Contagious Bovine Pleuropneumonia Isolation and Seroprevalence in Khartoum State, Sudan

By
Amira Shareef Farh Shareef
(B.V.Sc. 2004)
University of Khartoum

Supervisor
Dr. Suliman Mohamed El Hassan

A thesis submitted to the University of Khartoum in partial fulfillment for the requirement of the degree of Master of Science in Microbiology

Department of Microbiology
Faculty of Veterinary Medicine
University of Khartoum

October 2009
DEDICATION

To soul of my father
To my mother
To my brothers and sisters
To all my friends
ACKNOWLEDGEMENTS

First, I thank greatly Allah for his guidance throughout the period of the study.

I wish to express my gratitude to Dr. Suliman Mohamed El Hassan for his close supervision and guidance.

Sincere thanks are due to Dr. Ameera Mahgoub Mohammed Ahmed for close follow up and permission to do this research work at the Department of Mycoplasma, Central Veterinary Research Laboratories, Soba.

My thank are due to Dr. Maha Shareef Farh for advice and encouragement.

My thanks are also due to Mr. Eltayeb Elsadig, Mrs. Nadia Abdelwahab, Mr. Awad Elseed and the other members of Mycoplasma Department, Central Veterinary Research Laboratories for their great assistance in the experimental and laboratory work.

My great thanks also due to staff members of Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum.

I thank also the staff members of slaughterhouses in Khartoum State.

Finally, I would like to thank everyone who contributed directly or indirectly in this study.
# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>i</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>ARABIC ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER ONE: LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>1.1 History and distribution of disease</td>
<td>3</td>
</tr>
<tr>
<td>1.2 Taxonomy</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Aetiology</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Host range</td>
<td>9</td>
</tr>
<tr>
<td>1.5 Transmission of the disease</td>
<td>9</td>
</tr>
<tr>
<td>1.6 Symptoms of the disease</td>
<td>10</td>
</tr>
<tr>
<td>1.7 Pathology and post-mortem findings</td>
<td>11</td>
</tr>
<tr>
<td>1.8 Diagnosis</td>
<td>13</td>
</tr>
<tr>
<td>1.8.1 Clinical diagnosis</td>
<td>13</td>
</tr>
<tr>
<td>1.8.2 Differential diagnosis</td>
<td>13</td>
</tr>
<tr>
<td>1.8.3 Histopathology</td>
<td>13</td>
</tr>
<tr>
<td>1.8.4 Isolation of the organism</td>
<td>16</td>
</tr>
<tr>
<td>1.8.5 Biochemical tests</td>
<td>17</td>
</tr>
<tr>
<td>1.8.6 Serology</td>
<td>18</td>
</tr>
<tr>
<td>1.8.6.1 Complement fixation test (CFT)</td>
<td>19</td>
</tr>
<tr>
<td>1.8.6.2 Enzyme linked immunosorbent assay (ELISA)</td>
<td>19</td>
</tr>
<tr>
<td>1.8.6.3 Immunoblotting tests (IBT)</td>
<td>20</td>
</tr>
<tr>
<td>1.8.6.4 Slide agglutination test</td>
<td>20</td>
</tr>
<tr>
<td>1.8.7 Molecular diagnosis</td>
<td>21</td>
</tr>
<tr>
<td>1.9 Disease control and eradication</td>
<td>22</td>
</tr>
<tr>
<td>1.9.1 Stamping out policy</td>
<td>22</td>
</tr>
<tr>
<td>1.9.2 Treatment</td>
<td>22</td>
</tr>
<tr>
<td>1.9.3 Vaccination</td>
<td>23</td>
</tr>
<tr>
<td>1.10 Suitable age of vaccination</td>
<td>24</td>
</tr>
<tr>
<td>CHAPTER TWO: MATERIALS AND METHODS</td>
<td>25</td>
</tr>
<tr>
<td>2.1 Sterilization</td>
<td>25</td>
</tr>
<tr>
<td>2.1.1 Collection of the samples</td>
<td>25</td>
</tr>
<tr>
<td>2.1.2 Method of collection of samples</td>
<td>25</td>
</tr>
<tr>
<td>2.2 Medium</td>
<td>28</td>
</tr>
<tr>
<td>2.2.1 Fresh yeast extract stock solution</td>
<td>28</td>
</tr>
<tr>
<td>2.2.2 Yeast extract stock solution</td>
<td>28</td>
</tr>
<tr>
<td>2.2.3 Penicillin stock solution</td>
<td>28</td>
</tr>
<tr>
<td>2.2.4 Thallium acetate stock solution</td>
<td>29</td>
</tr>
<tr>
<td>2.2.5 Glucose solution</td>
<td>29</td>
</tr>
<tr>
<td>2.2.6 Preparation of horse serum</td>
<td>29</td>
</tr>
<tr>
<td>2.2.7 Liquid medium</td>
<td>29</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mycoplasma mycoides cluster</td>
</tr>
<tr>
<td>2</td>
<td>The advantages and disadvantages of the methods of diagnosis</td>
</tr>
<tr>
<td>3</td>
<td>Sources of lung tissue samples collected from different slaughterhouses in Khartoum State.</td>
</tr>
<tr>
<td>4</td>
<td>Distribution of bovine serum samples according to localities</td>
</tr>
<tr>
<td>5</td>
<td>Detection of antibodies of Mycoplasma mycoides subsp mycoides small colony type of bovine sera by competitive ELISA</td>
</tr>
</tbody>
</table>

LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distribution of sera and Mab.</td>
</tr>
<tr>
<td>2</td>
<td>Colonial morphology of Mycoplasma mycoides subsp. mycoides small colony type isolate grown on heart infusion agar after 8 days incubation (x 40)</td>
</tr>
</tbody>
</table>
This research was carried out to study the current situations of contagious bovine pleuropneumonia (CBPP) in Khartoum State by isolation and serological technique.

Fifty-five pneumatic lung tissue samples, out of 750 lungs examined in slaughterhouses in the State were collected. Six pneumatic lung tissue samples were also collected from animals showing typical signs and post-mortem lesions of CBPP in Alkadaro, Khartoum North. Two hundred serum samples were collected randomly from animals in different areas of the State and screened for antibodies against *Mycoplasma mycoides* subsp. *mycoides* (small colony type) using competitive ELISA test. Two Mycoplasmas were isolated from infected cases in Alkadaro. The isolates were recovered from pneumatic lungs showing typical post-mortem lesions of the disease which included fibrinonecrotic pneumonia with yellowish fluid and sequestra. The two isolates were identified as *Mycoplasma mycoides* subsp. *mycoides* by their cellular morphology, cultural characteristics and growth inhibition tests. One hundred and fourteen, out of the 200 randomly collected serum samples, were seropositive for CBPP with competitive ELISA test.

The isolation and identification of the causative agents and serosurveillance findings of this study confirmed high prevalence (57%) of CBPP in Khartoum State.
Mycobacterium anitratum infection was also observed in the examined areas. The prevalence of Mycoplasma mycoides subsp. mycoides SC in the examined areas was determined by competitive ELISA. A total of 114 out of 124 samples were positive for Mycoplasma mycoides subsp. mycoides SC. The prevalence of Mycoplasma mycoides subsp. mycoides SC was 92.2% in the examined areas. The results showed that the infection rate was higher in the areas with higher humidity and lower temperatures. The infection rate was also higher in areas with higher air pollution levels.
INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a severe disease affecting cattle as well as buffaloes. It creates great economic losses in endemic regions and is declared by the Office International des Epizootic (OIE) to be disease of list A.

Mycoplasmas are the smallest free living microorganisms found so far. The mycoplasmas are widely distributed and many of them are pathogenic to humans, animals and plants (Dienes, 1994). The range of animal hosts varies from nonhuman primates to domestic animals. Animals mycoplasmas are essentially extracellular parasites with special affinity to mucous membranes in respiratory system, the urogenital tract, the mammary glands or serous membranes. Many mycoplasmas are non-pathogenic (commensals), while others are pathogenic to a widely varying degree. The pathogenic mycoplasmas are largely host specific, but closely related animals species may share the same mycoplasmas flora. The most common types of animal diseases caused by mycoplasmas are pneumonia, arthritis and mastitis.

The most important animal mycoplasmas belong to Mycoplasma mycoides cluster. Two members of Mycoplasma mycoides cluster are of particular importance, Mycoplasma mycoides subsp mycoides small colony type (MmmSC) causing contagious bovine pleuropneumonia (CBPP) and Mycoplasma capricolum subsp. capripneumoniae (Mccp) causing the contagious caprine pleuropneumonia (CCPP).

CBPP was eradicated from most continents during the 19th century but remained endemic in Africa. In Sudan CBPP remains to be persistent constrain of animal production. The disease is endemic in many parts of the country. It causes serious economic losses in the form of exhaustion
of the affected animal and finally death. Moreover, it affects adversely the country livestock trade and export to foreign markets and especially to traditional markets in the Middle East.

The current research was carried out to study the situation of CBPP in Khartoum State. This was fulfilled by:

1. Detection of CBPP in slaughterhouses and suspected areas by isolation and identification of the causative agents.

2. Serological screening of animals in the state for antibodies against *Mmm* (SC) using competitive enzyme linked immunosorbent assay.
CHAPTER ONE
LITERATURE REVIEW

1.1 History and distribution of disease

There is no full solid information about the time and place where contagious bovine pleuropneumonia (CBPP) was reported for the first time in the world. According to Carrason (1942), the disease was first observed in Italy and France in the middle of the sixteenth century. The development of the international trade in middle of the nineteenth century helped greatly the spread of the disease to England and Scandinavian countries. By 1803, the disease was reported in the United State of America (Jasper, 1967). The disease was introduced in South Africa in 1854 from Britain (Hutgra et al., 1971). In 1958 the disease was carried by sea to Australia through infected cattle (Turner, 1954).

Due to strict control measurement at the end of the nineteenth century and at the beginning of the twentieth century, the disease was eradicated from the USA, 1852 and most of the European countries e.g. England, 1898 and Russia 1940 (Jasper, 1967).

It was reported as late as 1958 in Portugal (Ferronha et al., 1990), further outbreaks occurred in 1961 in Spain and then in the Department des Pyrenees-Orunatales in France in 1967 (Anon, 1967, 1980, 1982 and 1984, Provost et al., 1987) where some mortality was recorded in three herds (Turner, 1961).

Infection in Portugal was reported to be widespread in 1983 with serological evidence suggesting that the disease had been present for months, may be years before (Ayling et al., 1999). CBPP was endemic in northwestern parts of Portugal around Porto, but outbreaks
subsequently decreased significantly. Spain began reporting cases of CBPP from 1989. Whilst the first cases occurred around Madrid and Segovia, the majority of outbreaks were in the northern coastal areas broadening the Bay of Biscay (Turner, 1961).

In 1990 Italy reported its first outbreak for over 100 years in Piedmont in the north. The disease quickly spread to most major cattle areas of Italy. However, as a result of abattoir surveillance, movement control linked to serological monitoring, and slaughter of infected and in contact animals, no cases have been reported since September 1993. For the first time for over 20 years, no outbreaks were reported in Europe in a year 2000.

The sources of outbreaks of CBPP in Italy (1990-1993) was never successfully traced, although France was heavily implicated by virtue of the large number of cattle exported to Italy from there and because they had experienced the disease in the previous decade, immunological evidence was presented by Poumarate and Solsona (1995) that most strains from Italy were different from those of outbreaks in Western Europe leading to the suggestion that "contamination of Italy did not arise from exportation of CBPP from Southwestern Europe" but from a resurgence in Italy itself or from some unknown foci elsewhere in Europe.

Outbreak of CBPP usually begins as the result of movement of an infected animal into a naive herd. It is widely believed that recovered animals which harbor the causative agent within pulmonary sequestra might become active shedder when stressed (Windson and Masiga, 1977).

The disease is still present in most African countries south of the Sahara, India and China (Hudson, 1971). Recently, there are
reports on severe outbreaks of CBPP in Kenya, Uganda, Ethiopia, South Africa, Asia and North Portugal (OIE, 2001).

In the Sudan the disease was first observed in 1875 in Darfur Province and later spread to Khartoum Province where it caused great losses among cattle (Anon, 1952). The disease disappeared during the Mahdi wars in 1889 and it reappeared again in Kordofan Province in 1912.

From there the disease spread quickly southward and eastwards of the province. In 1913 the disease was reported in Nuba Mountains, the White Nile, Upper Nile and Bahr El Gazal Provinces. In 1914 the disease reached Khartoum Province and then spread to Berber Province in 1913 and Kassala Province in 1917 (Anon, 1925).

The Pan African control of Epizootics (PACE) in 2003 showed that the disease is currently endemic in most parts of the Sudan especially in areas below 15°N latitude with varying degree of severity and with highest prevalence incidence in the south Equatoria States (Bahr Al Gabel State and Kaboita in east Equatoria), Upper Nile States (Renk, Fashoda, Elnasir, Malakal. Akopo and in Goungali State), Bahr Al Gazal State (Wau, Raja, Nahr Al Gor, Awil, and Rumbek) and Warab Lakes States.

The disease is frequently reported from eastern states near Ethiopian border in Doka and Al Galabat in Gedarif State.

Al Managel (Gezira State), Al Dinder and Singa (Sinnar State), Al Rossires and Al Angasana (Blue Nile State) and Algabalin and Kosti (White Nile State) are the endemic areas in the Central States.

Southern Kordofan State is also endemic area and reports were received from Al Delling, Kadogli and Abu Gebiha in the Southern
Kordofan State up Babanosa, Almoglad and Abeyei in the southern part of the Western Kordofan State. CBPP is widely spread in Southern Darfur State specially areas of El Dain, Buram and Radom.

Sporadic cases were reported in Khartoum State (Mageed, 2003). The disease was not reported in the northern Sudan (River Nile and Northern and Red Sea States) for more than 15 year.

1.2 Taxonomy

The mollicutes represent a class of unique cell wall-less prokaryotes that include members of the genus *mycoplasma*.

According to International Committee on Systemic Bacteriology-Subcommitte on the Taxonomy of Mollicutes, the class contain the following orders, families and genera

Order I: Mycoplasmatales

Family I: Mycoplasmataceae

Genus II: Mycoplasma

Order II: Entomoplasmatales

Family I: Entomoplasmataceae

Genus I: Entomoplasma

Genus II: Mesoplasma

Family II: Spiroplasmataceae

Genus I: Spiroplasma

Order III: Acholeplasmatales

Family I: Acholeplasmataceae

Genus I: Acholeplasma

Order IV: Anaeroplasmatales

Family I: Anaeroplasmataceae

Genus I: Anaeroplasma

Genus II: *Asteroplasma*
The members of this class are eubacteria that are bound by a single membrane. The major class characteristics are the lack of cell wall, the tendency to form fried-egg type colonies on solid media, the filterability of cells through 450 and 220 nm pore-size membrane filters, the presence of A-T-rich genome, and the failure of the wall-less to revert to walled bacteria under appropriate conditions.

Taxonomy within orders and families is based on sterol requirement, cell and colony morphology, optimum growth temperature and requirement for oxygen. Differences at genera and species levels are based on the above mentioned parameters beside the host origin, genome size and cultural and biochemical properties. More than 102 Mycoplasma species have been discovered. The most important animal pathogens belong to Mycoplasma myciodes cluster (Freundt and Edward, 1979).

1.3 Aetiology

CBPP is caused by Mycoplasma mycoides subsp. mycoides (Mmm SC) (Nocard et al., 1898). The causative agent (Mmm SC) is a member of the mycoplasma mycoides cluster which include 6 biochemically and antigenically similar mycoplasmas of animal importance (Table 1) (Haraswa, 2000). Species and subspecies within this cluster are difficult to distinguish by morphological and conventional biochemical tests. Although Mmm SC was considered to be host specific but it has been isolated from pneumonic lungs of sheep and goats (Brando, 1995; Thiaucoart et al., 1999). This may constitute a difficulty in the control of the disease in areas where cattle, sheep and goats are raised together.
Table 1: *Mycoplasma mycoides* cluster

<table>
<thead>
<tr>
<th>Mycoplasma</th>
<th>Type strain</th>
<th>Principal animal host</th>
<th>Disease</th>
<th>Other animal host</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. mycoides</em> subsp. <em>mycoides</em> type</td>
<td>PG1</td>
<td>Cattle</td>
<td>CBPP</td>
<td>Buffaloes, goats, sheep</td>
</tr>
<tr>
<td><em>M. mycoides</em> subsp. <em>capri</em></td>
<td>PG3</td>
<td>Goats</td>
<td>Pleuropneumonia arthritis</td>
<td></td>
</tr>
<tr>
<td><em>M. mycoides</em> subsp mycoides LC</td>
<td>Y-goat</td>
<td>Goats</td>
<td>Pleuropneumonia</td>
<td>Sheep, cattle</td>
</tr>
<tr>
<td><em>M. capricolum</em> subsp <em>capripneumoniae</em></td>
<td>F38</td>
<td>Goats</td>
<td>CCPP</td>
<td>Sheep</td>
</tr>
<tr>
<td><em>M. capricolum</em> subsp <em>capricolum</em></td>
<td>California Kid</td>
<td>Goats</td>
<td>Mastitis, arthritis</td>
<td>Sheep, cattle</td>
</tr>
<tr>
<td><em>Mycoplasma</em> sp. Bovine group 7 (leach)</td>
<td>PG50</td>
<td>Cattle</td>
<td>Arthritis</td>
<td>Goats, sheep</td>
</tr>
</tbody>
</table>

*Mycoplasma mycoides* subsp. *mycoides* was classified into small colony type (SC) and large colony type (LC) according to their morphology, cultural and biochemical characteristics (Cottew and Yeast, 1978). The large colony type activity digests casein, liquefies serum and survives at 45°C. These together with differences in growth characteristics and others permitted differentiation of the two types.

### 1.4 Host range

Contagious bovine pleuropneumonia is predominantly a disease of the Bovidae of the kind Bos (*Bos taurus* and *Bos indicus*). There is a variation in susceptibility to infection; in general European breeds tend to be more susceptible than indigenous African breeds (Provost *et al.*, 1987). Animal less than 3 years of age are less resistant to experimental challenge (Masiga *et al.*, 1978). The susceptibility of cattle to infection with *M. mycoides* depends on many factors such as the type of animal husbandry, individual resistance and other factors (Shallali, 1997).

In 2002 the infection was recorded in bison and yak. Although it has been reported that the domestic buffalo (*Bubalus bubalis*) is susceptible, the disease is difficult to reproduce experimentally in this species (Provost, 1988).

### 1.5 Transmission of the disease

Spread of the disease from infected to susceptible animals is mainly through inhalation of infected droplets which may be carried to 10-20 meters or more by the air currents (Turner, 1954). Factors, which influence the infection rate, are closeness of contact, intensity of infection and the level of individual susceptibility (Turner, 1954).
Even under extensive condition the gathering of animals at watering places will lead to outbreaks and will maintain the disease in herds (Coetzer et al., 1994).

The disease may not be detected for several weeks or months after infected animals have entered an area. Some animals also have a degree of resistance to the disease and those surviving CBPP are even more resistant (Coetzer et al., 1994). Outbreaks usually begin as a result of movement of an infected animal into a naive herd. It is widely believed that recovered animals harboring infectious organisms within pulmonary sequestra may become active when stressed. Although this may be a factor in some outbreaks, it has not been proved experimentally (Windsor, 1977).

Asymptomatically and chronically infected animals are very important in the spread of the disease to new areas. Chronic carriers are apparently healthy animal that have a localized focus of infection sequestered in a fibrous capsule in their lungs. The organisms can persist in such lesion for months, and in time the fibrous capsule may breakdown, allow viable organisms to escape by the bronchi and so infect susceptible in contact animals. This is particularly proved to occur when chronic carrier animals are subjected to stress such as when mustered or walked for long distances.

*Mycoplasma* can pass through the placenta from the infected dams to the off-spring (Stone, 1969).

1.6 Symptoms of the disease

The incubation period of the natural disease may range from 5 to 207 days. Although Turner and Campbeel (1937) reported a range of 29-58 days and Provost *et al.* (1987) stated 20 to 40 days. In
experimental infection, Regalla et al. (1994) reported that the disease symptoms appear in cattle 40 days after contact with inoculated animals and these symptoms lasted for 20 days.

The disease may be either clinical or sub-clinical. The clinical form may be acute, sub-acute or chronic (Turner, 1954).

The clinical signs in a typical acute case include a rise in body temperature (up to 40°C), shallow and rapid respiration in the early stage of the disease, which soon becomes abdominal with painful granting. The neck is extended. There is an excess mucous in the nostrils and frothy saliva around the mouth and occasionally soft cough is heard (Bygrave et al., 1968; Hudson, 1971).

In sub-acute cases lesions are localized in small part of the lung, the position of which cannot be easily located by percussion and auscultation. The only symptom is a rare cough, sometimes new foci of infection are created and acute symptoms set in (Henning, 1965).

In chronic cases the only symptom is a cough that usually appears when the animal rises or when it suddenly passes from a warm stable to cold weather or when it drinks cold water (Henning, 1965).

Sub-clinical and chronic cases of CBPP constitute more than 50% of animals involved in an epizootic (Bygrave et al., 1968). In calves about one year old or less the clinical picture of the disease in primarily characterized by arthritis and the chest may be free from any lesion (Moulton et al., 1956; Turner and Trethewic, 1961; Simmons and Johnson, 1963; Harbi and Salih 1979 and Elmahi, 1980).

1.7 Pathology and post-mortem findings

Pathological lesions are confined to the thoracic cavity. Different degree of pathological changes may be found in an animal.
Lesions are usually unilateral without a preference for the left or right side. The lesion is always localized in the diaphragmatic lobe. The cranial lobe seldom affected in the acute stage. Many liters of serous fluid are usually present in the thoracic cavity. The fluid is clear yellow-brown and may contain pieces of fibrin. A thick caseous fibrinous deposit is frequently present on the visceral and parietal pleura which is a pathognomonic sign. Another pathognomonic sign of CBBP is that the interlobular septa are distended by amber-coloured serous fluid which gives the lung the marbling appearance. The hepatized lung lobules have different colours varying from relatively normal to deep red or yellow-gray due to acute fibrinonecrotic pneumonia (OIE, 2001).

In the chronic stage, an adhesion connects the thickened visceral and parietal pleura. When the thoracic cavity is opened pieces of lung adhere to chest wall. The fluid in the thoracic cavity may disappear. The lung parenchyma appears oedematous with red consolidation. The interlobular septa are distended with lymph fluid (Turner, 1959; Hudson, 1971 and Liyloid, 1970). A necrotic portion or portions of the lung are walled off by a fibrinous tissue capsule forming what is known as sequestrum (Turner, 1959 and Liyloid, 1970). Sequestra vary in size from a centimeter in diameter to 20-30 cm in their greatest diameter (Hudson, 1971).

Nevertheless, the classical picture of the disease showing marbling appearance, consolidation, adhesion of the pleura and considerable amount of pleural fluid was reported in naturally infected calves (El Tahir et al., 1988).
1.8 Diagnosis

1.8.1 Clinical diagnosis

Tentative diagnosis depends on clinical signs, PM findings and demonstration of *Mmm* (SC) in pleural fluid of infected animal by dark field microscopy. But other confirmatory tests are necessary. The methods of diagnosis have advantages and disadvantages (Table 2).

1.8.2 Differential diagnosis

In carrying out CBPP clinical diagnosis, some sources of confusion may occur. Therefore, it is very important to differentiate this disease from other diseases which may have similar clinical signs or lesions.

1.8.3 Histopathology

Early in the course of the disease, the CBPP lesion comprise a bronchiolar necrosis and oedema which progress rapidly to an exudative serofibrinous bronchiolitis with extension to the alveoli and uptake of alveolar fluid into tissue spaces, lymph vessels and ultimately septal lymphatics (Done *et al.*, 1995). These rapidly reach saturation and the process is extended centrifugally to the tracheobronchial lymph nodes and centripetally to the pleural lymphatics. The mediastinal, sternal, aortic and intercostal lymph nodes may then become enlarged, oedematous or even haemorrhagic. With stasis, lymph vessels become thrombosed and ultimately fibroosed (Buttery *et al.*, 1980). The pulmonary lobules become consolidated with alveolar oedema, fibrin and inflammatory cells. Coagulative necrosis is common. *Mmm* SC can be demonstrated in these lobules by immunohistochemistry.
Table (2): The advantages and disadvantages of the methods of diagnosis.

<table>
<thead>
<tr>
<th>Method of diagnosis</th>
<th>Some advantages</th>
<th>Some disadvantages</th>
<th>Sample types</th>
<th>Preservation and storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation and identification</td>
<td>diagnosis very sure</td>
<td>Slow and requires viable pathogenic agents</td>
<td>Lungs, pleural, liquid</td>
<td>Under cold in order to guarantee the viability of the pathogenic agent</td>
</tr>
<tr>
<td>Serology (detection of the antibodies)</td>
<td>Speed and simplicity</td>
<td>Some tests are not very sensitive and for the majority do not allow to differentiate the post infectious antibodies from the vaccine antibodies</td>
<td>Blood in a tube without anticoagulant (to harvest serum)</td>
<td>Under cold to avoid the degradation of the antibodies</td>
</tr>
<tr>
<td>Molecular biology</td>
<td>Speed, sensibility and effective even when the pathogenic agent is not viable any more in the sample</td>
<td>Expensive and requires a suitably equipped laboratory and adequately trained personnel</td>
<td>Pleural, liquid, lungs, nasal swabs</td>
<td>Does not require any particular precaution</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Simplicity and does not require the conservation of the samples under cold temperature</td>
<td>Presumptive diagnosis, requires some heavy equipment and adequately trained personnel</td>
<td>Tissues (lungs)</td>
<td>In 10% of neutral formal saline</td>
</tr>
</tbody>
</table>

Razin and Tully (1981)
Perivascular organization foci or 'organizing centers', found in the interlobular septa, are considered pathognomonic for CBPP (Ferronha et al., 1988). They consist of a center occupied by a blood vessel with proliferation of connective and inflammatory cells. Two types of foci have been recognized (Numes Petisca et al., 1988). Type I foci contains more proliferative cells in the central zone which is larger than the peripheral zone and probably corresponds to the success of the host immune response in resolving the infection; immunoreactive antigen is associated with macrophages. In type II foci, the proliferative cells are scarce and the peripheral zone is relatively larger. Immunoreactive antigen can be seen in the central zone inside blood vessels and it is thought that Type II foci indicate failure of the immune response leading to aggravation of symptoms.

In an immunocytochemical study of CBPP infected Italian cattle, Scanziani et al. (1997) showed that the severity of lung lesions correlated with the severity of changes in the lymph nodes. In the acute stage of the disease specific antigen was detected in the lobular periphery and in the cytoplasm of alveolar macrophages. In chronic lesions, immunoreactivity was in the fibrotic areas and in macrophages located in the lobular septa; necrotic debris and macrophages located in the inner part of the sequestra were specifically stained. Immunoreactive material was also seen in the centrofollicular areas of the broncho-associated lymphoid tissue structures and in the lymph node follicles. Furthermore, electron microscopy of the mediastinal lymph nodes of a chronically affected calf showed degenerating mycoplasmas and a few apparently intact mycoplasmas in the macrophages.
The histological section of the lung in acute stage of the disease showed edema in the lymphatics of the interlobular septa and interstitial tissue and massive infiltration of fibrin, macrophage and neutrophils into the alveolar lumen (Bygrave et al., 1968). Also there was presence of lymphocytes and alveolar macrophages around the lymphatic vessels and septa margin (Jubb and Kennedy, 1963). In chronic cases the main lesions are the sequestra and fibrinous scars (Jubb and Kennedy, 1963; Bygrave et al., 1968).

1.8.4 Isolation of the organism

If the animal died, pleural fluid or affected lung can be collected aseptically for cultural examination in selective media (Gourlay, 1973). Sterile paper can be soaked with pleural fluid or allowed to absorb fluid from lung lesion (Turner, 1954).

When the animal died, pleural fluid or affected lung can be collected aseptically for cultural examination in a selective medium. The organism can be cultivated in different liquid or solid media depending on the availability of facilities in each laboratory. Different media are available for the isolation and cultivation of *Mmm* SC. Basically they are composed of two parts; the basic medium and the supplement. The basic part is a broth of lean meat heart muscle or liver (Bennett, 1932; Campbell, 1938; Dafalla, 1961; Gourlay, 1962; El Nasri, 1972 and Buttley, 1967). The supplement consists of equine or swine serum, yeast extract, DNA, glucose and also penicillin and thallium acetate as bacterial and fungal inhibitors.

Nowadays many basic commercial media as PPLO and brucella media are available for isolation and propagation of mycoplasma.
Growth of this organism in liquid medium culture becomes apparent as uniform faint turbidity within 3-6 days after aerobic incubation at 37°C. The organism is described as pleomorphic occurring as round ovoid, ring and filamentous forms in liquid media (Edward, 1950 and Turner, 1954). Colonies on solid media grow slowly in 3-6 days and are different in diameter and difficult to be seen by naked eye.

1.8.5 Biochemical tests

a) Glucose fermentation

Some strains of mycoplasma ferment glucose and produce acid (Erno and Stipkovits, 1973).

b) Hydrolysis of ariginine:

Some strains of mycoplasma, hydrolize arginine and shift the medium to alkaline (Erno and Stipkovits, 1973). This is done by the action of arginine deaminase as a character associated with non-fermentative and some of the fermentative mycoplasma.

c) Phosphotase activity:

It was done by incorporating phenolphthalein diphosphate in agar media as described by Eron and Stripkovits (1973). The activity is manifested by change of the colour of media to pink.

Film and spots phenomenon was first described by Edward (1950) and Edward and Fitzgerald, (1954). This test is of diagnostic value and is used to indicate the lipolytic activity of certain mycoplasmas containing the enzyme lipase. Characteristic, wrinkled, pearly film with tiny black spots appear on the surface of the medium that contains horse serum or egg yolk. The film consist of cholesterol
and phospholipids and the spots contain calcium and magnesium salts of fatty acids.

e) **Serum digestion:**

The method was described by Eron and Stripkovits (1973). The proteolytic activity is determined by the ability of the organism to digest coagulated serum.

f) **Terazolium reduction:**

This was usually done in liquid media (Erno and stripkovits, 1973) but others use solid media (Senterfit, 1973). It shows the ability of some mycoplasma to reduce 2, 3, 5 Triphenyl tetrazolium chloride to brick red formozan. The ability of the strains to reduce tetrazolium was noticed to be higher under anaerobic than aerobic conditions.

g) **Haemolysis:**

The test is performed on agar or in broth culture, both showing various degree of haemolyosis due to the production of peroxide by the organism.

h) **Hemadsorption:**

The observation of the adsorption of erythrocytes to mycoplasma colonies is detected microscopically. This property is used to measure the ability of mycoplasma to adhere to eukaryatic cells, hence it is related to the pathogenicity of the organism (Shmuel and Tully, 1983).

1.8.6 **Serology**

Two groups of serological tests are used. Specific identification of isolated mycoplasma relies heavily of growth inhibition test (GIT) and immunofluorescence (IF).
Serological diagnosis of CBPP depends on antibody detection using different tests e.g. CFT, ELISA, Agglutination tests… etc.

1.8.6.1 Complement fixation test (CFT)

Complement fixation test is currently the official and the most widely used serological diagnostic test. It is thought to be specific and sensitive in the acute phase of the disease. However, the test is reported to detect only about 70% of clinically infected animals and seem not to detect asymptomatic animals in the early stage of infection (Nicolas et al., 1996).

Although it is specific, CFT lacks sensitivity with a positive reaction result being only at 1/10 or higher. CFT is also far from robust, in a thorough examination of CFT in which over 33000 sera from healthy herds were tested between 1991-1994 in Italy. Bellini et al. (1998) reported that CFT was 98% specific. Regarding sensitivity, based on nearly 600 cattle with specific lesion from 11 infected herds, only 64% of animals were positive. Isolation of the causative agent from the affected animals was even more insensitive at 54%.

Surprisingly, during the Italian outbreaks, abattoir surveillance detected nearly as many cases as serological monitoring, while clinical examination was much less useful (Regalla et al., 1996). It followed that by using CFT as a screening test, some CBPP affected cattle, in the early or later stages of infection were missed, accounting for the persistence of the disease in Portugal.

1.8.6.2 Enzyme linked immunosorbent assay (ELISA)

The ELISA was used to detect antibodies against *M. mycoides* (Onoviran and Tayler Robenson, 1971). The test was found more sensitive than the CFT, slide agglutination serum test and agar gel
immunodiffusion test. It could detect *M. mycoides* antibodies in sera of cattle at least 19 months of infection and 25 months after vaccination.

A competitive ELISA (C-ELISA) was developed at CIRAD-EMVT, Montpellier. The test may have advantages interim of ease of testing and standardization of results, but it has sensitivity levels similar to that of CFT and thus will enable the persistence of disease as seen with CFT (Le Goff and Thiaucourt, 1998).

**1.8.6.3 Immunoblotting tests (IBT)**

Immunoblotting test is used generally to compare between isolated *Mmm* SC strains. Gonclaves *et al.* (1998) used it to detect five different antigens (110, 98, 95, 62/60 and 45 KDa). These antigens were highly characteristic of sera from Portuguese herds affected by CBPP (170 cattle).

There was 79% agreement between CFT and IBT. In a study of 88 cattle with CBPP lesions, IBT detected 80 positive animals and CFT detected 72 (Ayling *et al.*, 1991). Abdo *et al.* (1998; 2000) identified a 48 KDa protein, named LPPQ. It was found in the type strain and European, African and Australian field strains. They used the protein in an immunoblotting test for the sero-detection of *Mmm* SC in experimentally infected cattle.

**1.8.6.4 Slide agglutination test**

The slide agglutination test using serum (Pricestly, 1951) or blood (Newing and Field, 1953) is sensitive in early stages of the disease and suitable for establishing of preliminary diagnosis when large number of cattle are involved and for selection. So it is
recommended to be used as a herd test rather than an individual animal.

The most important test for detecting animals particularly those with sequestra is CFT (Campbell and Turner, 1937; Lindley, 1960; Huddart, 1963 and Pearson and Mcpherson, 1966). The advantage of CFT is the low number of false positive and negative reaction (Hudson, 1971). The main disadvantage of CFT is that it fails to detect infected animals in the incubation period of the disease.

1.8.7 Molecular diagnosis

A powerful diagnostic system based on PCR has been developed for rapid detection, identification and differentiation between members of the *M. mycoides* cluster. An arbitrarily primed PCR (AP-PCR) of *Mmm* SC, with Mlip 1 and Mlip 4 primers produced a fingerprint with little genomic polymorphism and thus of limited epidemiological use. Two bands of 900 bp and 100 bp for strains PGI, PO, KH3J and Fatick were produced, although the later had a faint band at 400 bp, in contrast to the five *M. caprivolum* strains tested which produced four different patterns (Rawadi *et al.*, 1995). These test strains were designed from sequences of unknown functions or from known genes (Bashiraddin *et al.*, 1994b; Dedieu *et al.*, 1994; Hotziel *et al.*, 1996; Niserez *et al.*, 1997; Rodriguez *et al.*, 1997; Person *et al.*, 1999). With many of these tests, confirmation of the presence of *Mmm* SC-type or production of the expected amplification product was possible by the digestion of the product with specific restriction enzyme. In some cases the standard detection of PCR products by agarose gel electrophoresis was replaced with enhanced methods which improved the sensitivity.
These methods have been used for the identification and detection of *Mmm* SC-type from culture and clinical materials including nasal swab, mucous, pleural fluid, lung tissues, lymph nodes, kidneys, spleen and semen from cattle (Bashiruddin *et al.*, 1994a, 1994b; Nicholas and Palmer, 1994; Bashiruddin *et al.*, 1999a; 1999b; Stradaioli *et al.*, 1999) and from milk and respiratory tract of small ruminants (Brandao, 1995). In some cases they had superior diagnostic sensitivities compared with conventional diagnostic tests. In particular, the sensitive nested PCR system has been used for the detection of *Mmm* SC from culture and clinical material where the target organism may be in low numbers such as in nasal swab samples (Hotzel *et al.*, 1996; Niserez *et al.*, 1997).

1.9 Disease control and eradication

Control of CBPP can be based on:

1.9.1 Stamping out policy:

This policy is a general method when infection is detected early. In the Sudan in which the mode of animal husbandry is nomadic this can not be done but cattle owner are persuaded to kill their clinically sick animals (Abdalla, 1975), so when the disease is reported in the herd its movement is restricted and segregated in grazing and watering areas.

1.9.2 Treatment

Another method of control is chemotherapy with broad spectrum antibiotics. This is only recommended for control of severe local reaction at the vaccination site since its use on actual case of CBPP could lead to a high incidence of carrier animals with squeuestra in their lungs. In 1967 the FAO/OIE OAU panel unanimously opposed therapeutic treatment for the actual case of CBPP and
strongly recommended that mass drug or antibiotic treatment of CBPP to be discouraged (FAO Report, 1967).

1.9.3 Vaccination

Vaccination against CBPP in Sudan has been practiced since 1914 (Anon 1914). In those early days infected pleural exudates from a sick animal was inserted under the skin of the tail tip. This method usually caused severe swellings.

Broth culture vaccine prepared from field strain of *M. mycoides* subsp *mycoides* was produced in the Veterinary laboratory in Khartoum in 1925 (Anon 1925) and in 1926 (Anon 1926). F strain isolated in 1944 from pulmonary exudates of naturally infected case and was subcultured in glycerine broth (Anon, 1950) was used for vaccine production in the Sudan and the recommended dose was 1 ml (Abdalla, 1975).

T₁ strain broth culture vaccine production originated in East Africa and was tested in the laboratory and the field in Sudan (Lindley and Abdalla, 1967). Comparative studies showed that this vaccine has superior immunizing power over F strain (Lindley and Abdalla, 1967, Daleel and Lindley, 1970). Cattle vaccinated with this vaccine were immune for more than 12 months (Daleel, and Lindley, 1970).

The other alternative and most practical way is the use of lyophilized vaccine. The research for the production of a dried vaccine started in the mid fifties (Gray and Turner, 1954). Lindley (1971) emphasized that the T₁ lyophilized vaccine is most valuable tool which can be used for controlling of CBPP, being more stable than broth culture vaccine (Brown *et al.*, 1965). Later Shallali *et al.* (1997) has successfully prepared lyophilized T₁/44 vaccine and has been used
in the field since that time. $T_{1/44}$ /SR strain is streptomycin resistant and dependant strain (Anon, 1988). Vaccine made from this strain is safe and gives satisfactory immunity for about 14 month (Provost, 1988).

1.10 Suitable age of vaccination

Colostrums is the source of antibodies which pass from calf gut to its blood stream and persist for up to 60 day (Stone, 1969). On the other hand Turner and Trethewie (1961) found that calves born to unvaccinated dams were highly susceptible to joint involvement within the first week of life. It was found that calves born to recently vaccinated dams showed antibodies one week post-parturition. Titers reached maximum at one month and then gradually declined. By the six months, 71% of the tested calves sera were found negative. On the basis of these results it is suggested that six months is the suitable age to start vaccination of calves from recently vaccinated dams (Shallali et al., 1998).
CHAPTER TWO
MATERIALS AND METHODS

2.1 Sterilization

All glassware used were soaked for an hour in warm 10% detergent solution, scrubbed and rinsed in running warm water. The glassware were then rinsed in distilled water and were put on clean surface to drain dry. Graduated and capillary pipettes, Petri dishes, forceps, scissors, glass syringes, needles and cotton swabs were sterilized in hot air oven at 160ºC for 2 h. Vials such as bijou, universal, and Seitz-filter were sterilized by autoclaving at 121ºC (15Ib/inch²) for 15 minutes.

2.1.1 Collection of the samples

Lung tissue samples were collected from different slaughterhouses (Ganawa, Al Sabaloga, Alkadaro, El Huda) in Khartoum State between January – May 2008. A total of 790 animals were investigated and only 55 lungs showed pneumonic lesions (Table 3).

Pneumonic lung tissues and sera were also collected from 6 animals showing typical clinical signs and post-mortem lesions of CBPP at one of Alkadaro Dairy Farms, Khartoum North.

A total of 200 serum samples were collected randomly from dairy farms and cattle herds in Khartoum State (Table 4).

2.1.2 Method of collection of samples

Specimens (5 cm³) were cut and placed in sterile plastic bags, labeled and transported in ice box at to the Central Veterinary Research Laboratory, Soba.
Table 3: Sources of lung tissue samples collected from different slaughterhouses in Khartoum State.

<table>
<thead>
<tr>
<th>Slaughter house</th>
<th>No. of animals examined</th>
<th>No. of Pneumonic lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkadaro</td>
<td>240</td>
<td>15</td>
</tr>
<tr>
<td>Al Huda</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>Alsbaloga</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>Ganawa</td>
<td>300</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>790</td>
<td>55</td>
</tr>
</tbody>
</table>
Table 4: Distribution of bovine serum samples according to localities

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>No. of sample collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkadaro</td>
<td>40</td>
</tr>
<tr>
<td>Soba</td>
<td>80</td>
</tr>
<tr>
<td>Hilat Kuku</td>
<td>15</td>
</tr>
<tr>
<td>Kafori</td>
<td>25</td>
</tr>
<tr>
<td>Omdurman</td>
<td>20</td>
</tr>
<tr>
<td>Shambat</td>
<td>15</td>
</tr>
<tr>
<td>Different samples</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>200</strong></td>
</tr>
</tbody>
</table>
For collection of serum, 10 ml of blood samples were collected in sterile plain vacutainers. Samples were left for 1h at room temperature then kept overnight in refrigerator at 4ºC. Samples were then centrifuged at 3000 rpm for 10 min. The separated serum was aspirated with sterile pipette, transferred into sterile containers and stored at -20ºC till used.

2.2 Medium

A basic medium of beef heart infusion (Oxoid) supplemented with yeast extract (fresh and powder), horse serum, glucose, in addition to penicillin and thallium acetate was used in the study. The medium was used as broth or solid medium.

2.2.1 Fresh yeast extract stock solution

Two hundred and fifty grams of pure baker’s dry yeast were added to 1L of distilled water, heated to boiling with stirring for 30 min. The mixture was centrifuged at 6000 rpm for 10 min, then sterilized by filtration through 0.2 um pore size filter (Seitz-filter). The filtrate was distributed into 100 ml volume and stored at -20ºC until used.

2.2.2 Yeast extract stock solution

Twenty five grams of yeast extract powder (Oxoid) were added to 100 ml distilled water and dissolved using magnetic stirrer. The preparation was sterilized by filtration through 0.2 um pore size filter (Seitz- filter). The filtrate was distributed in 20 ml quantities into sterile universal bottle, stored at -20ºC and used within one year.

2.2.3 Penicillin stock solution

One million IU Benzyl Penicillin (Alembic) was dissolved in 20 ml sterile distilled water, stored at (-) 20ºC and used within one year.
2.2.4 Thallium acetate stock solution

Thallium acetate 10% was prepared and sterilized by autoclaving at 121ºC for 15 min. The sterilized solution was stored at 4ºC and used within one year.

2.2.5 Glucose solution

Fifty grams of glucose were dissolved in 100 ml distilled water and sterilized by autoclaving at 110ºC for 5 min. The sterilized solution was stored at 4ºC.

2.2.6 Preparation of horse serum

Blood was collected aseptically from the jugular vein of a healthy horse into bottles contained 20% trisodium citrate. It was kept on the bench overnight. The plasma was then separated and measured. To each 100 ml plasma, one ml CaCl₂ was added. The mixture was shaken until fibrin was separated, then the serum was put in water-bath at 50ºC for half an hour to inactivate the complement and then sterilized by filtration through 0.2 µm pore size filter (size-filter). Aliquots of 50 and 100 ml serum were prepared and stored at 4ºC until used.

2.2.7 Liquid medium

Heart infusion broth:

PART A: Heart infusion broth-base (Oxoid):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth</td>
<td>40 g</td>
</tr>
<tr>
<td>Tryptose</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>880 ml</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.8
PART B:

- Horse serum: 100 ml
- Fresh yeast extract: 20 ml
- Glucose: 2 ml
- Penicillin: 0.33 ml
- Thallium acetate: 0.25 ml

Solid ingredients in part A were dissolved by boiling and the pH was adjusted to 7.8. Part A was then sterilized by autoclaving at 121°C for 15 min. After cooling part B was added aseptically and the mixture was distributed in 5 ml amounts into sterile test tubes. The distributed medium was incubated at 37°C overnight to check sterility and then stored at 4°C till used.

### 2.2.8 Solid medium

Heart infusion agar:

**Part A:** Heart infusion agar-base (Oxoid):

- Heart infusion agar: 50g
- Tryptose: 10g
- Sodium chloride: 5g
- Distilled water: 880 ml
  
  pH was adjusted to 7.8

**Part B:**

- Horse serum: 100 ml
- Yeast extract 30%: 20 ml
- Glucose 100.000 IU: 0.00 ml
- Thallium acetate 10%: 0.25 ml

Solid ingredients in part A were dissolved by boiling and the pH was adjusted to 7.8. Part A was then sterilized by autoclaving at 121°C for 15 min. After cooling to 45-50°C, part B (pre-incubated at
45°C) was then added aseptically to part A. The mixture was distributed immediately in 15 ml amounts into sterile Petri dishes and allowed to solidify on leveled surface.

2.3 Method of isolation

Lung tissue samples were pulverized in broth medium with antibiotics. The suspension was then diluted through tenfold steps (to $10^{-3}$) to minimize bacteria contamination. Dilutions were plated on solid medium. The pleural fluid was inoculated directly without previous dilution onto solid medium.

2.3.1 Incubation

The inoculated broth cultures were incubated at 37°C aerobically for three to ten days. The inoculated solid medium was incubated aerobically at 37°C in a humid chamber for 3 to 15 days. Further incubation for up to 21 days was continued if no visible growth was seen.

2.3.2 Detection of growth

The inoculated liquid medium was examined daily. Tubes showing faint turbidity (compared with the control medium) after day 3 of incubation were subjected to further investigation by dark field microscopy for presence of filamentous microorganism or bacterial contamination.

2.3.3 Purification and storage of isolate

Broth cultures were streaked on solid medium. The plates were then incubated as mentioned before. The surface of solid medium was examined under (x40) power lens of stereomicroscope for fried-egg type colony. When a fried egg appearance colony appeared after 3 days, agar cube containing single colony was incised and used to
inoculate broth medium. After aerobic incubation at 37ºC and when faint turbidity appeared, the culture was then inoculated on solid media without inhibitors to exclude the L-form bacteria. Fried-egg types of colonies were then used to inoculate broth media. When inoculated medium showed growth, the culture was stored at -20ºC.

2.4 Identification of the isolates

2.4.1 Microscopy

Broth cultures showing signs of growth were investigated by dark filed microscopy. A drop of culture was put on clean slide and covered with a cover slip. The slide was then examined under oil emersion lens. Short or long filaments indicated the presence of Mycoplasma.

2.4.2 Cultural characteristics

Growth of the isolate into liquid medium and on solid medium and the growth conditions were fully described.

2.4.3 Biochemical tests

2.4.3.1 Digitonin test:

Seventy five mg of digitonin were added to 5 ml ethanol 95% in a screw-capped tube and the solution was heated gently in flask of boiling water to dissolve the digitonin completely. Several sterile filter paper disks approximately 6 mm in diameter were placed in sterile plastic Petri dish and 0.025 ml of digitonin solution were added to each disk. The disks were dried overnight at 37ºC and stored at 4ºC until use.

2.4.3.1.1 Method of digitonin test

A solid medium was inoculated by the drop technique. A drop of culture was placed on the surface of medium and allowed to run in
one way. Then a disk of digitonin was placed aseptically on the line of culture. After aerobic incubation at 37°C for 3-10 days, inhibition of growth only around the disk indicated the sensitivity of the isolate to digitonin.

2.4.4 Serological test

2.4.4.1 Growth inhibition test

Growth inhibition test (GIT) was used for the identification of isolates and was performed as described by Wallace and Clyde (1983).

2.4.4.1.1 Materials

Sterile paper disks (6-7 mm in diameter) were impregnated with antisera against *Mmm* (SC). The antisera were obtained from Central Veterinary Research Laboratory, Soba, Sudan.

- Broth cultures of isolates, 3 days old.
- Heart infusion agar plates.
- Hundred ml – volume automatic pipettes and sterile tips.
- Sterile forceps.
- Ethanol 95%.

2.4.4.1.2 Method of growth inhibition test

Agar plates were dried in the incubator at 37 °C for 30 min. Then 0.1 ml of the broth culture was deposited on the agar surface and allowed to run across the plate. The plate was left at room temperature until the entire inoculum was absorbed into the medium. The sterile disk (containing *Mmm* SC-type antisera) was then placed carefully on the path of the drop using alcohol flamed forceps. The plate was incubated aerobically in a humid chambers at 37°C for 3-5 days. The surface of the plate was then examined using stereomicroscope at low
magnification for evidence of a zone without colonies encircling the disk *i.e.* zone of growth inhibition. The result was recorded by measuring the width of the zone of inhibition from the edge of the paper disk to the nearest colonies.

### 2.4.4.2 Competitive ELISA

Serum samples were tested using competitive ELISA. All samples as well as controls were tested by ELISA in duplicates (Avrameas *et al.*, 1978, Engvall and Perlmann, 1972).

#### 2.4.4.2.1 Principle of the test

It is an assay to determine the presence of anti-CBPP antibody in serum. It is based on the competition between the anti-CBPP monoclonal antibody and the antibodies in the serum sample binding to the CBPP antigen. The presence of antibodies CBPP in the serum sample will block reactivity of the monoclonal antibody resulting in reduction in expected colour following the addition of conjugate and substrate chromogen solution. As this is a solid phase assay, wash step are required between each step to ensure removal of unbounded reagents.

Competitive ELISA kit was that of CIRDO/Institut POURQUIER, CBPP serum competition ELISA - Version PO5410/01- Page 1/6. The technique described sheet fact accompanied the kit was followed.

#### 2.4.4.2.2 The components of the kit

- Monowell coated microplates.
- Wash concentrate (20x).
- Dilution buffer 24.
- Control sera.
- Monoclonal antibody 117/5 (anti-Mmm SC) (Mab).
- Monoclonal anti-mouse IgG peroxides conjugate.
- Revelation solution 3 (TMB).
- Stop solution (H₂SO₄ 0.5M solution).

2.4.4.2.3 Instruments
- ELISA plates.
- Microplates covers (lid, aluminium foil).
- Adjustable micropipettes with different volumes and tips.
- An incubator with shaker.
- ELISA reader with computer.
- Disposable Petri dishes.

2.4.4.2.4 Test procedure

The test was carried out according to manufacture instructions as follows:

1- Deposit of the sera

The samples were diluted and incubated with the monoclonal anti-Mmm SC antibody (Mab) in pre-dilution plate.

a) Dilution of the sera

- Hundred µl of dilution buffer 24 were dispensed in all wells of Pre-Plate.
- Eleven µl of the 3 control samples CP++ were dispensed in B₁, B₂, C₁, C₂ and CP+ in D₁, D₂, E₁, E₂ and CN in H₁, H₂.
- Eleven µl of dilution buffer 24 were dispensed in the wells A₁, A₂.
- Eleven µl of the samples in the other wells (A₃ to H₁₂). According to the plate layout (Figure 1).
**Figure 1: Distribution of sera and monoclonal antibody in the microplate in competitive ELISA**

Cc: Conjugate control (without serum, without Mab = 100% inhibition).
Cm: Monoclonal control (without serum = 0% inhibition).
CP++: Strong positive serum.
CP+: Weak positive serum.
CN: Negative serum.
1: Sample n° 1
2: Sample n° 2

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cc</td>
<td>Cc</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>CP++</td>
<td>CP++</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CP++</td>
<td>CP++</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>CP+</td>
<td>CP+</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CP+</td>
<td>CP+</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Cm</td>
<td>Cm</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Cm</td>
<td>Cm</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>CN</td>
<td>CN</td>
<td>71</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
b) Reconstitution and deposit of the monoclonal antibody (Mab)
   - The Mab was reconstituted with 1mL of distilled water.
   - The Mab was diluted to \( \frac{1}{20} \) in dilution buffer 24.
   - One hundred and ten \( \mu \)l diluted Mab was dispensed in all wells except A₁ and A₂.

C) Incubation of the mixture serum/ monoclonal antibody
   - Hundred \( \mu \)l of the mixture sample / Mab were transferred from pre-plate to the coated plate.
   - The plate was covered and incubated at 37°C on shaker for 1 h.

2- Washing:
   - The wash concentrate 20x was diluted in 1900 ml distilled water and this solution called wash solution.
   - The plate was washed 3 times with wash solution and dried.

3- Deposit of the conjugate:
   - The conjugate was diluted to 1/100 in dilution buffer 24.
   - Hundred \( \mu \)l of the diluted conjugate were dispensed in all wells.
   - The plate was covered and incubated at 37°C on shaker for 30 ± 3 min.

4- Washing
   The plate was washed 3 times with wash solution and dried.

5- Revelation
   - Hundred \( \mu \)l of the revelation solution 3 were dispensed in all wells.
   - The plate was incubated for 30 min at 37°C in the dark.
   - The reaction was stopped by addition of 100 \( \mu \)l stop solution to all wells.

Finally the plate was read at 450 nm.
The reader was connected to a computer loaded with ELISA Data Interchange (EDI) software, which was used to automate the reading and calculation of percentage of inhibition (PI) values. The optical density (OD) values were converted to percentage inhibition by using the following formula:

\[ P1 = 100 \times \frac{\text{ODcm} - \text{OD test}}{\text{ODcm} - \text{ODCc}} \]

Sera showing \( P1 \) equal to or lower than 40% were considered negative and those showing \( P1 \) equal or greater than 50% were considered positive.
CHAPTER THREE

RESULTS

3.1 Isolation of *Mycoplasma mycoides* subsp. *mycoides* small colonies

A total of 55 pneumonic lung samples were collected from different slaughterhouses in Khartoum State. Six samples were also collected from six animals showing clinical signs and post-mortem lesions of CBPP at Alkadaro. Post-mortem examination of the six clinical cases revealed restriction of lesions to thoracic cavity. Lesions included adhesions of the lungs to the chest cavity, lungs were covered with yellowish materials, increase of pleural fluid and marbling appearance.

No isolate was obtained from 55 pneumonic lungs collected from slaughter houses. However two isolates were obtained from the six lung samples collected from Alkadaro.

3.2 Identification of the isolates

3.2.1 Dark field microscopy

When smears prepared from cultures were examined under oil immersion lens of dark field microscope short and long filaments were seen only in case of the two samples for which the two isolates were obtained.

3.2.2 Cultural characteristics

The inoculated Petri dishes when examined by stromicroscope at low magnification after 8 days of incubation at 37°C, revealed fried egg appearance (Figure 2) in only two plates which were cultured by the two of the six clinical samples.
3.2.3 Digitonin test

An inhibition zone of 10 mm around digitonin disk was noticed in case of the two isolates from two of the clinical cases.

3.2.4 Growth inhibition test

The growth of the two isolates was inhibited by Mmm Sc antisera and inhibition zone around Mmm SC antisera disk was seen.

The two isolates from infected cases in Alkadaro were characterized and identified as *Mycoplasma mycoides* subsp *mycoides* (SC. Type) depending on dark – field microscopy, cultural characteristics, digitonion test and growth inhibition.

3.2.5 Serological identification

3.2.5.1 Competitive ELISA

Two hundred serum samples collected in this study were tested for presence of antibodies against Mmm SC with C-ELISA. Antibodies were detected in 114 (57%) serum samples (Table 5).
Table (5): Detection of antibodies of *Mycoplasma mycoides* subsp *mycoides* small colony type of bovine sera by competitive ELISA

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>Positive samples</th>
<th>Sero positive percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkadaro</td>
<td>25</td>
<td>62.5</td>
</tr>
<tr>
<td>Soba</td>
<td>45</td>
<td>56.3</td>
</tr>
<tr>
<td>Hilat Kuku</td>
<td>10</td>
<td>66.7</td>
</tr>
<tr>
<td>Kafori</td>
<td>16</td>
<td>64.0</td>
</tr>
<tr>
<td>Omdurman</td>
<td>9</td>
<td>45.0</td>
</tr>
<tr>
<td>Shambat</td>
<td>4</td>
<td>26.7</td>
</tr>
<tr>
<td>Different samples</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>Total</td>
<td>114</td>
<td>57.0</td>
</tr>
</tbody>
</table>
Fig. (2): Colonial morphology of *Mycoplasma mycoides* subsp. *mycoides* small colony type isolate grown on heart infusion agar after 8 days incubation (x 40)
CHAPTER FOUR
DISCUSSION

Sudan is a big country in Africa, approximately one million square miles in area. It shares borders with nine countries in central and east Africa. It has the largest and most species diverse livestock population in Africa that contribute greatly to the national economy.

Efforts have been made and continue to be made in the control of animal diseases in the country. This has been done usually by using therapeutics and vaccines. Some constrains facing this policy summarized as follows: continuous movement of animals, husbandry adopted in Sudan, availability of vaccines, lack of rapid and accurate diagnosis and treatment and instability due to war in the western region.

Contagious bovine pleuro pneumonia (CBPP) is considered as one of the most important animal diseases in Africa including Sudan after the eradication of rinderpest. The disease causes economic losses among cattle in the form of debilitation and death of infected animals. The Government of Sudan paid attention to this disease due to its direct effects on milk and meat production and therefore, the food intake of poor people. It also affects the external trade of livestock. CBPP vaccine production in Sudan started early in 1925 using a local strain. In 1970, T:44 was introduced in the form of broth culture vaccine and in 1999; a lyophilized form of this vaccine was produced. It is important to note that the quantity of this vaccine which produced in the country, was not sufficient to vaccinate cattle in Sudan. For this and other reasons, cattle owners used antibiotics to treat their animals infected with CBPP. In chronic cases, there will be consolidation of the lung and the septa with presence of sequestra. This sequestra
contains viable mycoplasma which may not be affected by antibiotics and breakdown of the sequestra capsules in chronically affected and recovered animals is a major factor in spread and persistence of CBPP in endemic areas.

In this study samples were collected from different slaughterhouses in Khartoum State revealed two isolation of Mycoplasma mycoides subsp. mycoides Sc type. The isolation was performed on beef heart infusion medium. The infected animals showed typical postmortem findings of CBPP. The isolates were identified as Mmm SC based on microscopic appearance, cultural characteristics, digitonin sensitivity and serological identification with GIT. This isolation confirms the presence of the disease and this agrees with many investigators surveyed the prevalence of CBPP (Ameera, 2002; Karam, 2005). They reported the presence of the disease in Khartoum state. Reports of the Administration of Animal Health and Epizootic Control and Ministry of Animal Resources (2003) stated that sporadic cases of CBPP were observed in certain areas of Khartoum State including Khartoum North.

Identification of the isolates was based mainly on serological identification with growth inhibition test. The test recommended by the OIE (1991). It is simple, specific, false negative and false positive results are rare. The isolates revealed a small inhibition zone around the disk and that can be attributed to many factors. Wallace and Clyde (1983) reported that the inhibition effect does not consist always of sharply defined zone surrounding antiserum disks. Strain variation and antiserum quality in addition to inoculum size and titer, may produce zones of suppressed (breakthrough colonies) rather complete zone of inhibition around the disks. Generally, any clear-cut suppression of growth can be considered as a positive result, assuming that antisera
are known to be mono specific and do not possess nonspecific inhibition properties.

Serum samples collected from 2 cases from which the isolates obtained were examined by C-ELISA and showed presence of Mmm Sc-type.

One hundred and fourteen out of 200 serum samples collected randomly from cattle in different areas of Khartoum State gave positive result with competitive ELISA. Higher antibody titers were detected in sera from Hilat Kuku while low titres were detected in sera from Shambat.

The OIE reference method for CBPP serology is the complement fixation test (CFT). This technique was used in the past for CBPP eradication in many countries. However, it presents some disadvantages, mainly the difficulties of antigen production, standardization and the existence of non-specific positive results. For these reasons the CIRD-EMTV (FAO World Reference Center for CBPP) has developed a second test, a competitive ELISA based on a monoclonal anti- Mmm SC antibody named 117. This test is an alternative to CFT for the OIE and can be used for the official CBPP controls. For both ELISA and CFT, the disease in the incubation stage cannot be detected.

CFT or C-ELISA can be use to evaluate efficient control of vaccination. However, as the post vaccination antibodies do not persist more than 3 months, CFT or C-ELISA can be used for detection of natural infection, even in areas where vaccination is used (Ayling et al., 1999).

Results of this study are clear confirmation that CBPP is persistent in Khartoum State and prompt action is needed to find out all infected focal areas and is find a control measure.
CONCLUSION AND RECOMMENDATIONS

Conclusions

- *Mycoplasma mycoides* subs *mycoides* SC was isolated from infected lungs.
- C-ELISA detected the presence of Mmm SC antibodies in 57% of cattle examined.

Recommendations

- More isolates of *Mycoplasma mycoides* subsp. *mycoides* should be isolated to help in epidemiological studies and control programmes of CBPP.
- Extensive and intensive vaccination should be carried out in all endemic areas.
- Further investigation have to be done to examine the possibility of use gene detection method for diagnosis of CBPP in Sudan.
REFERENCES


Anon - Annual Report of the Sudan Veterinary Service (1914).


