intradermal comparative tuberculin test (SICTT) and a questionnaire to determine the risk factors. Assessment of the risk factors was based on comparisons of the reactivity of cattle to the tuberculin test.

The overall prevalence of bovine TB in the investigated herds was 1.5%. Statistically, significant association was observed between poor body condition (P =0.001) and large herd size (P=0.027) on one side and prevalence of bovine tuberculosis on the other side. Additionally, among the negative animals, 1.9% showed reaction to avian PPD due to exposure to environmental mycobacteria.

To study the zoonotic implications of BTB, 102 sputum samples were collected from patients admitted to the Tuberculosis Reference Laboratory, El Shaab Teaching Hospital and Abu Anga Hospital during the period January 2005 – January 2006. The 102 samples were stained by Ziehl-Neelsen and all revealed acid-alcohol-fast bacilli, 6 (5.9%) were fragmented acid-fast bacilli.

Out of 102 sputum samples, 79 (77.5%) showed visible growth on Löwenstein-Jensen medium (LJ) when incubated aerobically at 37° C for up to 8 weeks. All the samples that showed fragmented acid-fast bacilli failed to grow on LJ medium. One sample showed visible growth after 6 days and was considered as rapid grower whereas 78 samples showed visible growth after 2 weeks and were considered as slow growers.

The 79 mycobacterial isolates were tentatively differentiated by biochemical tests. One isolate was catalase positive and thus identified as mycobacterium other than tuberculosis (MOTT). Most of the isolates were nitrate positive and were resistant to thiophen-2-carboxylic acid hydrazide (TCH) and hence identified as Mycobacterium tuberculosis.

Nested polymerase chain reaction (nPCR) was performed to differentiate the 79 mycobacterial isolates using the primer pair MTUB-f and MTUB- r for M.
tuberculosis complex specific amplification of the 1,020-bp fragment of the gyrB gene. 77 (97.5%) isolate were positive for gyrB-PCR1 and thus identified as members of M. tuberculosis complex (MTBC) and 2 (2.6%) isolates were negative and identified as MOTT.

The 77 MTBC isolates were further differentiated using a set of specific primers MTUB-756-Gf and MTUB-1450-Cr that allowed selective amplification of the gyrB fragment specific for M. tuberculosis. All the MTBC isolates 77 (100%) were positive for the gyrB-PCR2 and thus confirmed as M. tuberculosis strains.

To evaluate the gyrB PCR-RFLP technique, the DNA polymorphisms in the 1,020-bp gyrB fragment for 7 M. tuberculosis strains confirmed by nPCR as well as 2 reference strains; M. tuberculosis H37Rv and M. bovis BCG were analyzed with the restriction enzyme RsaI. All the M. tuberculosis isolates showed the typical M. tuberculosis specific RsaI RFLP patterns (100,360,560-bp) while 360 and 480-bp fragments were generated from M. bovis BCG.
(Arabic Abstract)

الخلاصة

أعدت هذه الدراسة لنقصي وبائية مرض الدرن في الأبقار وتحديد العوامل التي تساعد
علي انتشاره في ولاية الخريطوم في الفترة من فبراير 2005م وحتى فبراير 2006م. شملت
الدراسة 35 حبزة للماعز تحتوي على 587 رأس. الوسائل التي استخدمت في الدراسة هي
اختبار التيوبركلين المقارن واعدة لتحديد العوامل التي تساعد في حدوث مرض الدرن في
الأبقار. تحدد هذه العوامل بني على مقارنتها لجهازية الأبقار لاختبار التيوبركلين المقارن.

أظهرت نتائج اختيار التيوبركلين في الأبقار أن نسبة 1.5% من الأبقار مصابة بالدرن. حلت هذه
النتائج إحصائيا وأظهرت علاقة كبيرة بين حالة الهزاز للأبقار و كبر حجم القطع من جهة
 وإنشار المرض من جهة أخرى. كما أظهرت الدراسة وجود استجابية غير نوعية بنسبة 1.9%
والتي تعلو إلى وجود المتغيرات البيئية.

في إطار دراسة وبائية مرض الدرن في الأبقار ومقارنة ذلك بدي إنتشاره في الإنسان في ولاية
الخرطوم، تم جمع 102 عينة قش عش من المعمل المرجعي للدرن ومستشفى الشعاب التعليمي
ومستشفى أبو عينجة في الفترة من يناير 2005م وحتى يناير 2006م.

تم تجهيز شرائح من كل العينات وصبغها باستعمال طريقة زيل-نلسن. كل العينات وجدت صامدة
للحمض الكحولي عندما تم فحص الشرايين مجهريا و6 (6.9%) فقط منها أظهرت عصيات
متفشة.

تسع و سبعون (77.5%) من جملة 102 عينة استطاعت أن تنمو في منبت لونشتاين-
جنسن عندما حضنتها في درجة حرارة 37°م حتى 8 أسابيع. كل العينات التي أظهرت
عصيات متفشة لم تستطيع النمو. عينة واحدة اعتبرت متقلبة سريع النمو وذلك لظهور
مستاعراتها بعد 6 أيام من استزراعها في منبت لونشتاين-جنسن، بينما 78 عينة اعتبرت
متفشة بطبيعة النمو وذلك لظهور مستاعراتها بعد إسبوعين من استزراعها.

تسع و سبعون عينة تم تعريفها ميدانيا باستخدام الاختبارات الكيميائية. عينة واحدة فقط كانت
موجهة لاختبار الكاتالاز وبالتالي عرفت على أنها متقلبة غير سلبية. أغلب العينات كانت موجهة
لحث (TCH ) thiophen-2-carboxylic acid hydrazide لاختبار النمو في مادة
الإختبار اختزال النتائج وبالتالي عرفت على أنها متقلبة سلبية.
أخضعت كل العزلات لاختبار التفاعل السلسي البوليمرزي الحضني تمييزها، 77 (97.0%)
كانت موجبة للتفاعل الأول وعرفت على أنها متقلبات ضمن مجموعة المتقلبة السلبية، بينما
2 (2.6%) كانت سالبة وعرفت على أنها متقلبات غير سلبية.
في التفاعل السلسي البوليمرزي الثاني كل العزلات الموجودة في التفاعل
الأول 77 (100%) كانت موجبة للاختبار وعرفت على أنها متقلبات سلبية.
لتقييم اختبار تم استخدام الاختبار على 7 عزلات تم تعريفها مسبقاً
M. tuberculosis بالتفاعل السلسي البوليمرزي الحضني بالإضافة الى عينتين مراجعتين، كل عزلات المتقلبة السلبية أعطت النمط المحدد للمتقلبة السلبية
M. bovis BCG، H37R بينما أعطت المتقلبة البقرية النمط المحدد للمتقلبة البقرية. وأظهرت العينات السبعة تطابق مع
التفاعل السلسي البوليمرزي كما ظهرت مقدرة اختبار RFLP على تمييز أعضاء مجموعة
المتقلبة السلبية استناداً على مقدراته للتمييز بين المتقلبة السلبية والمتقلبة البقرية.
INTRODUCTION

Bovine tuberculosis (BTB) is a disease of economic and public health importance, mainly caused by the intracellular bacterium, *Mycobacterium bovis* (Whelan *et al*., 2003). *M. bovis*, a member of the *M. tuberculosis* complex (MTBC), has a wide host range compared to those of other species in the disease complex, including humans and it is the most often isolated species from tuberculous cattle (Waters *et al*., 2004). Thus, infection of cattle with *M. bovis* constitutes a human health hazard as well as an animal welfare problem (Villarreal – Ramos *et al*., 2003).

An estimated 2 billion persons are currently infected with *M. tuberculosis* and other *Mycobacterium* species, and an estimated 3 million people worldwide die annually from complications of this disease. There are an estimated 8 million new cases each year, 95% of which occur in developing countries (Davies and Grange, 2001). The World Health Organization (WHO) estimates that nearly 400 cases per 100000 population occurred in Sub Saharan Africa (WHO, 2006). The rates of morbidity and mortality are rising as multidrug-resistant strains of several *Mycobacterium* species have emerged, primarily on the heels of the onset of the acquired immunodeficiency syndrome (AIDS) epidemic (Koneman *et al*., 1997).

Bovine tuberculosis still occurs in many developing nations and epidemiological data on the impact of this disease on human health is scanty but, in the light of the increasing incidence of tuberculosis
worldwide, it is urgently needed (WHO, 1994; Bonsu et al., 2000; Grange, 2001; Etter et al., 2006).

In the Sudan, the first mention of tuberculosis in cattle was in the Annual Report of the Veterinary Department for the year 1915, which stated that the disease was reported in Egypt, among cattle exported from Sudan. The first attempt in the Sudan to use tuberculin test was made in Wau city in 1955. In 1956, the scheme of tuberculin testing was extended to include different herds from various regions including Khartoum State to detect infection in exposed herds and then ensuring their early removal (Karib, 1962). Previous studies conducted in the Sudan confirmed the presence of the disease. Depending on the tuberculin test followed by the slaughtering of positive reactors, Karib (1962) concluded that the incidence of the bovine tuberculosis was high in southern Sudan and low in the dry northern zone. Moreover, Suliman and Hamid (2002) and Manal et al (2005) isolated *M. bovis* from caseous lesions collected from slaughtered cattle from different slaughterhouses in Khartoum State.

The five members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti* and *M. microti*) share 85-100% DNA-DNA relatedness. Differentiation among the members is currently based mainly on analysis of phenotypic characteristics such as colony morphology, growth rate and biochemical tests. In part, because of their close genetic relatedness and slow growth, species assignment is time-consuming and difficult (Sreevatsan et al., 1996).

In the past few years molecular approaches to diagnosis have been transforming the investigation of tuberculosis, especially in human
medicine. The introduction of PCR and nucleic acid hybridization has greatly reduced identification time and improved the level of detection in clinical specimens (Vitale et al., 1998). In addition, the application of molecular methods has permitted new insights into the molecular characteristics and phylogeny of MTBC species to be obtained (Niemann et al., 2004).

The increased rates of human tuberculosis in the Sudan (El Sony et al., 2000) and the possibility of the *M. bovis* as a causative agent encouraged us to undertake the present study, therefore, the aims of the present work were:

- to investigate the presence of bovine tuberculosis in Khartoum State, Sudan using the single comparative intradermal tuberculin test (SCITT) among dairy herds and to analyze some of the risk factors that affects the occurrence of BTB.
- to assess the role of *M. bovis* as tuberculosis causative agent in human TB patients.
- to determine the diagnostic value of the gyrB-based methods to differentiate the closely related strains of the *M. tuberculosis* complex (MTBC).
CHAPTER ONE
LITERATURE REVIEW

1.1 Definition

Bovine tuberculosis (BTB) is a chronic bacterial disease of animals and humans (Cagiola et al., 2004) characterized by progressive development of specific granulomatous tubercles in lung tissue, lymph nodes or other organs (Ayele et al., 2004). Bovine tuberculosis is caused by *Mycobacterium bovis*, a Gram positive rod does not show branching, typically acid-fast, non-motile, non-sporing and doesn’t produce aerial hyphae (Barrow and Feltham, 1995). Bovine tuberculosis is a well known zoonotic disease which affects cattle world-wide and considered to be of socio-economic and public health importance and of significance to the international trade of animals and animal products (Cousins, 2001).

1.2 Historical background

Tuberculosis is a disease of great antiquity, having been found in the mummies of ancient Egypt (Stablefoth and Gallowy, 1959). *M. tuberculosis* was probably first seen in tissues by Baumgarten and Koch in 1882. Koch cultivated *M. tuberculosis* and reproduced the disease in the period from 1882-1884 (Carter et al., 1986).

1.3 Epidemiology

1.3.1 Transmission

Inhalation is the most probable and important route of bovine infection. Infection by ingestion from infected animals or from
contaminated pastures, water or fomites is considered secondary to respiratory spread. Congenital infection and vertical transmission to calves by drinking infected milk are uncommon in regions where intensive eradication programs operate, as are infections from the udder. Genital transmission can occur if the reproductive organs are infected, but this extremely rare (Pollock and Neill, 2002).

### 1.3.2 Occurrence

*M. bovis*, the cause of bovine-type tuberculosis, has an exceptionally wide host range. Susceptible species include cattle, humans, non-human primates, goats, cats, dogs, pigs, buffaloes, badgers, possums, deer and bisons. Many susceptible species, including man, are spillover hosts in which infection is not self-maintaining (Daborn and O’Reilly, 1996). The presence of the disease in animals is usually signaled by detection in carcasses at abattoirs (Radostits *et al*., 2000).

### 1.3.3 Source of infection

Cattle-to-cattle transmission (CCT) plays a part in the entry of infection into herds, through purchased infected animals or contiguous spread (Goodchild and Clifton-Hadley, 2001). Infected cattle are the main source of infection for other cattle. Animals with gross lesions that communicate with airways, skin or intestinal lumen are obvious disseminators of infection, while cattle in the early stages of the disease, may also excrete viable mycobacteria in nasal and tracheal mucus (Radostits *et al*., 2000).
Ocepek et al. (2005) reported that animal attendants with active pulmonary TB represent an important source of *M. tuberculosis* for animals, spreading the mycobacterium via sputum, urine or feces.

### 1.3.4 Risk factors

#### 1.3.4.1 Host risk factors

Natural resistance to tuberculosis in cattle has usually been considered to be confined to zebu breeds of cattle (*Bos indicus*) (Pollock and Neill, 2002). The same authors considered susceptibility of cattle to BTB to be dependant upon the dose and route of infection, with little influence from other factors such as host genetics or nutritional status. Susceptibility to *M. bovis* infection increases with age of cattle (Phillips *et al.*, 2002).

#### 1.3.4.2 Pathogen risk factors

Inhalation of small numbers of mycobacteria can initiate lesions; however, the size, viability and consistency of aerosolized droplets also appear to be of crucial importance in establishing infection (Pollock and Neill, 2002).

The causative organism is moderately resistant to heat, desiccation and many disinfectants, but destroyed by direct sunlight unless it is in a moist environment. In warm, moist, protected positions, it may remain viable for weeks (Radostits *et al.*, 2000).

#### 1.3.4.3 Environmental risk factors

Although the prevalence of the disease within a country varies from area to area, the highest incidence of BTB is generally observed
where intensive management is most common (Pollock and Neill, 2002). Herd size is a risk factor for the incidence of TB, both in herds and in individual cattle (Goodchild and Clifton-Hadley, 2001).

1.3.5 Wildlife reservoirs

In a report from the Office International des Epizooties (OIE), 22% of countries have detected bovine tuberculosis in wildlife in the last 10 years. The potential role of wild animals in the maintenance and spread of M. bovis infection in domestic livestock is of particular importance in countries where eradication programs have substantially reduced the incidence of BTB (Aranaz et al., 2004). The spread of the infection from affected to susceptible animals is most likely to occur when wild and domesticated animals share pasture or territory (Cosivi et al., 1998). The best-known examples of such spread include infection in European badger (Meles meles) in the United Kingdom and the Republic of Ireland, the possum (Trichosurus vulpecula) in New Zealand, the white-tailed deer (Odocoileus virginianus) in the United States, the African buffalo (Syncerus caffer) in Africa (Aranaz et al., 2004) and water buffaloes (Bubalis bubalis) in Australia (Radostits et al., 2000). Wild animal TB represents a permanent reservoir of infection and poses a serious threat to control and elimination programs (Cosivi et al., 1998).

1.3.6 Zoonotic importance

The global prevalence of human TB due to M. bovis has been estimated at 3.1% of all human TB cases, accounting for respectively 2.1% and 9.4% of pulmonary and extra-pulmonary TB cases (Cosivi
et al., 1998; Ayele et al., 2004). Infection of humans with *M. bovis* may occur by inhalation of aerosols from infected cattle or through consumption of milk contaminated with bacilli (Wedlock et al., 2002). The same authors reported that, in countries where bovine TB has been eradicated from cattle a small number of cases are still occurring in elderly people as a result of reactivation of dormant infections.

A number of factors maintain the threat of bovine TB to human health, including the increase in the number of immunocompromised individuals, the emergence of strains of *M. bovis* resistant to known drugs and in some countries, an increasing prevalence of disease in cattle.

In developed countries pasteurization of milk and control of bovine TB by test- and slaughter-based control measures have dramatically reduced the transmission of *M. bovis* infection from cattle to human, and *M. bovis* is now predominantly an occupational zoonosis with potential risk for workers on farms, in abattoir and in zoos. In contrast, human TB caused by *M. bovis* is still a major health issue in many developing countries (Chambers et al., 2002).

Evidence of transmission of *M. bovis* between humans is considered rare, and the rate of transmission seems insignificant compared to animal-to-animal or animal-to-human infection (Ayele et al., 2004).

1.3.7 Economic importance

It has been estimated that more than 50 million cattle are infected with *M. bovis* worldwide and that the resulting economic losses are
approximately US $3 billion annually (Fend et al., 2005) and bovine TB is also of major importance as an economic cost, requiring massive annual expenditures for its control and eradication (Aagaard et al., 2003). In Argentina, the annual loss due to bovine TB is approximately US $63 million (Ayele et al., 2004), while Whelan et al. (2003) reported that in United Kingdom the cost of control measures exceeds £25 million per annum.

The economic implications in terms of trade restrictions and productivity losses have direct and indirect implications for human health and the food supply (Villarred-Ramos et al., 2003).

1.4 Mycobacteriology
1.4.1 Genus Mycobacterium

The genus Mycobacterium is the only genus in the family Mycobacteriaceae. Mycobacteria are aerobic, non-spore forming, non motile, slightly curved or straight rods. A filamentous or mycelium-like growth sometimes occurs but, on slight disturbance, easily fragments into rods or coccoid elements (Metchock et al., 2003).

Mycobacteria are Gram-positive and characteristically acid-fast. A comparatively slow growth rate is a characteristic of the mycobacteria, with generation times ranging from 2-20 hours.

The genus includes animal and human pathogens as well as saprophytic members, which are often referred to as atypical, anonymous or non-tuberculous mycobacteria. Some of these can occasionally cause disease in animals. The mycobacteria are most closely related to the
genera Nocardia and Rhodococcus and all three have a similar cell wall type (Quinn et al., 2002).

The cell wall peptidoglycolipid contains mycolic acids, glycolipids and variety of others. Mycolic acids, together with free lipids, provide a hydrophobic permeability barrier. The cell wall also contains fatty acids like waxes. These cell components are unique for a number of members of the Actinomycetales (Metchock et al., 2003).

1.4.2 Morphology and staining

Mycobacteria are thin rods of varying lengths (0.2-0.6 x 1.0-10.0 µm) (Quinn et al., 2002). Typically the human tubercle bacilli are slender, straight or slightly curved rods which may show beading, while the bovine type is rather short and thick. They remain uncolored with simple stains, but show acid-fast staining with warm carbol fuchsin followed by decolourization with 20% H₂SO₄ or by 3% HCl in 95% ethanol and this is known as Ziehl-Neelsen staining method (Collee et al., 1989).

1.4.3 Cultural Characteristics

The tubercle bacillus is an obligate aerobe and grows optimally at 35-37°C. Growth is slow and colonies are not visible before 2-3 weeks up to 6-8weeks (Collee et al., 1989).

Colony morphology varies among species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigmented (photo-chromogens or scotochromogens) (Metchock et al., 2003).
The egg-based Löwenstein-Jensen and Stonebrinks are the most commonly used media. The media are prepared as solid slants in screw-capped bottles. Malachite green dye is commonly used as the selective agent. *M. tuberculosis*, *M. avium* and many of the atypical mycobacteria require glycerol for growth. However, glycerol is inhibitory to *M. bovis* while sodium pyruvate enhances its growth. The luxuriant growth of *M. tuberculosis* and *M. avium* on glycerol–containing media, giving the characteristic rough, tough and buff colonies, is known as eugenic while the sparse, thin growth of *M. bovis* is called dysgenic (Quinn *et al.*, 2002).

1.4.4 MTB complex

The *Mycobacterium tuberculosis* complex (MTBC) consists of five closely related groups, *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* (Grange and Yates, 1994) and *M. canetti* (Chimara *et al.*, 2004).

Members of MTBC are the causative agents of tuberculosis in humans and animals. Despite their genetic relationship, the species of the MTBC show a large variability in their phenotypic properties, epidemiology and importance for human TB (Kubica *et al.*, 2003).

1.4.4.1 *M. tuberculosis*

*M. tuberculosis* is considered primarily a human pathogen but its infection has been reported in a wide range of domestic or wildlife animal species, most frequently living in close contact with humans (Ocepek *et al.*, 2005).
1.4.4.2 *M. bovis*

*M. bovis* principally affects cattle, but it can cause disease in a range of wild, domesticated animals and also humans (Smith *et al*., 2004). Cervical lymphadenopathy, intestinal lesions, chronic skin TB (Lupus vulgaris) and other nonpulmonary forms are also due to *M. bovis* (Cosivi *et al*., 1998).

The clinical picture of pulmonary TB caused by *M. bovis* is identical to TB due to *M. tuberculosis* (Kubica *et al*., 2003). Both subspecies of *M. bovis*, *M. bovis* subsp. *bovis* and *M. bovis* subsp. *caprae* are reported to infect humans, while the vaccine strain *M. bovis* BCG is more frequently used for bladder cancer immunotherapy (Richter *et al*., 2004). Resistance to Pyrazinamide (PZA) is a major criterion for the differentiation of *M. bovis*, but some studies report susceptibility to PZA among *M. bovis* isolates (Niemann *et al*., 2000).

1.4.4.3 *M. africanum*

Since its first description in 1968, *M. africanum* has been found in several regions of Africa, where it represents up to 60% of clinical strains from patients with pulmonary tuberculosis. Two major *M. africanum* subgroups have been described, according to their biochemical characteristics, these subgroups correspond to their geographic origins in West Africa (subtype І) or East Africa (subtype ІІ). Numerical analysis of biochemical characteristics revealed that *M. africanum* subtype І is more closely related to *M. bovis*, whereas subtype ІІ more closely resembles *M. tuberculosis* (Niemann *et al*., 2004).
1.4.4.4 *M. microti*

The exact nature of *M. microti* is not currently known, but morphological and serological studies suggest it to be in the tuberculosis complex (Gunn-Moore *et al*., 1996). *M. microti*, the vole or dassie bacillus, causes tuberculosis in small rodents, and although it has been reported to cause infection in cats, pigs and llama, it is not considered to be an important human pathogen (Liébana *et al*., 1996).

1.4.4.5 *M. canetti*

All *M. canetti* cases have been reported from Africa and this support the hypothesis that *M. canetti* might be more abundant on the African continent. With its smooth, round and glossy colonies, it differs from all other members of MTBC (Metchock *et al*., 2003).

1.4.5 Other clinically significant mycobacteria

Many of mycobacteria species are innocuous free-living saprophytes, but some are inherently pathogenic for animals and humans (Table 1).

1.5 Pathogenesis and pathology

The local manifestation depends upon the route of invasion. In pulmonary form, the infection is acquired by inhalation and becomes localized in lungs and associated lymph nodes. In extra-pulmonary form, it is usually through ingestion and localized in the mesenteric nodes and intestinal wall (Carter *et al*., 1986). When tubercle bacilli are initially implanted in tissue, they are phagocytosed by macrophages, and if the resistance of the macrophages is adequate, the bacilli are eventually
<table>
<thead>
<tr>
<th>Species</th>
<th>Host (s)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. africanum</em></td>
<td>Humans</td>
<td>Human tuberculosis (Africa)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Humans, dogs, canaries and psittacine birds</td>
<td>Human tuberculosis (worldwide)</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Many animal species and humans</td>
<td>Bovine tuberculosis</td>
</tr>
<tr>
<td><em>M. microti</em></td>
<td>Voles</td>
<td>Vole tuberculosis. Localized lesions seen in rabbits, calves, and guinea-pigs</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>Deer, pigs, and cattle</td>
<td>Tuberculosis-like disease. Isolated from lungs and lymph nodes</td>
</tr>
<tr>
<td><em>M. simiae</em></td>
<td>Humans and to (monkeys)</td>
<td>Isolated from lymph nodes of healthy monkeys. Pulmonary disease in man</td>
</tr>
<tr>
<td><em>M. marinum</em></td>
<td>Marine fish, aquatic mammals and amphibians</td>
<td>Fish tuberculosis: granulomtous and disseminated disease.</td>
</tr>
<tr>
<td><em>M. vaccae</em></td>
<td>Saprophytic</td>
<td>Non-pathogenic</td>
</tr>
<tr>
<td><em>M. scrofulaceum</em></td>
<td>Domestic and wild pigs, cattle and buffaloes</td>
<td>Tuberculous lesions in cervical and intestinal lymph nodes</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>Poultry and wild birds and pigs and others</td>
<td>Avian tuberculosis. Generalized form rare in mammals. Lesions in cervical lymph nodes. Intestinal lesions (rare)</td>
</tr>
<tr>
<td><em>M. intracellulare</em> (Battey bacillus)</td>
<td>Poultry and wild birds and pigs and cattle</td>
<td>Avian tuberculosis. Saprophyte in soil and water can be present in intestinal lymph nodes. Granulomatous enteritis (resembles Jone’s disease)</td>
</tr>
<tr>
<td><em>M. ulcerans</em></td>
<td>Cats</td>
<td>Nodulo-ulcerative skin lesions</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>Cats, pigs</td>
<td>Nodulo-ulcerative skin lesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tuberculous lesions lymph nodes of the alimentary tract.</td>
</tr>
<tr>
<td><em>M. fortuitum</em></td>
<td>Cattle</td>
<td>Granulomatous lesions lymph nodes and mammary glands.</td>
</tr>
</tbody>
</table>
Table 1. (cont.)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Host(s)</th>
<th>Lesion/Manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. phiel</em></td>
<td>Cats</td>
<td>Nodulo-ulcerative lesions of skin</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>Cattle</td>
<td>Granulomatous mastitis</td>
</tr>
<tr>
<td><em>M. paratuberculosis</em></td>
<td>Cattle, sheep, goats and other ruminants</td>
<td>Paratuberculosis (Johnne’s disease) chronic, progressive, intestinal, wasting disease.</td>
</tr>
<tr>
<td><em>M. lepraemurium</em></td>
<td>Cats and rodents</td>
<td>Feline and murine leprosy (respectively). Not yet isolated on conventional media</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>Humans and 9-banded armadillo</td>
<td>Leprosy in humans. Replication in armadillos. Not isolated <em>in vitro</em></td>
</tr>
<tr>
<td>Unidentified acid-fast bacterium</td>
<td>Cattle</td>
<td>Skin tuberculosis (lymphangitis)</td>
</tr>
</tbody>
</table>

*Quinn et al., 2002.*
killed (Jubb et al., 1985). If not, the bacilli proliferate and are released from killed macrophages, and subsequently interact with cells involved in innate and acquired immune responses in tissue. This often results in nonvascular nodular Granulomas known as tubercles.

Characteristic tuberculous lesions occur most frequently in lungs, retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, serous membranes, pleura and other organs (Ayele et al., 2004).

Pulmonary lesions are small and not encapsulated in earliest lesions but yellowish caseated and calcified. On the other hand, miliary lesions are small, grayish tubercles, translucent at first but soon become caseous and centrally calcified (Jubb et al., 1985). The caseous centre of tubercle is usually dry, firm and covered with a fibrous connective capsule of varying thickness and fixed tissues are not easily removed (OIE, 2000).

Microscopically, epithelioid and giant cells form the centre of young tubercles and the periphery is a narrow zone of lymphocytes, plasma cells and monocytes. As the lesion progresses, the classical tubercle develops peripheral fibroplasia and central necrosis (Jubb et al., 1985).

1.6 Diagnosis of tuberculosis
1.6.1 Clinical findings

In the case of a less advanced infection, there may be no clinical signs, but in advanced tuberculosis, the characteristic signs include weakness, anorexia, dyspnoea, enlargements of lymph nodes and cough,
but these signs are also observed in other diseases (OIE, 2000). Moreover, progressive emaciation, fluctuating temperature, pharyngeal obstruction, reproductive disorder and mastitis are also included (Radostits et al., 2000).

1.6.2 Microscopical examination

Sputum smear microscopy to detect acid–fast bacilli (AFB) is a rapid, inexpensive, and highly useful tool for identifying persons with active tuberculosis (Laserson et al., 2005). The sensitivity of this technique is reported to vary from 60 to 80% when assessed against culture (Freeman et al., 2001). Factors influencing smear sensitivity include the amount of sputum submitted to the laboratory, type of specimen examined, staining techniques, reader experience and whether the smear has been done with or without pretreatment (indirect or direct smear). A minimum of 300 microscopic fields should be examined for the presence of AFB before the smear is reported as negative (Metchock et al., 2003).

Katoch (2004) reported that the sputum smear microscopy requires 10,000 to 100,000 organisms/ml and suggested that smear microscopy can be made much more convenient by using various fluorochrome dyes such as auramine and rhodamine.

1.6.3 Isolation and identification

Culture is more effective than smear, in detecting as few as 10 - 100 viable organisms/ml of specimen. Off the egg–based media, Lowenstein-Jensen medium is most commonly used in clinical laboratories, and colonies may be observed in 18 to 24 days, in contrast
to 10-12 days with agar-based media (Middlebrook 7H10 OR 7H11) (Metchock et al., 2003).

Recently, more rapid and reliable methods for culture have been regarded as worthy and of absolute priority (Tortoli et al., 1999).

MB Redox is a new culture system combining a liquid medium and a redox indicator which enables an easy macroscopic vision of growth. MB Redox tubes contain 4 ml of Kirchner medium enriched with glycerol, serum, vitamins and antibiotics. The tubes also contain an invisible tetrazolium salt which appears as red – violet particles when reduced by the growth of mycobacteria (Cambau et al., 1999). Heifets et al. (2000) evaluated the time to recovery in this medium as was significantly shorter than that on L-J medium or 7H11 agar.

Mycobacteria Growth Indicator Tube (MGIT) is one of the nonradioactive broth-based culture methods were also introduced. MGIT contains 4 ml of modified Middlebrook 7H9 with oxygen quenching based fluorescent sensor. The oxygen initially dissolved in the broth quenches the fluorescence, but the growth of mycobacteria, is accompanied by the consumption of the oxygen, which allows the indicator to fluoresce under 365-nm UV light (Somoskövi and Magyar, 1999).

Katoch (2004) described that the Septi-Check is a biphasic medium system (Roche) consisting of an enriched selective broth and a slide with nonselective Middlebrook agar on one side and with two sections on other side, one with NAP (p-nitro-α-acetylamino-ß-hydroxy propiophenone) and egg containing agar, second with chocolate agar for
detection of contamination. This system is useful for rapid detection of mycobacteria.

A radiometric culture method such as the semi-automated BACTEC 460 system employing a liquid Middlebrook medium. Radio-labeled carbon dioxide ($^{14}C\text{O}_2$), obtained from the metabolism of $^{14}C$-labelled palmitic acid incorporated into the culture medium, is measured in an ion chamber system as an indicator of bacterial growth (Ayele et al., 2004). This system offers a NAP test, which discriminates the M. tuberculosis complex from MOTT (Hasegawa et al., 2002).

Mycobacteria are usually preliminarily identified by traits such as growth rate, pigmentation and selection of biochemical tests (Metchock et al., 2003).

1.6.4 Tuberculin test

Tuberculin tests have been used for the diagnosis of tuberculosis in cattle for more than 100 years (Costello et al., 1997).

1.6.4.1 Allergy

Allergy is a state of tissue hypersensitivity of specific immunoresponsiveness acquired by an animal as a result of exposure to an antigen. An allergic test is one that elicits a sensitivity response in an animal following the injection into its tissues of an antigen in the form of a protein derivative of the specific micro-organism with which the animal is, or has been infected (Kelly, 1984).
1.6.4.2 Tuberculin

Tuberculins were preparations made from the heat-treated products of *M. tuberculosis*, *M. bovis* (AN5 or Vallee strains) (OIE, 2000) and *M. avium* (D4ER or TB56 strains) cell lysates (Monaghan *et al.*, 1994). Due to their higher specificity and easier standardization, purified protein derivative (PPD) products have replaced heat concentrated synthetic medium tuberculins (HCSM) and old tuberculins (OT). PPD tuberculin is an extract of production strain which has been cultivated on synthetic media, subsequently being broken down and purified (OIE, 2000).

1.6.4.2.1 Types of tuberculin test

Sensitivity to tuberculin, which is employed to identify animals with sub-clinical tuberculosis, can be determined by applying different types of tuberculin test (Kelly, 1984).

1.6.4.2.1.1 Subcutaneous test

Tuberculin is injected subcutaneously and the rectal temperature record is made to measure the response. Temperature records are made before injection of tuberculin and at 9, 12, 15 and 18 hours thereafter. The frequency of observation makes it an unsuitable test when large numbers of herds are under examination (Stablefoth and Galloway, 1959).

1.6.4.2.1.2 Ophthalmic test

Ophthalmic test is inferior to the subcutaneous and intradermal tests for detection of tuberculosis (Stablefoth and Galloway, 1959).
Because of the low specificity or susceptibility to extraneous factors which reduce reactivity, ophthalmic test has been discarded (Kelly, 1984).

1.6.4.2.1.3 Intra-dermal test

Single intradermal tuberculin test (SIT) involves injection of bovine tuberculin purified protein derivative (Bovine PPD) intradermally and measuring the subsequent swelling at the site of injection 3 days later, while the single intradermal comparative tuberculin test (SICTT) with bovine and avian tuberculin is used mainly to differentiate between animals infected with *M. bovis* and those sensitized to tuberculin due to exposure to other mycobacteria or related genera.

The choice of which of the two tests to use depends on the prevalence of tuberculosis infection and on the environmental level of infection with other sensitizing organisms. The tuberculin test is usually performed on the mid-neck site (Stablefoth and Galloway, 1959) but the test can also be performed in the caudal fold of the tail (OIE, 2000).

1.6.4.2.1.3.1 Interpretation of the SICTT

The reaction is usually considered to be positive if the bovine reaction is positive and more than 4 mm greater than the avian reaction. The reaction is considered to be inconclusive if the bovine reaction is positive and from 1-4 mm greater than the avian reaction. The reaction is considered to be negative if the bovine reaction is negative or if the bovine reaction is positive, but equal to or less than a positive avian reaction. The difference in the skin thickness in each site is based on
observation and recorded increases in skin-fold thickness in the single intradermal tuberculin test (SIDT). The reaction is considered to be negative if only limited swelling is observed, with an increase of no more than 2mm and without clinical signs, such as diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes. The reaction is considered to be inconclusive if the increase in skin fold thickness is more than 2mm and less than 4mm and if none of these clinical signs are observed. The reaction is considered to be positive if clinical signs are observed or if there is an increase of 4mm or more in skin fold thickness (OIE, 2000). This standard interpretation scheme is used in European Union (EU) countries and is recommended in Council Directive 64/432/EEC. Sometimes a more stringent interpretation is used (severe interpretation).

Cattle classified as inconclusive reactors should be retested after an interval of 60 days; in the event that they are again classified as inconclusive they should be considered positive and removed from the herd. Otherwise a first-time inconclusive reactor may be removed from the herd under an official permit for slaughter following which a post-mortem examination for evidence of tuberculosis is performed. When the existence of tuberculosis has been established in a herd or group of cattle any inconclusive reactors should be considered positive and removed as reactors and this is called severe interpretation (Kelly, 1984).

**1.6.4.2.1.3.2 Sensitivity of SICTT**

The sensitivity of single intradermal comparative tuberculin test (SICTT), when using avian and bovine tuberculin, ranges from 90-99%.
False negative reactions to tuberculin test may arise for number of reasons. Recently infected animals may fail to show a reaction for 18-50 days after being infected. In addition, it has been recognized that some animals, including many with severe or generalized disease, may not react to the tuberculin test. Also the use of tuberculins of lower or reduced potency, the injection of insufficient tuberculin, desensitization following a tuberculin test, immunosuppression during the early post partum period, incorrect identification of animals and variations between observers, increase false-negative rates. The sensitivity of the test also depends on the cut-off value used to define a positive result. Altering this value increase or decrease the test’s sensitivity which will have an inverse effect on its specificity (Costello et al., 1997).

1.6.5 Serological tests

Serological tests including complement fixation, fluorescent antibody, direct bacterial agglutination, precipitin and hemagglutination tests have been developed but have little potential value for the routine diagnosis of tuberculosis (Radostits et al., 2000). Kämpfer et al. (2003) suggested that serodiagnosis of TB in badgers can be enhanced with the new Dach TB-ELISA utilizing multiple antigens as targets instead of an indirect ELISA of *M. bovis* specific antibodies to the serodominant antigen in TB in badgers due to the presence of other antigens during the course of the disease.

Fend *et al.* (2005) described the electronic nose (EN) approach as a straightforward alternative to conventional methods of TB diagnosis, and it offers considerable potential as a sensitive, rapid and cost-effective
means of diagnosing *M. bovis* infection in cattle and badgers. Electronic Nose is the colloquial name for an instrument made up of chemical sensors combined with a pattern recognition system which is able to classify and memorize odors and thus detecting volatiles in *M. bovis* infected animals.

### 1.6.6 Gamma interferon test

The whole blood interferon-γ (IFN-γ) assay was developed for the detection of bovine TB (Buddle *et al.*, 2003). Indeed, the IFN-γ assay has proven to be a practical ancillary assay for re-testing skin test-positive cattle, and has been shown to have a sensitivity of 85% and specificity of 93% in cattle reacted positively in the caudal fold skin test. The test is based on the detection of IFN-γ liberated in whole blood cultures incubated with PPD tuberculins, and IFN-γ in the supernatant plasma is then measured by enzyme immunoassay (capture ELISA) (Wedlock *et al.*, 2002; Buddle *et al.*, 2003). IFN-γ test has turned out to be the best, because it measures the in vitro proliferation of stimulated T cells from *M. bovis* infected animals on the basis of production of the cytokine IFN-γ, which is predominantly released by T cells after antigenic stimulation (Cagiola *et al.*, 2004).

### 1.6.7 Bacteriophage assay

For the rapid diagnosis of tuberculosis and susceptibility studies, a low-cost diagnostic technology currently under investigation is mycobacteriophage based technique (McNerney *et al.*, 2004). The mycobacteriophage assay uses *M. tuberculosis* complex-specific mycobacteriophages to report the presence of viable TB bacilli within a
sample. The target bacteria are rapidly infected by the target-specific bacteriophage. A selective virucide is added which, destroys all exogenous phages that have not infected the bacilli. The phages protected within the \textit{M. tuberculosis} complex organisms replicate and form clear areas (plaques) in a lawn of rapidly growing host helper cells. The number of plaques is related to the number of viable \textit{M. tuberculosis} complex cells and results from specimens can be read by eye after 48h (Alcaide et al., 2003).

\textbf{1.6.8 Molecular diagnosis}

The greatest breakthrough for the entire field of infectious diseases came from biotechnology, with the introduction of nucleic acid amplification techniques (NATs) (Roth et al., 1997). Identification using molecular techniques provides two primary advantages when compared to phenotypic identification: a more rapid turn around time and improved accuracy in identification (Roth et al., 1997; Harmsen et al., 2003).

Nucleic acid probes are selected segments of DNA or RNA sequences that have been labeled with enzymes, antigenic substrates or radioisotopes and are chemically easy to synthesize. Hybridization of nucleic acid probes to a specific target is considered the state of the art for the rapid culture confirmation of \textit{M. tuberculosis} or \textit{M. avium} complex (Roth et al., 1997) and these probes are available as commercially prepared kits for this purpose (Accu Probe; Gene Probe, Inc., SanDiego, Calif.) (Liébana et al., 1996; Richter et al., 2004).

The amplification of specific sequences of DNA by the polymerase chain reaction (PCR) technique has been reported for the
detection of *M. tuberculosis* complex organisms in human specimens (sputum) and detection of *M. bovis* in bovine tissues (Aranaz *et al*., 1996). Zanini *et al*. (2001) concluded that PCR showed the highest efficiency, compared to bacteriological culture and microscopic examination when used to diagnose *M. bovis* in bovine tissue samples and the success of PCR depends on the availability of DNA, free of contaminants that impair the amplification process. PCR has been used for the rapid identification of mycobacteria with different gene targets. The genomic fragments most commonly chosen for species-specific amplification of the *M. tuberculosis* complex are the insertion sequences IS6110, IS1081, MPB70 gene (Liébana *et al*., 1996), 65kDa heat shock protein gene, MPB64 protein gene, 16S rDNA, 35 kDa protein gene and 23S rRNA (Roth *et al*., 1997).

In the nested polymerase chain reaction (nPCR), two pairs of PCR primers are used for a single locus. The first pair amplifies the locus, while the second pair of primers (nested primers) binds within the first PCR product and produce a second PCR product that will be shorter than the first one (Viljoen *et al*., 2005). Miyazaki *et al*. (1993) reported that the (nPCR) was compared with the conventional smear and culture methods for detection of *M. tuberculosis* and the reaction showed excellent specificity, sensitivity and agreement with the conventional methods, indicating a contribution to the rapid diagnosis of mycobacterial infectious diseases.

Extensive studies based on DNA homology have reported 95-100% DNA relatedness between members of the complex. Sequencing of the 16S rRNA gene and sequencing of the more variable internal
transcribed spacers (ITSs) between 16S and 23S rRNA showed that, there are no sequence differences between the members of the complex. Furthermore, sequence analysis of \( rpoB \), \( katG \), \( rpsL \) and \( gyrA \) genes have revealed a very strong identity among bacteria of the \emph{M. tuberculosis} complex (Aranaz \textit{et al.}, 1999).

\subsection*{1.6.8.1 MTBC typing}

Despite the close genetic relatedness, the members of the MTBC differ in their epidemiology, pathology and antibiotic response (Aranaz \textit{et al.}, 1999; Niemann \textit{et al.}, 2000) and development of molecular techniques to differentiate strains of the \emph{M. tuberculosis} complex has helped to understand the epidemiology of TB (Gutiérrez \textit{et al.}, 1997).

The earliest strain typing method applied successfully to the MTBC was bacterial restriction enzyme analysis (REA) which produced a highly discriminating typing most notably \emph{M. bovis} but, due to difficulties in accurately reproducing the technique, recording and data basing patterns, the REA technique has not gained widespread acceptance (Skuce and Neill, 2001).

A modification of the REA technique is pulsed Field Gel electrophoresis (PFGE), where rare cutting restriction enzymes are used to digest genomic DNA. The DNA fragments are then separated by electrophoresis in a specific apparatus. This method discriminates the strains with low \textit{IS6110} copies (Narayanan, 2004). The same author described another tool for typing members of MTBC, the whole genome fingerprinting technique, fluorescent amplified fragment length
polymorphism (FAFLP). This technique has been developed and applied to *M. tuberculosis* isolates for discriminating low copy number strains.

Identification of repetitive DNA, such as variable number tandem repeats (VNTRs) in the genome sequences of *M. tuberculosis* strains H37Rv and CDC 1551 and *M. bovis* AF2122/97 has been exploited recently in strain typing of MTBC (Roring *et al.*, 2004).

Pavlik *et al.* (2002) reported that spacer oligonucleotide typing (spoligotyping), is a PCR-based method that reveals the polymorphism of the direct repeat region by detecting the presence or absence of specific spacer sequences. Spoligotyping is an excellent method for differentiation of MTBC members with low amounts of DNA (dead isolates stored in liquid or on solid media for several years) so it can be used in epidemiological studies of bovine tuberculosis in the countries with low incidence and prevalence of the infection.

Restriction fragment length polymorphism using the insertion sequence IS6110 (IS6110/RFLP) is considered to provide the best discrimination of MTBC isolates because of its variability in copy numbers but lacks sensitivity for the majority of *M. bovis* due to the low number of IS6110 copies (Gutiérrez *et al.*, 1995; Aranaz *et al.*, 1996; Gibson *et al.*, 2004).

Yamamoto and Harayama (1995) have proposed that *gyrB* could be a suitable phylogenetic marker for the identification and classification of bacteria. They have shown that the divergence of *gyrB* sequences reflected the taxonomical relationships in the genera *Acinetobacter* and *Pseudomonas*. They have also shown that the average base substitution rate of 16S rDNA was 1% per 50 million years, while that of the *gyrB*
genes at synonymous sites was 0.7-0.8 per one million years. The \textit{gyrB} analyses of other bacterial genera have also resolved closely related strains. The \textit{gyrB} gene encodes the B subunit of DNA gyrase (topoisomerase II), an enzyme universally distributed and essential for bacterial replication (Fukushima \textit{et al.}, 2003; Chimara \textit{et al.}, 2004).

Recently, Kasai and co-workers (2000) reported DNA sequence variations in the \textit{gyrB} gene that may be useful for species differentiation of slowly growing mycobacteria and even for the differentiation of members of the MTBC (Niemann \textit{et al.}, 2000). They developed a method of PCR and PCR-Restriction Fragment Length Polymorphism analysis (PCR-RFLP) to differentiate these species (Fukushima \textit{et al.}, 2003) (Table 2).
Table 2. DNA sequences of the four discriminatory regions in the gyrB gene described by Kasai et al., 2000 and of one new region found by Niemann et al., 2000 *.

<table>
<thead>
<tr>
<th>REFERENCE SEQUENCE</th>
<th>region 1 (675)</th>
<th>region 2 (756)</th>
<th>region new (1311)*</th>
<th>region 3 (1410)</th>
<th>region 4 (1450)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em></td>
<td>GGGTA C GAGT</td>
<td>AACG GT G CGG</td>
<td>GGCCGC T GTGA</td>
<td>TGTAA C GAAC A</td>
<td>CCGAC G CGAA</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> subsp. bovis</td>
<td>GGGTA C GAGT</td>
<td>AACG GT A CGG</td>
<td>GGCCGC T GTGA</td>
<td>TGTAA T GAAC A</td>
<td>CCGAC T CGAA</td>
</tr>
<tr>
<td><em>M. bovis</em> subsp. caprae</td>
<td>GGGTA C GAGT</td>
<td>AACG GT A CGG</td>
<td>GGCCGC G GTGA</td>
<td>TGTAA C GAAC A</td>
<td>CCGAC T CGAA</td>
</tr>
<tr>
<td><em>M. bovis</em> subsp. c</td>
<td>GGGTA C GAGT</td>
<td>AACG GT A CGG</td>
<td>GGCCGC G GTGA</td>
<td>TGTAA C GAAC A</td>
<td>CCGAC T CGAA</td>
</tr>
<tr>
<td><em>M. africanum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. africanum</em> subtype I</td>
<td>GGGTA C GAGT</td>
<td>AACG GT G CGG</td>
<td>GGCCGC T GTGA</td>
<td>TGTAA C CAAC A</td>
<td>CCGAC T CGAA</td>
</tr>
<tr>
<td><em>M. africanum</em> subtype II</td>
<td>GGGTA C GAGT</td>
<td>AACG GT G CGG</td>
<td>GGCCGC T GTGA</td>
<td>TGTAA C CAAC A</td>
<td>CCGAC G CGAA</td>
</tr>
<tr>
<td><em>M. microti</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. microti</em> type IIAMA</td>
<td>GGGTA T GAGT</td>
<td>AACG GT G CGG</td>
<td>GGCCGC T GTGA</td>
<td>TGTAA C CAAC A</td>
<td>CCGAC T CGAA</td>
</tr>
<tr>
<td><em>M. microti</em> type vole</td>
<td>GGGTA T GAGT</td>
<td>AACG GT G CGG</td>
<td>GGCCGC T GTGA</td>
<td>TGTAA C CAAC A</td>
<td>CCGAC T CGAA</td>
</tr>
</tbody>
</table>
CHAPTER TWO
MATERIALS AND METHODS

2.1 Investigation in Cattle

2.1.1 Study area

The study was conducted in different areas of Khartoum State (Figure 1). The State is located between 15° 36N and 32° 33E. The altitude is 380 m above sea level. The mean minimum and maximum temperatures are 18.5°C and 33.9°C, respectively. The rainfall fluctuates between trace (April) and 77.6 mm (July) (National Meteorological Service, 2005).

The Animal population in the State is comprised of cattle (222,000), sheep (445,000), goats (726,000) and camels (5,000) (Ministry of Agriculture, Animal Wealth and Irrigation, 2005).

2.1.2 Study design

2.1.2.1 Study population and sampling method

During the period February 2005-February 2006, a total of 587 dairy cattle (25 indigenous, 404 crossbred and 158 pure Holstein) from 35 randomly selected dairy farms were examined. Cattle in the sampled herds were of both sexes and above 6 months of age.

Dairy farms were located in different geographical areas of Khartoum State and animals were sampled according to accessibility and willingness of the owner. The calculation of sample size was based on a
Figure 1. Distribution of the sampled herds in Khartoum State, Sudan
previous prevalence of 0.5% of bovine tuberculosis among slaughtered animals in Khartoum State (Manal, 2003) with an absolute precision of 5% for a 95% confidence interval using the following formula (Thrusfield, 1995):

\[ n = \frac{1.96^2 \cdot P_{\text{exp}} \cdot (1 - P_{\text{exp}})}{d^2} \]

Where:

- \( n \) = required sample size
- \( P_{\text{exp}} \) = expected prevalence
- \( d \) = desired absolute precision

Determination of expected prevalence \( (P_{\text{exp}}) \) depended on previous information on BTB in Khartoum State.

### 2.1.2.2 Questionnaire survey

The data for the study were obtained by questioning the animal owners. The questionnaire covered data on sex, age, breed, body condition, and pregnancy/lactation, and other data related to different risk factors associated with bovine tuberculosis such as herd size, housing, feeding, stocking rate, hygiene, degree of ventilation, degree of exposure to sunlight, existence of wild or domesticated animals, number of feeding and drinking troughs and previous history of tuberculosis in the herd (Appendix 1). The questionnaire was done to each individual cattle using a sample form (Appendix 2).

### 2.1.3 Tuberculin Test

Comparative intra-dermal tuberculin test was carried out, following the manufacturer’s instructions, on cattle older than six months.
using bovine and avian purified protein derivative (PPD) (Synbiotics, France).

All selected cattle were inoculated with 20000 IU bovine PPD and 25000 IU avian PPD. For inoculation, two sites 12-15 cm apart on the middle third of the neck (Plate 1) were shaved, cleansed and examined for presence of any gross lesion.

The skin thickness was measured (in millimeters) with calliper and recorded before the injection of tuberculin. Point one milliliter of avian tuberculin was injected intradermally into the upper site and an equivalent dose of bovine PPD was injected into the lower site of the neck (Plate 2) using a short needle and a graduated syringe.

The skin thickness of each injection site was remeasured 72 hours after injection and recorded. Test procedure and interpretation of test results were made according to Office International des Epizooties (OIE) as follows:

\[
\text{bv}_2 - \text{bv}_1 = \text{bvd} \\
\text{av}_2 - \text{av}_1 = \text{avd}
\]

Then:
- \(bvd\) is positive and \(bvd - avd > 4\text{mm}\) = positive reaction
- \(bvd\) is positive and \(bvd - avd\) between 1mm-4mm = inconclusive
- \(bvd\) is negative or positive but \(\text{bvd} = avd\)
- \(bvd < avd\) = negative

Key:
- \(\text{av}_1\) = skin thickness before injection of avian PPD
- \(\text{av}_2\) = skin thickness 72 hours after injection of avian PPD
- \(\text{bv}_1\) = skin thickness before injection of bovine PPD
- \(\text{bv}_2\) = skin thickness 72 hours after injection of bovine PPD
- \(\text{avd}\) = skin thickness difference of avian PPD
- \(\text{bvd}\) = skin thickness difference of bovine PPD

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Plate 1. Inoculation sites of Avian PPD and Bovine PPD on the middle third of the neck of the individual cattle
Plate 2. Avian PPD (upper site) and Bovine PPD (lower site) are 12-15 cm apart from each other on the middle third of the neck.
2.2 Investigation in Man

2.2.1 Collection of specimens

One hundred and two positive sputum specimens were collected randomly from TB patients at the Tuberculosis Reference Laboratory (National Health Laboratory), El Shaab Teaching Hospital and Abo Anga Hospital in Khartoum state during the period January 2005-January 2006. Sputum specimens were collected in wide-mouthed, screw-capped, plastic sputum containers and processed immediately.

All the mycobacteriological methods were performed inside the biological safety cabinet (BSC) class II – Tuberculosis Reference Laboratory- National Health Laboratory (Stack).

2.2.2 Media and stains

2.2.2.1 Media

Modified Lowenstein-Jensen (LJ) medium was used for the cultivation and differentiation of Mycobacterium species, specially *M. tuberculosis* and *M. bovis* (IUT, 1955).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer salt solution</td>
<td></td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>7</td>
</tr>
<tr>
<td>Disodium hydrogen orthophosphate</td>
<td>4</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Citric acid</td>
<td>3</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>10</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

Preparation

Lowenstein-Jensen medium was prepared as described by the International Union Against Tuberculosis as follows:
All the base components were added at one time and dissolved in distilled water, autoclaved for 15 minutes at 121°C and cooled to 50°C.

600 ml of the buffer salt mixed with 1000 ml sterile whole-egg homogenate containing 40 ml 1% malachite green to give a homogenous mixture avoiding formation of air bubbles. 5ml amounts were distributed into sterile 25ml screw-capped bottles, then laid horizontally in an inspissator and heated at 85°C for 45 minutes.

Lowenstein-Jensen pyruvate medium was made by substituting sodium pyruvate for the glycerol in the mineral salt and egg fluid mixture of the standard medium.

2.2.2.2 Ziehl-Neelsen stain

Ziehl-Neelsen (ZN) stain was prepared and used to stain smears from clinical materials and from cultures (IUAT, 1978).

2.2.2.2.1 Reagent
Solution A: Saturated alcoholic solution of fuchsin

| Basic fuchsin | 3 g |
| Ethanol 96%   | 100 ml |

Solution B: Phenol solution, 50g/L (5%), aqueous

| Phenol crystals | 10g |
| Distilled water | 200 ml |

Then:

| Solution A | 10 ml |
| Solution B | 90 ml |
Formula for decolorizing agent

- Ethanol 96% 970 ml
- Hydrochloric acid 30 ml

Formula for counterstaining solution

- Methylene blue 0.3 g
- Distilled water 100 ml

2.2.2.2 Method of Staining

Fixed smears on slides were flooded with strong carbol-fuchsin, heated gently until vapor raised and left for 5 minutes. Slides were then washed gently under running water, decolorized by 3% acid alcohol until all macroscopically visible stain has been washed, rinsed again under running water and then flooded with methylene blue for 1 minute. Slides were washed gently under running water and allowed to dry, then examined at a magnification of x100 (oil immersion) (IUAT, 1978).

2.2.3 Decontamination of sputum specimen

Sputa were decontaminated by modified Petroff’s method without centrifugation (Mackie and McCartney, 1989). The specimen was transferred into a sterile centrifuge tube, vortexcid with an equal volume of 1N sodium hydroxide (NaOH 4%) and kept at room temperature for 20 minutes. 1 N hydrochloric acid with phenol red was added to neutralize the alkaline reaction until the solution becomes yellow to clear. The sediment was used for microscopy and culture.

2.2.4 Microscopy

All decontaminated specimens were microscopically examined by Ziehl-Neelsen staining.
2.2.5 Isolation and identification

2.2.5.1 Isolation

Two slants of Lowenstein-Jensen medium; one with sodium pyruvate and the other with glycerol were inoculated with the sediment from the processed sputum specimen and were then incubated aerobically at 37°C. The slants were examined within 3-7 days after incubation for detection of rapidly growing Mycobacteria and contaminated cultures. All cultures were incubated for 8 weeks with weekly examination for evidence of growth.

2.2.5.1.1 Growth rate

The growing organism was identified as slow-growing or rapid-growing by monitoring the growth in the primary isolation. Mycobacteria forming colonies within 7 days were considered rapid growers, while those requiring longer periods were considered slow growers (Metchock, et al., 2003).

2.2.5.1.2 Colony morphology

Cultures were identified from their morphology as eugenic or dysgenic, smooth or rough according to known criteria (Metchock, et al., 2003).

2.2.5.1.3 Morphology of the acid-fast organisms

Colonies were examined for acid-fastness by the Ziehl-Neelsen staining method and morphological characteristics such as shape (rods or bacilli) were recorded.
2.2.5.2 Identification tests

2.2.5.2.1 Catalse test

Catalse test was done as described by WHO (1998).

2.2.5.2.1.1 Reagents

0.067M phosphate buffer solution, pH 7.0

Solution 1: 0.067M solution

\[ \text{KH}_2\text{PO}_4 \quad 9.7 \text{ g} \]
\[ \text{Distilled water} \quad 1000 \text{ ml} \]

Solution 2: 0.067M solution

\[ \text{Na}_2\text{HPO}_4 \text{ anhydrous} \quad 9.07 \text{ g} \]
\[ \text{Distilled water} \quad 1000 \text{ ml} \]

Solution 1 and solution 2 were added to each other to provide 0.067M phosphate buffer solution, pH 7.0.

Hydrogen peroxide, 30%

Tween 80, 10%

\[ \text{Tween 80} \quad 10 \text{ ml} \]
\[ \text{Distilled water} \quad 90 \text{ ml} \]

Tween 80 was mixed with distilled water and autoclaved at 121°C for 10 minutes.

Complete catalse reagent (Tween peroxide mixture)

Immediately before use, equal parts of 10% Tween 80 and 30% hydrogen peroxide were mixed.

2.2.5.2.1.2 Test procedure

The test was done by using the 68°C at pH 7 (indicates loss of catalse activity due to heat) as follows:
Point five milliliter of 0.067M phosphate buffer solution, pH 7.0 was added aseptically, with a sterile pipette, to screw-capped tubes. Several loopfulls of each test cultures were suspended in the buffer solutions using sterile loops. Tubes containing the emulsified cultures were placed in water bath at 68°C for 20 minutes. Tubes were removed from heat and cooled to room temperature. 0.5 ml of freshly prepared Tween peroxide mixture was added to each tube and caps replaced loosely. Positive result was recognized by formation of bubbles on the surface of the liquid.

2.2.5.2.2 Nitrate reduction test

Test procedure and interpretation of the test was done according to WHO (1998).

2.2.5.2.2.1 Reagents

Sodium nitrate substrate in buffer

Solution 1 (0.022 M solution)

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 3.02 \text{ g} \\
\text{Distilled water} & \quad 1000 \text{ ml}
\end{align*}
\]

Solution 2 (0.022 M solution)

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 3.16 \text{ g} \\
\text{Distilled water} & \quad 1000 \text{ ml}
\end{align*}
\]

611 ml of solution 2 was added to 389 ml of solution 1 and mixed well to provide solution 3, pH 7.0.

Complete sodium nitrate substrate buffer

\[
\begin{align*}
\text{NaNO}_3 & \quad 0.58 \text{ g} \\
\text{Solution 3} & \quad 1000 \text{ ml}
\end{align*}
\]
The sodium nitrate was dissolved in the buffer, sterilized by autoclaving at 121°C for 15 minutes.

Hydrochloric acid solution

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated HCl</td>
<td>10 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Concentrated HCl was added to distilled water to obtain 1:1 dilution.

Sulfanilamide solution, 0.2%

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfanilamide</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Sulfanilamide was dissolved in distilled water.

N-naphthylethylene-diamine, 0.1%

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-naphthylethylene-diamine</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

N-naphthylethylene-diamine was dissolved in distilled water.

**2.2.5.2.2.2 Test procedure**

Two loopfulls of a 4 week old culture were suspended in 0.2 ml sterile saline in screw-capped tubes. 2 ml of NaNO₃ substrate was added, shaken well and the tube was incubated at 37°C in water bath for 3 hours. Tubes were removed and 1 drop HCl, 2 drops 0.2% sulfanilamide and 2 drops 0.1% N-naphthylethylene-diamine were added and tubes examined for a pink to red color. A positive reaction was indicated by a red color.

**2.2.5.2.3 Susceptibility to thiophen-2-carboxylic acid hydrazide (TCH)**

Löwenstein-Jensen medium containing 5 mg/liter TCH was prepared. A loop full of homogenized culture suspension was inoculated
into slope of TCH medium and incubated at 37°C for 2-3 weeks. A positive result (resistance to TCH) was recognized by the presence of growth compared with that on TCH-free control medium (Grange and Yates, 1994).

2.2.6 Molecular identification

Further identification of all isolated mycobacterial strains (79 isolates) was performed by using PCR methods on the *gyrB* gene (Kasai *et al.*, 2000; Niemann *et al.*, 2000; Chimara *et al.*, 2004).

2.2.6.1 Nested polymerase chain reaction (nPCR)

The PCR kit was purchased from Bioline, UK.

2.2.6.1.1 Preparation of mycobacterial DNA

DNA extractions were performed as described by Roth *et al.* (1998) with some modifications. A loopful of colony from each isolate was suspended in 500µl deionized distilled water in a 1.5 ml screw-capped microcentrifuge tube. The bacterial suspensions were vortexed then boiled at 100 ºC for 20 min in a thermoblock (Biometra-Germany) to release DNA. The heat-treated samples were then centrifuged at 13,000 rpm for 15 min. The supernatants containing the extracted DNA were transferred to a sterile microcentrifuge tube and stored at -20°C until used for amplification.

2.2.6.1.2 Amplification of the *gyrB* gene by PCR (*gyrB*-PCR1)

The target DNA for amplification was 1,020-bp fragment of the *gyrB* gene, which used to identify members of the *M. tuberculosis* complex. The primers used were MTUB-f (5'- TCG GAC GCG TAT GCG ATA
TC-3') and MTUB-r (5'-ACA TAC AGT TCG GAC TTG CG-3') (Niemann et al., 2000; Chimara et al., 2004). The 50-µl reaction mixture contained 5µl 10x PCR buffer (10 mM Tris-HCL (pH 8.3) and 50 mM KCL), 1.5 µl of 1.5 mM MgCl₂, 1µl of 10 mM of each deoxynucleoside triphosphate (dNTP), 1µl of 20 pmol of each primer, 0.2 µl of 1U of Taq DNA polymerase and 2µl of template DNA. The mixture was covered with 25 µl of light mineral oil to prevent evaporation.

Thermal profiles were performed in an automated PROGENE thermal cycler. After initial denaturation for 5 min at 94°C, 35 amplification cycles were completed, each consisting of 1min at 95°C, 1 min at 65°C and 2 min at 72°C. A final extension of 10 min at 72°C was applied.

A positive control containing chromosomal DNA of M. tuberculosis H37Rv (National Reference Laboratory) and a negative control without template DNA were included in each run.

2.2.6.1.3 Electrophoresis

Amplified products of the gyrB gene (1020-bp) were electrophoretically separated at a constant voltage of 80V for 45 min on 1.5 % agarose gel, which was prepared as follows:

0.6 g agarose gel were dissolved in 40 ml 1x TBE buffer (Tris-HCl, Boric acid, EDTA) under heat, then 0.4 µl of ethidium promide was added before pouring the liquid agarose into the gel electrophoresis apparatus. 3 µl of the PCR product from each sample were mixed with 3 µl loading dye, then the mixture was loaded onto gel of 1.5 % and electrophoresed together with 5 µl of DNA ladder (100 bp marker) as
DNA size marker. The gel electrophoresis apparatus was connected to a power pack (Biometra-Germany). Gels were visualized under UV light with transilluminator (Biometra-Germany), photographed and stored as a soft copy (Gel documentation system (BIODOG ANALAYZ, BIOMETRA, Germany).

2.2.6.1.4 gyrB-based species specific PCR for M. tuberculosis complex (gyrB-PCR2)

MTBC isolates confirmed with gyrB-PCR1 were analyzed further by species specific PCR using the specific primer set MTUB-756-Gf (5'-GAA GAC GGG GTC AAC GGT G) and MTUB-1450-Cr (5'-CCT TGT TCA CAA CGA CTT T CGC-3') for selective amplification of the gyrB fragments from M. tuberculosis.

Three µl of the first amplification product were transferred to a PCR tube containing 2.5 µl reaction buffer, 0.3 µl of 1.25 U of Taq DNA polymerase, 0.2 µl of 10 mM of each deoxynucleoside triphosphate (dNTP), 1 µl of 100 pmol of each primer. Deionized distilled water was added to each PCR tube to obtain a total volume of 20 µl. The mixture was covered with 10 µl of light mineral oil to prevent evaporation.

The thermal cycling profiles were as follows: 5 min incubation at 94°C, followed by 35 cycles at 95°C for 1 min and annealing-extension step at 72°C for 1.5 min. A final extension at 72°C for 10 min was carried out to ensure complete synthesis of the expected PCR products. Positive and negative control reaction in which PCR mixes were inoculated with M. tuberculosis H37Rv DNA and sterile distilled water
without PCR product, respectively, were performed with each set of reactions. PCR products for 77 isolates were analyzed and sized as previously described using 1.5% agarose gel.

2.2.6.2 PCR-restriction fragment length polymorphism (RFLP) analysis of the gyrB DNA polymorphisms

The DNA polymorphisms in the 1,020-bp gyrB fragment amplified with the primer pair MTUB-f and MTUB-r for 9 isolates were analyzed by restriction with RsaI as instructed by the manufacturer (Promega, USA) and as described by Niemann et al. (2000). The analysis was performed for 9 MTBC isolates, including 2 reference strains; *M. tuberculosis* H37Rv and *M. bovis* BCG (National Reference Laboratory) and 7 clinical isolates.

Five µl of the amplified product were added to a mixture containing 12 µl deionized distilled water and 2 µl of the restriction 10x buffer. The preparation was mixed gently by pipetting and then 1 µl 10u/µl of the RsaI was added to obtain a total volume of 20 µl. The mixtures were incubated in the thermal cycler for 90 min at 37°C to complete digestion. Samples were prepared by adding 4 µl of the digest with 3 µl of the loading dye with 5 µl of DNA ladder (100 bp marker) as DNA size marker and the restriction fragments were separated in 2% agarose gel by electrophoresis at a constant voltage of 90V for 45 min.

2.3 Statistical analysis

2.3.1 Animal investigation

Microsoft Excel (Windows 2003) and Stata (Windows 98/95/NT) were used for data analysis. Chi-square (\(\chi^2\)) was used for assessing the
statistical associations of various risk factors with BTB. The logistic regression model was employed to obtain the odds ratios only for those factors which gave statistical significant with regard to BTB. Interpretation of the results was done as follows: exposure could not be associated with BTB if the odd ratio (OR) = 1, could be a risk factor if the OR > 1 and could not be a risk factor if the OR < 1. To determine the significance of the association, 95% Confidence Interval (CI) was used.

2.3.2 Human investigation

Descriptive statistics (percentages) were used to analyze the human data.
CHAPTER THREE
RESULTS

3.1. Animal investigation

In total, 587 dairy cattle from 35 randomly selected dairy farms were investigated for the presence of bovine tuberculosis using the single Intradermal Comparative Tuberculin Test (SICTT). Nine animals (1.5%) gave positive or inconclusive reactions. Negative reactors and reactors to avian PPD were (98.5%). Tables 3 and 4 show the outcome of these reactors according to the standard interpretation and the severe interpretation respectively. The prevalence of reaction to avian PPD was 1.9% (Table 5).

The statistical association of various risk factors and prevalence of BTB was assessed using Chi-square ($\chi^2$) test. The results of the factors related to the animal are summarized in Table 6. The poor body condition was significantly associated with the prevalence of BTB ($\chi^2$ =10.519; P-value =0.001). To quantify this agreement, logistic regression model (Table 7) showed that the body condition could not be a risk factor for BTB (OR= 0.106). On the other hand, no significant statistical association was found between the prevalence of the disease and most of the risk factors related to the animal.

The results of the factors related to the husbandry and management and prevalence of BTB are presented in Table 8. A Positive correlation ($\chi^2$ =7.215; P-value = 0.027) was recorded between the herd size and occurrence of BTB. Logistic regression model (Table 9) showed that
herd size could be a risk factor for BTB (OR = 1.947). The percentages of the stocking rate by prevalence of BTB are shown in figure 2.

Table 3. Outcome of specific reactors to SICTT according to standard interpretation

<table>
<thead>
<tr>
<th>Study site</th>
<th>No. of cows Investigated (%)</th>
<th>Specific reactors (%)</th>
<th>Doubtful reactors (%)</th>
<th>Negative reactors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shargeneel</td>
<td>230</td>
<td>0(0.00)</td>
<td>1(0.43)</td>
<td>229(99.57)</td>
</tr>
<tr>
<td>Khartoum</td>
<td>153</td>
<td>0(0.00)</td>
<td>0(0.00)</td>
<td>153(100)</td>
</tr>
<tr>
<td>Khartoum North</td>
<td>41</td>
<td>0(0.00)</td>
<td>1(2.44)</td>
<td>40(97.56)</td>
</tr>
<tr>
<td>Omdurmann</td>
<td>163</td>
<td>1(0.61)</td>
<td>6(3.68)</td>
<td>156(95.71)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>587</strong></td>
<td><strong>1(0.17)</strong></td>
<td><strong>8(1.36)</strong></td>
<td><strong>578(98.5)</strong></td>
</tr>
</tbody>
</table>
Table 4. Prevalence of bovine tuberculosis in Khartoum State according to severe interpretation (SICTT)

<table>
<thead>
<tr>
<th>Study site</th>
<th>No. of cows investigated (%)</th>
<th>Specific reactors to bovine PPD (%)</th>
<th>Negative reactors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shargelneel</td>
<td>230</td>
<td>1(0.43)</td>
<td>229(99.57)</td>
</tr>
<tr>
<td>Khartoum</td>
<td>153</td>
<td>0(0.00)</td>
<td>153(100)</td>
</tr>
<tr>
<td>Khartoum North</td>
<td>41</td>
<td>1(2.44)</td>
<td>40(97.56)</td>
</tr>
<tr>
<td>Omdurmann</td>
<td>163</td>
<td>7(4.29)</td>
<td>156(95.71)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>587</strong></td>
<td><strong>9(1.5)</strong></td>
<td><strong>578(98.5)</strong></td>
</tr>
</tbody>
</table>
Table 5. Prevalence of reaction to avian PPD

<table>
<thead>
<tr>
<th>Study site</th>
<th>No. of cows investigated</th>
<th>Avian positive reactors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shargelneel</td>
<td>230</td>
<td>5(2.17)</td>
</tr>
<tr>
<td>Khartoum</td>
<td>153</td>
<td>4(2.61)</td>
</tr>
<tr>
<td>Khartoum North</td>
<td>41</td>
<td>0(0.00)</td>
</tr>
<tr>
<td>Omdurmann</td>
<td>163</td>
<td>2(1.23)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>587</strong></td>
<td><strong>11(1.9)</strong></td>
</tr>
</tbody>
</table>
Table 6. Relationship between prevalence of BTB and some factors related to the animal

<table>
<thead>
<tr>
<th>Factor</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.175</td>
<td>0.676</td>
</tr>
<tr>
<td>Age</td>
<td>1.469</td>
<td>0.480</td>
</tr>
<tr>
<td>Breed</td>
<td>4.582</td>
<td>0.101</td>
</tr>
<tr>
<td>Body condition</td>
<td>10.519</td>
<td>0.001**</td>
</tr>
<tr>
<td>Pregnancy/Lactation</td>
<td>3.498</td>
<td>0.321</td>
</tr>
<tr>
<td>Existence of domesticated animals</td>
<td>0.057</td>
<td>0.811</td>
</tr>
<tr>
<td>Previous history of BTB</td>
<td>0.597</td>
<td>0.440</td>
</tr>
<tr>
<td>Avian PPD</td>
<td>0.175</td>
<td>0.676</td>
</tr>
</tbody>
</table>

** Highly significant P<0.01
$\chi^2$: Chi-square
P-value: significant level

Table 7. Logistic regression model for demonstration of the association between prevalence of BTB and body condition

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>SE</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body condition</td>
<td>0.106*</td>
<td>0.089</td>
<td>0.020</td>
</tr>
</tbody>
</table>

*The factor could not be a risk factor OR<1.
OR=odd ratio
SE=standard error
CI=confidence interval
Table 8. Relationship between prevalence of BTB and some factors related to the husbandry and management

<table>
<thead>
<tr>
<th>Factor</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd size</td>
<td>7.215</td>
<td>0.027*</td>
</tr>
<tr>
<td>Housing</td>
<td>3.222</td>
<td>0.073</td>
</tr>
<tr>
<td>Exposure to sun light</td>
<td>3.366</td>
<td>0.067</td>
</tr>
<tr>
<td>Hygiene</td>
<td>1.021</td>
<td>0.312</td>
</tr>
<tr>
<td>Feeding</td>
<td>1.374</td>
<td>0.241</td>
</tr>
</tbody>
</table>

*significant P<0.05  
$\chi^2$: Chi-square  
P-value: significant level

Table 9. Logistic regression model for demonstration of the association between prevalence of BTB and herd size

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>SE</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd size</td>
<td>1.947*</td>
<td>0.846</td>
<td>0.830</td>
</tr>
</tbody>
</table>

*The factor could be a risk factor OR>1  
OR=odd ratio  
SE=standard error  
CI=confidence interval
Figure 2. The relationship between presence of TB and stocking rate

Chi-square ($\chi^2$) = 5.727 \hspace{1cm} P-value = 0.057 (P > 0.05, not significant)
3.2. Human investigation

3.2.1. Sputum samples

One hundred and two sputum samples were collected randomly from TB patients. 61(59.8%) samples were collected from Tuberculosis Reference Laboratory, 27(26.5%) were from El Shaab Teaching Hospital and 14(13.7%) were from Abu Anga Hospital in Khartoum State during the period January 2005 to January 2006.

3.2.1.1. Microscopic examination

All One hundred and two sputum smears which were stained by Ziehl-Neelsen stain, revealed acid-alcohol fast organisms (Plate 3) (Table 10). Out of 102, 6 (5.9%) samples showed fragmented acid fast bacilli.

3.2.1.2. Isolation

Seventy nine (77.5%) out of 102 samples showed visible colonies on Lowenstein-Jensen medium incubated aerobically at 37°C for up to 8 weeks (Plate 4). All the samples, which showed fragmented acid fast bacilli failed to grow on LJ medium.

3.2.1.3. Identification of isolates

3.2.1.3.1. Cultural and morphological characteristics

The growth rate of the isolates ranged between 6 days to 8 weeks in the primary isolation. Most of the isolates showed visible growth after 2 weeks and were considered as slow growers whereas 1 sample showed visible growth after 6 days and was considered as rapid grower (Table 10). All isolates that showed growth were confirmed as acid-fast bacilli by ZN staining.
Plate 3. Tuberculosis bacilli in sputum, Ziehl-Neelsen (x100).
3.2.1.3.2. Biochemical tests

Biochemical analysis of the isolates included nitrate reduction, catalase test and growth in the presence of thiophene carboxylic acid hydrazide (Table 10).

3.2.1.3.3. Molecular identification
3.2.1.3.3.1. Nested polymerase chain reaction (nPCR)
3.2.1.3.3.1.1 Amplification of the *gyrB* gene from clinical isolates (*gyrB*-PCR1)

Seventy nine mycobacterial isolates were investigated by PCR to confirm their identity as member of the *M. tuberculosis* complex using the primer pair MTUB-f (5'- TCG GAC GCG TAT GCG ATA TC-3') and MTUB-r (5'- ACA TAC AGT TCG GAC TTG CG-3') for MTBC-specific amplification of the 1,020-bp fragment of the *gyrB* gene. 77(97.5%) isolates were positive for PCR and identified as members of *M. tuberculosis* complex whereas 2(2.6%) isolates were negative and identified as MOTT.

The confirmations of the positive results were obtained following the analysis of PCR amplified product in agarose gel electrophoresis. The products gave very clear bands that matched well with the DNA size marker (Figure 3). The presence of the single band of 1,020-bp was taken to denote positive *gyrB*, which is specific for *M. tuberculosis* complex. The positive result of the *gyrB* gene was demonstrated by comparing it with a positive control containing *M. tuberculosis* H37Rv DNA (Figure 3, Lane 2) and negative control containing PCR reaction mixture without template DNA (Figure 3, Lane 10).
Table 10. Selected discriminatory phenotypic characteristics, biochemical tests and PCR amplified gyrB of the isolates investigated.

<table>
<thead>
<tr>
<th>strain</th>
<th>acid fastness</th>
<th>Growth rate</th>
<th>Colony morphology</th>
<th>Catalse test</th>
<th>Nitrate reduction test</th>
<th>TCH</th>
<th>PCR</th>
<th>Species</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>slow grower</td>
<td>Eugenic</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>M.tuberculosis</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>slow grower</td>
<td>&quot;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>M.tuberculosis</td>
</tr>
<tr>
<td>3</td>
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<td>+</td>
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<tr>
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<td>+</td>
<td>M.tuberculosis</td>
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<tr>
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<td>M.tuberculosis</td>
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- (+) Positive test result / (-) Negative test result / (ND) Not determined (Biochemical tests of the isolates could not be performed because of the limited growth on Löwenstein Jensen medium or decontamination).
Figure 3. Analysis of PCR-amplified 1,020-bp fragment of the *gyrB* gene in 1.5% agarose gel electrophoresis.

Lane 1: 100-bp Ladder, Lane 2: Positive control amplified from *M. tuberculosis* H37Rv, Lane 3-9: DNAs from clinical isolates, Lane 10: control without Mycobacterial DNA.
3.2.1.3.3.1.2 gyrB-based species-specific PCR for \textit{M. tuberculosis} complex (\textit{gyrB}-PCR2)

77 MTBC isolates confirmed with the \textit{gyrB} PCR1 were further differentiated by species-specific PCR using specific set of primers MTUB-756-Gf (5’-GAA GAC GGG GTC AAC GGT G) and MTUB-1450-Cr (5’-CCT TGT TCA CAA CGA CTT T CGC-3’) that allowed selective amplification of the \textit{gyrB} fragments from \textit{M. tuberculosis}. All the 77(100%) MTBC isolates were positive for \textit{gyrB} PCR2 and confirmed with the presence of a single band of 734-bp, which is specific for \textit{M. tuberculosis} (Figure 4). The positive result of the 734-bp was demonstrated by comparing it with a positive control containing \textit{M. tuberculosis} H37Rv DNA (Figure 4, Lane 2) and negative control containing PCR reaction mixture without PCR product (Figure 4, Lane 10).

3.2.1.3.3.2 \textit{gyrB} PCR-RFLP

To further confirm the differentiation system presented, reference strains \textit{M. tuberculosis} H37Rv and \textit{M. bovis} BCG as well as 7 clinical \textit{M. tuberculosis} strains confirmed by \textit{gyrB}-PCR2 were analyzed by \textit{gyrB} PCR-RFLP.

All \textit{M. tuberculosis} isolates showed the typical \textit{M. tuberculosis}-specific \textit{RsaI} RFLP patterns (Figure 5) (100, 360 and 560-bp) (Figure 5, Lane 2, 3, 4, 5, 6, 7, 8, 9), while 360 and 480-bp fragments were generated from \textit{M. bovis} BCG (Figure 5, Lane 10).
Figure 4. Analysis of PCR- amplified 734-bp fragment of the gyrB gene from *M. tuberculosis* in 1.5 % agarose gel electrophoresis.

Lane 1: 100-bp Ladder, Lane 2: Positive control amplified from *M. tuberculosis* H37Rv, Lane 3-9: DNAs from clinical isolates, Lane 10: control without Mycobacterial DNA.
Figure 5. RFLP patterns of PCR products obtained by *RsaI* digestion of the 1,020-bp *gyrB* PCR fragment. Lane 1: 100-bp Ladder, Lane 2: Positive control amplified from *M. tuberculosis* H37Rv, Lane 3-9: patterns of *M. tuberculosis* clinical isolates (100- 360 and 560-bp), Lane 10: patterns of *M. bovis* BCG (360 and 480-bp).
CHAPTER FOUR
DISCUSSION

Bovine tuberculosis has been well documented in cattle in the Sudan (Suleiman and Hamid, 2002; Manal et al. 2005). It has gained importance in the animal production industry because of the economic losses incurred from herds infections and possible human health hazards, particularly there are no control programmes for BTB and the risk of opportunistic infection with \textit{M. bovis} has increased with emergence of human immunodeficiency virus (HIV) (Cosivi et al., 1998). Hence, continued surveillance is required to identify and eliminate infected animals so that the public health hazard and the economic impact are minimized.

The study aimed to assess the prevalence rate of bovine tuberculosis in some farms in Khartoum State. The zoonotic implication of BTB in humans was also investigated in TB patients in Khartoum State. Moreover, the \textit{gyrB}-based PCR was applied to differentiate the members of the \textit{M. tuberculosis complex} (MTBC).

The present study documented a prevalence rate of 1.5\% of bovine tuberculosis in Khartoum State depending on the investigation conducted using the single intradermal comparative tuberculin test (SICTT). Previous studies had reported BTB in Khartoum State and elsewhere in the Sudan. In addition, there are occasional reports of evidence of infection at post mortem examination among cattle, depending on these facts the severe interpretation scheme was used to display results of SICTT. This result is in agreement with that of Sanousi and Omer (1985)
who reported that standard interpretation is applied among herds with no recent history of tuberculosis or no evidence of infection at post-mortem of previous reactors while severe interpretation is considered among herds with recent tuberculosis problem or herds with one or more reactors of the current test in which tuberculosis is confirmed on post-mortem examination. In addition, Costello et al. (1997) reported that a positive or inconclusive result according to the standard interpretation specified in the EU directive was classified as reactors to the test. Moreover, Griffin and Dolan (1995) have supported this after an inconclusive reactor revealed tuberculous lesions, which yielded \textit{M. bovis} upon culture.

This individual animal prevalence (1.5\%) presented in this study is in agreement with the findings of Karib (1962) and Manal (2003) who recorded a prevalence rate of 3.9\% in northern herds and 0.5\% among slaughtered herds in Khartoum State, respectively.

The low prevalence of BTB in the present survey might be due to the fact that most of the cattle examined were not from semi-intensive or intensive management system, and in the case of the traditional animal husbandry system found in the dairy farms in Khartoum State, animals are kept in open-air, which is expected to minimize the rate of transmission of \textit{M. bovis}. It might also be ascribed to the number of positive reactions to avian PPD which may have diagnostic implications. The low prevalence of BTB might also be due to false negative reactions, which might occur in recently infected cattle, cattle with generalized severe tuberculosis and cattle under stress (malnutrition, gastrointestinal parasitosis concurrent infection and recent parturition). Animals under these circumstances are reported to be anergic and not reactive to PPD.
due to antigen excess and consequently immuno-suppression (Monoghan et al. 1994; Radostits et al. 2000; Ameni et al. 2003; Unger et al. 2003). Concurrent infections like tick-borne diseases and high burden of gastrointestinal parasites are common in the herds of the present study.

The prevalence of reactions to avian PPD was 1.9%. Recent findings have shown that the environmental infections with other mycobacteria could influence the immune response to *M. bovis* (Vordermeier et al., 2001; Buddle et al., 2002). Therefore, it is assumed that the low prevalence of *M. bovis* in the study population might not reflect the true epidemiological picture.

Results of the risk factors analysis showed that the poor body condition was significantly associated with the prevalence of BTB, but could not be a risk factor (OR<1). This is might be attributed to the nutritional deficiencies in animals with poor body condition which may be associated with increased susceptibility to *M. bovis* infection. Contrary to the result of the present study Cook et al. (1996) and Ameni et al. (2003) observed that a large number of reactors in animals with good body condition. This finding could be due to the fact that animals with good body condition have a better immunostatus and thus respond to any foreign protein better than those with poor body condition, which may be immunocompromised as the result of malnutrition and/or other stress factors.

The investigation on the factors related to the husbandry and management revealed that the herd size was a contribution factor for BTB occurrence. This finding is in agreement with Cook et al. (1996) and Omer et al. (2001). They observed that the herd size significantly
influenced BTB and non-specific infections. This might be due to the fact that risk of individual animal introducing tuberculosis infection into a negative herd may increase with the herd size and because the inhalation of aerosols is the most common route of transmission in housed cattle, thus the closer the animals are in contact the greater is the chance that the disease will be transmitted especially in large herds. In contrast, Shirima et al. (2003) reported that the herd size had no influence on the distribution of both bovine and avian reactors. Such difference could be attributed to the unequal number of herd examined.

In this study, no significant statistical association was observed between the risk factors related to the animals (sex, age, breed, pregnancy/lactation, existence of domesticated animals, previous history of BTB) and risk factors related to the husbandry and management (housing, exposure to sunlight, hygiene, feeding) and occurrence of BTB and this may be attributed to the small size of animals examined.

Reactors to avian PPD are mainly caused by infection either with \textit{M. avium} or \textit{M. paratuberculosis}. As a consequence to close antigenic relationship between \textit{M. avium} and \textit{M. paratuberculosis}, cattle with clinical John’s disease (caused by \textit{M. paratuberculosis}) were found to react to avian tuberculin (Quinn et al., 2002). This result may suggest the possible occurrence of John’s disease in the study area. Another assumption to explain the reactors to avian PPD occurrence observed is the circulation of \textit{M. avium} hold by poultry and possibly transmitted to cattle. Poultry keeping is commonly practiced in the dairy farms investigated.
Routine differentiation of the members of *Mycobacterium tuberculosis* complex (MTBC) is still based on a number of phenotypic characteristics and biochemical tests. These tests need sufficient bacterial growth, are time-consuming, do not allow an unambiguous species identification in every case and are not routinely performed by many laboratories. Hence, further methods allowing accurate and rapid species identification are urgently needed for clinical and epidemiological purposes.

In this study, sputum specimens were collected from TB patients and subjected to microscopical and bacteriological examination. Acid-alcohol fast bacteria (AFB) were detected in 102 (100%) samples using ZN staining method, 6(5.9%) of which were fragmented acid fast bacilli. Seventy nine (77.5%) of the samples gave growth when cultivated on Löwenstien-Jensen medium. The samples which showed fragmentation failed to grow on LJ medium. This is may be due to inhibition of growth by antibiotics since many of the patients were under treatment. Treatment is considered a type of disturbance which causes fragmentation.

In the present study, three biochemical tests, catalase test, nitrate reduction test and growth on the presence of thiophen-2-carboxylic acid hydrazide (TCH) were used to differentiate the mycobacterial isolates. Results of the biochemical tests tentatively indicated that most of the isolates were *M. tuberculosis* and 1 isolate was identified as mycobacteria other than tuberculosis (MOTT).

In this study, *gyrB*-based methods were performed to differentiate the mycobacterial isolates because of the high degree of sequence conservation among members of MTBC makes differentiation of species
in the clinical mycobacteriology laboratory a difficult task. Out of 79 mycobacterial isolates subjected to gyrB-PCR1, 77(97.5%) isolates were positive for PCR and identified as members of Mycobacterium tuberculosis complex and 2(2.6%) isolates were negative and identified as MOTT, no further molecular characterization was done to identify these two isolates to species level. This finding substantiated the findings of Kasai et al. (2000) and Niemann et al. (2000) that the primer pair MTUB-f and MTUB-r allow the MTBC-specific amplification of a part of the gyrB and may be also used for identification of MTBC isolates. The 77 MTBC isolates were examined by the gyrB-PCR2 for further differentiation. All the 77(100%) MTBC isolates were positive for the test and were identified as M. tuberculosis. This result is in agreement with that of Kasai et al. (2000) who designed PCR primers that allowed selective amplification of the gyrB fragment from each species of the M. tuberculosis complex.

To evaluate the clinical applicability of the gyrB PCR-RFLP, the target genes of two reference strains (M. tuberculosis H37Rv and M. bovis BCG) and 7 clinical isolates were amplified and the products were digested by the endonuclease Rsa1. M. bovis BCG showed the typical M. bovis Rsa1 RFLP pattern (360-480-bp) described by Niemann et al. (2000). All the M. tuberculosis strains showed the typical M. tuberculosis Rsa1 RFLP pattern (100,360 and 560-bp). This result is partially compatible with the finding of Niemann et al. (2000) who described that M. tuberculosis could be identified by their specific Rsa1 RFLP pattern (360 and 560-bp). Results presented in this study showed that there are bands which were not considered by Niemann et al. (2000), were clearly
visible. This observation is in agreement with Chimara et al. (2004), who developed a new diagnostic algorithm of *M. tuberculosis* specific *RsaI* RFLP pattern (100,385 and 560-bp).

Thus, the *gyrB* PCR-RFLP using the restriction enzyme *RsaI* presented in this study is rapid and easy to-use technique to discriminate between *M. tuberculosis/M. africanum* (360/560bp), *M. bovis* (360/480bp) and *M. microti* (360/660bp).

The low prevalence of BTB in Khartoum State is matched the absence of *M. bovis* infection in human at the time of the study. Possible explanations that there is no direct link between the human population investigated and the observed cases of BTB or there is a link, but the chosen study population in humans was still not sufficient to detect a relation between this low prevalence of BTB and human infection.

In conclusion, bovine tuberculosis among dairy herds in Khartoum State was found to be low (1.5%), and it has no public health implications. The DNA sequence polymorphism in the *gyrB* gene represents a unique marker that facilitates the differentiation of MTBC by DNA sequencing or species specific PCR or simple PCR-RFLP analysis.
RECOMMENDATIONS

1. The presence of bovine tuberculosis in cattle necessitates further investigations on the role of animal-derived tuberculosis in human health.

2. Similar studies should be conducted in different parts of the country (especially in southern area, where humans and animals are often share the same shelter) to establish the magnitude of the disease in both animals and man.

3. The single comparative intradermal tuberculin test (SCITT) is more efficient in medium or high-infected herds. In low infected populations it is therefore recommended to repeat the skin-test regularly to increase the sensitivity or use more sensitive test.

4. The use of (SCITT), although it is sensitive and inexpensive, is not simple and requires two field visits, therefore it would be desirable if future tests are simpler to perform.

5. Complementary post-mortem investigations of positive reactors in slaughterhouses would give further information concerning the prevalence of BTB.

6. Successful control of human tuberculosis should be accompanied by control of animal tuberculosis.

7. Public education on the cooking of meat and milk before consumption is recommended.
REFERENCES


APPENDICES
Appendix (1): General Questionnaire

Date………………………
Code No…………………..
Owner’s name……………………………………………………………………
Address………………………………………………………………………………
Herd number………………………………………………………………………

❖ Herd data

1. Housing
Classical……………….Intensive/Semi Intensive………………

2. Herd size
Number of cattle owned…………………………………………………………

3. Stocking rate
………………………………………………………………………………

4. Hygiene
Yes………………………………..No………………………………………..

5. Feeding
………………………………………………………………………………

6. Number of feeding and drinking troughs
………………………………………………………………………………

7. Degree of ventilation
Yes………………………………..No………………………………………..

8. Degree of exposure to sun light
………………………………………………………………………………

9. Existence of wild or domesticated animals
………………………………………………………………………………

10. Previous History of TB in the herd
Yes………………………………..No………………………………………..
Appendix (2): Sample Testing Form

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Date of test application</th>
<th>Date of measurement</th>
</tr>
</thead>
</table>

- **Animal data**

<table>
<thead>
<tr>
<th>Name/Number</th>
<th>Sex</th>
<th>Age</th>
<th>Breed</th>
<th>Body condition</th>
<th>Pregnancy/Lactation</th>
</tr>
</thead>
</table>

- **Measurement**

  - First reading
  - Second reading
  - Positive
  - Doubtful
  - Negative

- **Remarks**

  - 
  - 
  - 
  - 
Appendix 3.
Speciation within *Mycobacterium tuberculosis* complex (Metchock, *et al*., 2003; Grange and Yates, 1994)

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>GROWTH RATE</th>
<th>PIGMENTATION</th>
<th>OPTIMAL TEMP(°C)</th>
<th>COLONY MORPHOLOGY</th>
<th>NIAVIN</th>
<th>GROWTH ON TCH</th>
<th>NITRATE REDUCTION</th>
<th>CATALASE 68°C</th>
<th>PZA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M.tuberculosis</em></td>
<td>slow grower</td>
<td>N (100)</td>
<td>37</td>
<td>R</td>
<td>+(95)</td>
<td>(Res)</td>
<td>+(97)</td>
<td>-1</td>
<td>sens</td>
</tr>
<tr>
<td><em>M.africanum</em></td>
<td>slow grower</td>
<td>N</td>
<td>37</td>
<td>R</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>-</td>
<td>sens</td>
</tr>
<tr>
<td><em>M.bovis</em></td>
<td>slow grower</td>
<td>N (100)</td>
<td>37</td>
<td>Rt</td>
<td>-(4)</td>
<td>(Sens)</td>
<td>-(9)</td>
<td>-(2)</td>
<td>Res</td>
</tr>
<tr>
<td><em>M.bovis BCG</em></td>
<td>slow grower</td>
<td>N</td>
<td>37</td>
<td>R</td>
<td>-</td>
<td>(Sens)</td>
<td>-</td>
<td>-</td>
<td>Res</td>
</tr>
<tr>
<td><em>M.canetti</em></td>
<td>slow grower</td>
<td>N</td>
<td>37</td>
<td>Sm</td>
<td>+</td>
<td>(Res)</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

- N= nonchromogenic
- R= rough / Rt = rough and thin or transparent / Sm = smooth
- Positive (+) / Negative (-) / Variable reaction (V)
- Res = Resistance / Sens =Sensitive /ND = not determined
- The percentage of strains positive in each test is given in parentheses, and the test result is based on these percentages