INVESTIGATION ON BLACK QUARTER IN NUBA MOUNTAINS, SOUTH KORDOFAN STATE

By

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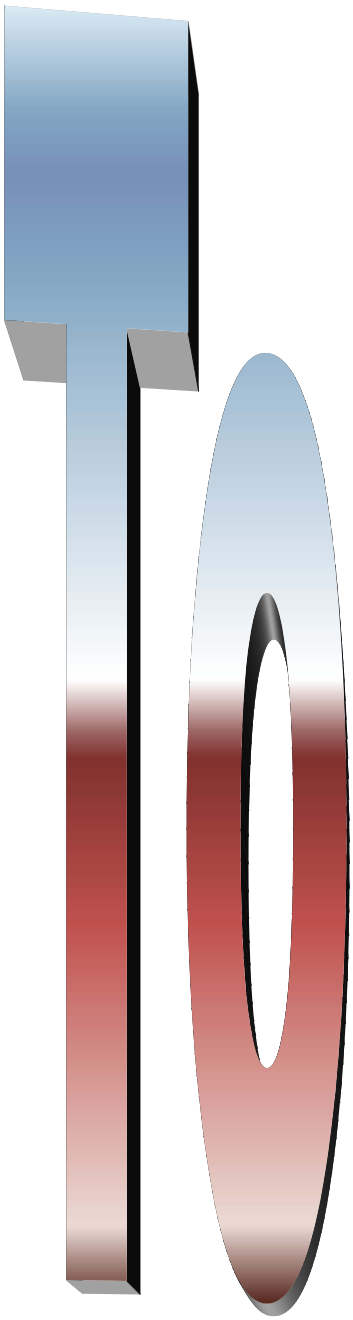
PREFACE

This work was carried out at the Department of Microbiology, Faculty of Veterinary Medicine, and University of Khartoum. Under supervision of Dr. Suliman Mohamed El Hassan.



Dedication

To soul of my father

To my Mother

To brothers, sisters, Uncle

and friends

To all whom I love

ACKNOWLEDGEMENTS

First l would like to thank god for giving me strength, confidence and patience to complete this study. I am deeply grateful to my supervisor Dr. Suliman Mohamed El Hassan for his help, guidance, suggestion, advice, patience, cooperation and encouragement throughout this work. Special thanks to Prof. S.M. El Sanusi for his great help and cooperation.

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ABSTRACT

The aim of this study was to isolate and identify, the causative agent of blackleg disease in Kadougli area, south-Kordofan, and to study the epidemiology and pathology of the disease. Clinical examination showed the affected cattle were 2-2.5 year's old and showed typical signs and symptoms of blackleg disease. Postmortem examination of affected cattle showed swelling, hemorrhage, and the muscle were black or darker in colour with gas and rancid odor. Specimens were collected from dead calve showing typical signs and lesions of the black leg disease, for bacteriological and histopathogical examination. The specimens were cultured onto blood agar and incubated anaerobically using anaerobic jar and Gas pack system. Isolates were reported from 2 calves. Cellular morphology, staining reaction, cultural characteristic and biochemical tests, identified the isolates as *C. chauvoei*. Histopathological examination showed inflammatory changes, haemorrhage, necrosis, and gas bubles. Pathogenicity test was done by inoculation of guinea pigs with one of the isolates. The inoculated guinea pig died within 48h. The postmortem finding showed swelling in injected leg, congestion, gas and hemorrhage in the internal parts. While histopathologyical examination showed necrosis, inflammatory changes and gas bubbles within the muscle.

The findings of the present investigation confirmed the outbreaks were blackleg outbreak and the causative agent of the disease outbreak was *C. chauvoei.*

ملخـص الأطروحـــة

الغرض من هذه الدراسة هو عزل والتعرف علي المسبب لوبـاء ذات الساق السوداء (أبو زقالة) الذي حدث في ولاية جنوب كردفان – كادقلي.

الفحص الاكلينكي أوضح أن العجول المصابة يتراوح عمرها ما بـين عامين إلي عامين ونصف وأظهرت علامات وأعراض مـرض ذات الـساق السوداء0 وأوضحت الصفة التشريحة للأبقار المصابة وجود تـورم ونـزف ولون اسود بالعضلات المصابة وغاز، تم جمع العينات من العجـول الميتـة والتي أظهرت الأعراض الاكلينيكية والصفه التشريحية الاصليه لمـرض ذات الساق السوداء وقد جمعت عينات للفحص البكتيري وكذلك جمعـت عينـات للفحص النسيجي وزرعت العينات للفحص البكتيري علي اقـار الدم وحضنت لاهوائياً باستخدام الجرة اللاهوائية وظرف غاز الهيدروجين.

وتم الحصول علي 2 عزله من العينات المزروعة وأوضـحت شـكل الخلايا الباكتيرية وتلوين صبغة الجرام ، الخصائص المزرعية والاختبـارات البايوكيميائية أن العزلات تنتمي إلي *باكتريـا المطثيـة الـشوفية* و الفحـص النسيجي أوضح وجود تغيرات التهابية ونزف ونخر وفراغات غازات.

وأوضح اختبار حيوانات التجارب موت الحيوان (خنزير غينيا) خلال 48 ساعة وأوضحت دارسة الصفة التشريحية وجود تورم بالرجل المحقونة ، احتقان ، غاز ، ونزف بالأعضاء الداخلية. أوضح الفحص النـسيجي وجـود نخر ونزف وتغيرات التهاب وفراغات غاز بالعضلات المصاب. أوضـحت هذه الدراسة أن المسبب للوباء المرضي هو باكتيريا *المطثية الشوفيه*.

INTRODUCTION

South Kordofan State in Western Sudan, occupies the area between (9^{o}.85-12^{o}.7) North and 29.6-33 East. It lies north of the assumed northern limits of the main African tsetse belt. Annual temperature gradient range between (37^{o}-40^{o}C), while rainfall is 350mm, 600mm and 850mm from north, middle and south part of the state respectively (GDOA.A.R., 2006).

The main ethnic group are Messyria Hummor, Messyria Zurug, Hammar, Aulad Himit, Dinka, Fulani and Nuba (Abed El Rahman *et al*., 1990).

South Kordofan has a large cattle population (Table, 1), most cattle are kept by the Baggara tribes who practice a transhumance way of herd management, and cattle are also owned by sedentary groups (Abed El Rahman *et al*., 1990).

Blackleg is a fatal disease of cattle, sheep and goats. It is caused by *Clostridium Chauvoei*.

In Sudan, the disease was first reported in 1939. In 1942 two outbreaks were reported one at Gadaref and the other in Rashad district of Kordofan state, where 890 death occurred. In 1943, cattle in the infected areas were vaccinated early in the year and no further death occurred in the vaccinated cattle (El Sawi, 1986). Although annual vaccination has brought the disease under control, sporadic cases are frequently reported, hence economical importance of treatment (El Sanousi *et al*., 1978).

Between 1987 and 1996 several outbreaks of black quarter disease occurred in different states of the Sudan involving cattle in all seasons of year and many strains were isolates (Musa *et al.*, 1998).

Blackleg disease is one of endemic disease in south Kordofan state it seems to occur during summer and after floods, since treatment can not be commoved immediately, the disease caused great losses on livestock and hence the great economical importance of the disease.

The aim of this work was

- To isolate and identify the causative agent
- To study the pathology of the disease.
- To examine the antibiotic sensitivity of the isolates.

Table (1): Animal population of Southern Kordofan State

Cattle	Sheep	Goats	Horses	camels
$7.05x10^6$	$4.3X10^6$	$4X10^6$	$0.895X10^6$	$0.780X10^6$

Source: (GDOAR. A.R. 2006), Government Direction of Agricultural and Animal Resource

CHAPTER ONE

1. LITERATURE REVIEW

1.1 Anaerobic infection

Anaerobic infections are those caused by organisms requiring low redox potential in their environment in order to multiply. This generally means marked reduction or complete absence of oxygen. With the exception of the genus clostridia, the role of anaerobic bacteria as aetiological agent in diseases of veterinary importance has been poorly established, and this was attributed to many reasons, these organisms have been considered difficult to cultivate (El Sawi, 1986).

The anaerobic bacteria which are isolated most frequently from human and animal material are clostridia, bacteriodes and anaerobic streptococci.

The best known of these anaerobes are the clostridia which include many species pathogenic to man and animal as shown in Table 2 (Hirsh and Bestrein, 2004)

1.2 Classification of clostridium

The classification of clostridium is presently based on their morphological, biochemical, serological and toxigenic characteristics. In many cases it is difficult to assign some organism to certain group.

A primary grouping of the genus clostridia was suggested soon after 1914-1918 war, it was based on position of the spore (El Sawi, 1986).

There are no convenient and outstanding characters for a detected subdivision of the genus, but some insight into the relation between the studying the composition of their cell walls, their nucleotide sequence in preparations of DNA, their nutritional requirement, the

Table (2): Selected members of the genus Clostridium and their usual source or associated condition

Species	Usual sources or associated condition
Clostridium botulinum	Botulism
C. chauvoei	Blackleg in ruminants, pigs
C. colinum	Enteritis and hepatitis
C. difficile	Antibiotic/stress-induced diarrhea in horses, dogs, cats
C. haemolyticum	Bacillary hemoglobinuria in rams, black disease in ruminants
C. novyi	Gas gangrene, big head in rams; black disease in ruminants.
C.perfringens	Gas gangrene, enterotoxaemia in ruminates, pigs, horses, necrotic enteritis in chickens; lamb dysentery, struck in sheep, pulpy kidney diseases in ruminants, diarrhea in dogs, cats and human patients
C. piliforme	Tyzzers disease
C. septicum	Malignant edema in ruminants, pigs; braxy in sheep necrotic enteritis in chickens
C. sordellii	Myositis and hepatitis in ruminants, horses
C. spiroforme	Mucoid enteritis of rabbits, antibiotic-induced enteritis in rabbits, guinea pigs, enterocolitis of foals
C. tetani	Tetanus

nature of amino acids utilized and their degradation products (Henry, 1969).

1.3 Genus clostridium

This genus is placed in those Gram positive, spore forming rods which are anaerobic, these bacilli are variable in size, and they are long slender rods (Hirsh and Bibestein, 2004).

Sporulation occurs in all species with a variable position of spores, central, subcentral and terminal. The type of disease caused by the clostridia can be classified as toxigenic or histoxic as shown in Fig I (Quinn *et al.*, 2002).

Many new clostridium species have been described recently, the genus now contains about 100 species, some of which are pathogenic and some of which are nonpathogenic (Kuhnert *et al*., 1996).

1.4 *Clostridium chauvoei*

Clostridium chauvoei sometime referred to as *C. feseri* which had been known since 1887 when it was demonstrated as the cause of black leg (black quarter) and it was named after J.B.A. Chauvoen, a French scientist of the 19th century (Smith and Holdman, 1968).

To resolve the phylogenetic position of *C. chauvoei* Kuhnert *et al.* (1996) determined its complete rrs gene sequence from DNA obtained by PCR amplification of genomic DNA from *C. chauvoei* type strain and a field isolate. In parallel, and they also determined the rrs gene sequence of the phenotypically very similar organism *C. speticum* (Fig, 2)

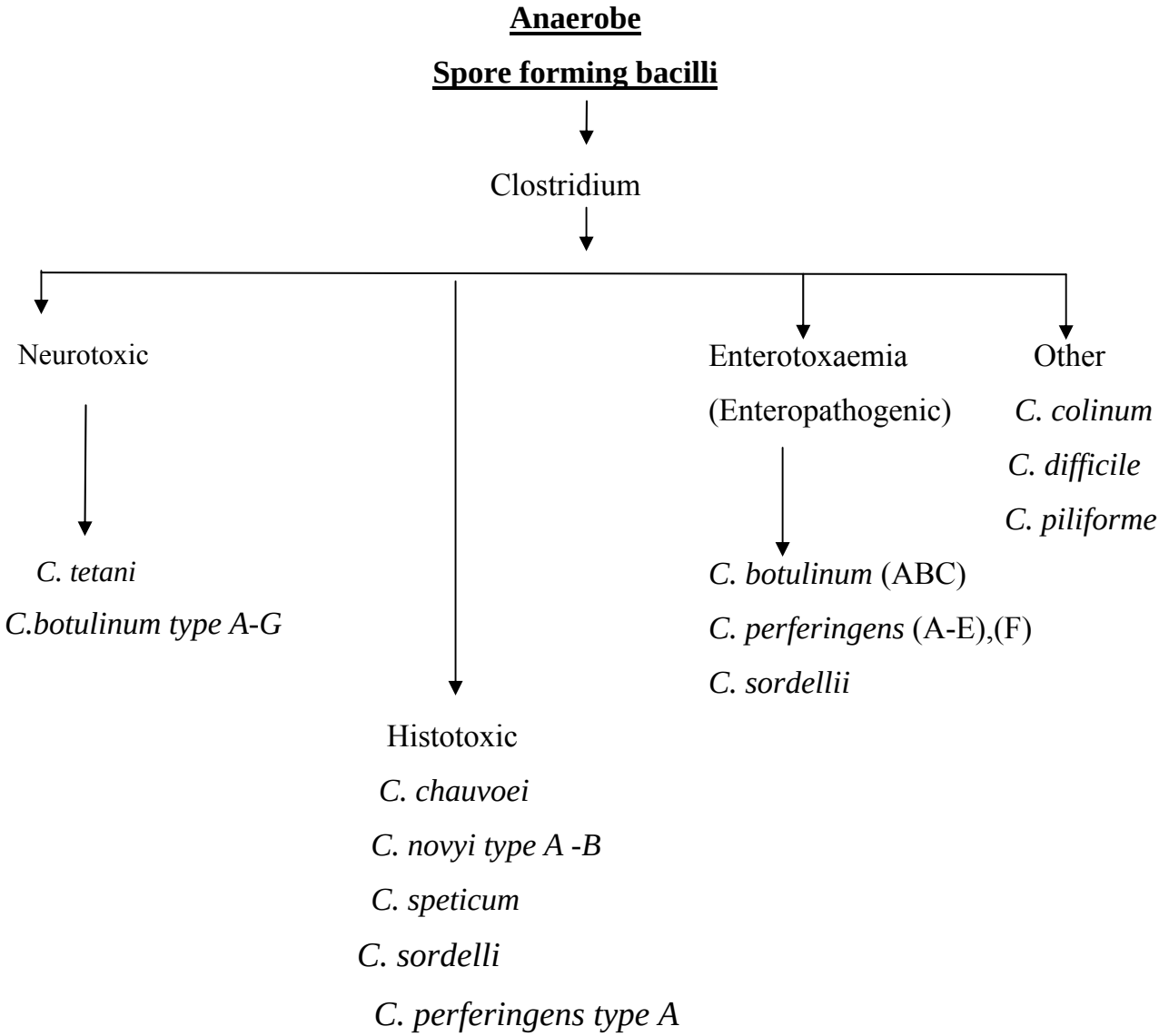


Fig (I): Pathogenic Clostridium

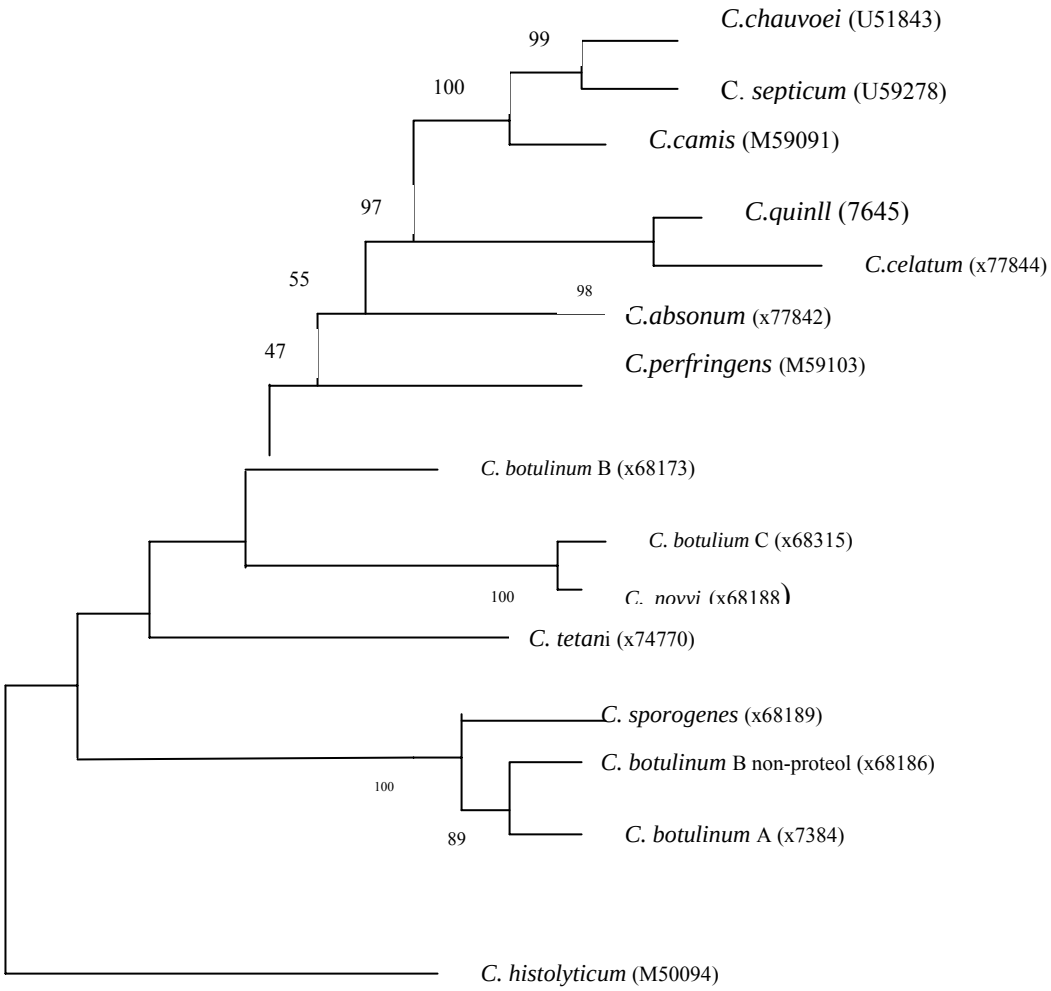


Fig (2): Phylogenetic relationships of *C. chauvoei* and other species of the genus clostridium

1.4.1 Habitat:

The organism is worldwide in its distribution in soil and pastures where its incidence may be particularly high in certain area.

The principle habitat of *C. chauvoei* seems to be the animal body. It has been isolated from the intestinal content, livers and spleens of apparently healthy cattle, from the muscle and liver of sheep (Kerry, 1964).

1.4.2 Morphology and cultural characteristics:

The organism is rod-shaped with rounded ends, 0.5-1.7x1.6-6.7μm in dimensions and occurs singly or in pair. Sometime it develops to a variety of polymorphic forms. *C. chauvoei* strains are Gram positive in tissues and fresh cultures but has a tendency to stain Gram negative when subcultured on laboratory media. It is non-capsulated, possesses peritrichous flagella and is motile. Occasionally non-motile variants do occur. Spores are oval, wider than the bacillus situated centrally or subterminally, and sporulation occurs readily both in the broth and on solid media surface colonies. On blood agar colonies are β. hemolytic, 0.5-3 mm in diameter, circular, low convex or raised, translucent to opaque, granular, shiny or dull, smooth and have entire margin. Cultures in broth are turbid with a smooth sediment and have a pH of 0.5-5.4 after incubation for 4 days.

The optimum temperature for growth is 37^{o}C. *C. chauvoei* ferment glucose, lactose, sucrose and maltose with acid and gas but most strains do not ferment salicin and mannitol. The organism is negative towards methyl red, indole and Vogues-Proskauer test. It neither reduces methylene blue nor it contains an enzyme catalase.

Ammonia is not evolved while nitrate is reduced and gelatin is liquefied.

Growth in cooked meat broth is slow, the meat is turned pink with rancid odour (Sneath *et al*., 1986).

1.4.3 Isolation and identification

The identification of members of the genus clostridium poses a problem. The biochemical test, routinely used in identification of clostridium species, they have to be repeated several times. On the other hand, serological test are hampered by difficulties in preparing clostridial antigen. This is because to which is a common feature in several members of the group and the cross serological reaction usually observed between the different species (El Sawi, 1986).

Detection in tissue or identification in culture can also be accomplished by using DNA primers designed to amplify (by using the polymerase chain reaction) species specific portions of the gene encoding the flagellar protein flagellin, which have been used successfully to differentiate *C. novyi* types A, and B, *C. chauvei, C. haemolyticum*, and *C. speticum* (Hirsh and Biberstein, 2004).

Identification and isolation of clostridia is rather difficult and a time consuming procedure. In Sudan studies were hampered by failure to isolate *C. chauvoei* from various outbreaks such failure was attributed by El Sanousi *et al.* (1978), partly to the inhibitory effect of metabolites of certain aerobic contaminats exerted on *C. chauvoei* in sample sent from the field to the laboratory.

Attempts to isolate organism of the genus clostrialium directly from specimens have been difficult due to the rapid putrefaction of the carcass or by the quick disposal of the carcass (El Sawi, 1986).

Properly selected samples arriving at a laboratory in good condition often make it possible to reach a precise diagnosis very quickly (El Sanousi, 2005).

1.4.4 Media for isolation:

El Sanousi (2005) described different media for anaerobic bacteria isolation like Reinforced clostidial agar (RCA), Reinforced clostridial media (RCM), Shahid and Ferguson (SF) and cooked meat medium which is best media for isolation of anaerobic bacteria.

On solid media the clostridium grow in thin, irregular colonies, but when the medium is moist, spreading occurs. Variation in colony size and shape exists under different conditions. Fluid media are rendered uniformly turbid. In blood agar, heamolysis is first of the alpha type, which later develops in the beta type (Carter, 1986).

Type and strains of *C. chauvoei* are capable of producing great quantities of gas in both protein and carbohydrate media (Hirsh and Biberstein, 2004).

1.4.5 Maintenance and preservation

Cooked meat medium is excellent for maintenance of stock cultures (particularly those of spore forming species) in which stock organisms remain viable when stored at room temperature for many months. Stock organisms should be subcultured once or twice a year to establish purity (Oxoid, 1976).

1.4.6 Resistant to physical and chemical agents:

Survival of anaerobic bacteria was affected by many factors which include, exposure to atmospheric oxygen, light, humidity and temperature. Spores of *C. chauvoei* are killed by steam in 40-50 minutes and in 20 minutes when autoclaved at 121^{o}C. It has also been

claimed that the spores are susceptible to 3% formalin for 15 minutes. (Haffamann and Justersen, 1980).

1.4.6.1 Effects of antibacterial agents on *C. chauvoei*

The effects of antibacterial agent on cultural growth and cellular morphology were studied. No striking difference was observed between culture grown on medium containing antibiotics, electron microscopy of culture containing antibiotics revealed intact cells and others devoid of flagella with increased cellular vacuoles. Partial and complete denaturing of the cell wall resulted in different pleomorphic forms and intense uptaking of stain. Shrunken cytoplasm and shredded protoplast were seen with flagella still attached to cytoplasmic membrane. Cell surviving antibiotic were found to be less pathogenic, this appears to be due to partial damage of cell wall and loss of invasiveness (El Sanousi and Mekki, 1978).

1.4.6.2 Antibacterial susceptibility

C. chauvoei was found to be highly sensitive to antibacterial agents, Aquacycline, Tetrramycin, Uvomycin and pencillin and moderately sensitive to sulfamethazine 33%, borgal and trivetrin (ELSanuosi and Mekki, 1978).

1.4.7 Biochemical tests:

Biochemical tests used for identification clostridium are not reliable and test must be repeated with great care several times before they can be accepted (El Azhari *et al*., 1981).

C. chauvei and *C. septicum* showed great similarity in biochemical reaction, and although they usually differ in fermentation of sucrose and salicin, analogous strain do occur (El Sawi, 1986).

Clostridium cultures typically emit putrid odors due to products of peptide catabolism, which is a common mode of energy production.

Most clostridium attack carbohydrates, proteins, lipids or nucleic acids. Biochemical reactions and their end products furnish a basis for species identification (Hirsh and Biberstein, 2004).

1.4.8 Strains of *C. chauvoei*:

C. chauvoei strains were isolated from outbreaks in different part of the Sudan (Musa *et al.*, 1998).

C. chauvoei: locally isolated in Sudan from outbreak of BQ in cattle:

1. *C. chauvoei*: strain kad_1 a local strain isolated from an infected cow in Kadougli (El Azhari *et al.*, 1981).
2. *C. chauvoei*: strain isolated from Kuku Dairy farm cattle, Khartoum North, September 1989.
3. *C. chauvoei* strain El Doeim (White Nile State) isolated from dairy cattle, September 1986.
4. *C. chauvoei* strain ElTies and Buram isolated from nomadic cattle in January 1996 (Musa *et al*., 1998).

1.4.9 Antigens

All strains of *C. chauvoei* have one common somatic antigen, but organisms are divisible in two groups on the basis of their flageller antigens, the organisms share a common spore antigen with *C. septicum.*

1.4.10 Toxins:

Clostridium chauvoei produces a number of protein exotoxins:

a. Alpha toxin: The alpha toxin of *C. chauvoei* is described as an oxygen stable hemolysin. This toxin is most likely comparable to the pore-forming, lethal alpha toxin of *C. septicum*.

b. Beta toxin: The beta toxin of *C. chauvoei* has DNase activity. The role played by this toxin in the pathogenesis of disease is undefined.

c. Gamma toxin: The gamma toxin of *C. chauvoei* has hyaluronidase activity. The role played by this toxin in the pathogenesis of disease is undefined.

d. **Delta toxin:** delta toxin of *C. chauvoei*, also known as chauveolysin, is a cholesterol binding cytolysin. Chauveolysin binds to cholesterol-containiong rafts in the eukaryotic cell membrane. Once bound, it forms a pore resulting in the death of the cell. The role played by this toxin in the pathogenesis of disease produced by *C. chauvoei* is indefined.

e. Neuraminidase (sialidase): The neuraminidase of *C. chauvoei* removes sialic acid residues from glycoconjugates on cell walls of eukaryotic cells resulting in disruptions of the intercellular matrix. The role played by this toxin in the pathogenesis of disease is indefined (Hirsh and Biberstein, 2004)**.**

1.4.11 Pathogencity:

C. chauvoei is the causative agent of black leg disease in cattle and sheep (El Sanousi *et al*., 1986). Black leg is endemic disease in Sudan affecting cattle under 4 year of age, the disease is sporadic and was believed to occur mostly in rainy seasons.

1.4.11.1 Black leg:

Black leg or black quarter (BQ is primarily a disease of the even toed unglates. The name "black leg" derives from the fact that the site of infection is often a leg muscle, and that the affected muscle is dark in colour (Wilson, 2004). Cattle are the most susceptible animals followed by sheep and goats and less often swines but rarely some other animals species which seem to have a great deal natural

immunity to infection by *C. chauvoei*. Man is probably entirely resistant. Horses, birds, dogs, cats and rabbits seem to have natural resistant to infection (Bushra, I986).

1.4.11.1.1 Mean of infection and pathogenesis

In most cases of BQ in cattle infection appears to be endogenous, for there are no wound or other breaks in the skin to explain the entry of the organism into the muscle masses, where infection first take place (Bruere, 1982).

Bacterial spores are eaten in contaminated feed or soil. The spore enters the bloodstream and lodge in various organs and tissues, including muscles. Here they lie dormant until stimulated, multiply, possibly by some slight injury to the animal. The injury reduces blood flow to the area, thereby reducing the supply of oxygen to the tissues. In the absence of oxygen, the spores germinate and multiply. As they grow, the bacteria produce toxins which destroy surrounding tissues. The toxins are absorbed into the animal's bloodstream which make the animal acutely sick and cause rapid death (Wilson, 2004). Seeding of tissue, especially skeletal muscle with spores precedes disease in cattle. Conditions favoruring spore germination, bacterial growth and toxins production cause formation of local lesions. The alpha (necrotizing), gamma (hyaluronidsase), delta (chaureolysin) toxins together with the neuraminidase are believed to be responsible for initial lesions. Bacterial metabolism, producing gas from fermentation, may be contributory. The lesions are marked by edema, haemorrhage and myofibrillar nearosis. The center of lesions becomes dry, dark and emphysematous due to bacterial

fermentation, while the periphery is edematous and haemorrhagic. A rancid butter odour is typcal (Hirsh and Bestrein, 2004).

1.4.11.1.2 Clinical signs:

Almost all outbreaks of BQ in cattle occur in animals that are on a high plane of nutrition and that are gaining weight. The disease is most commonly seen in calves 6 month and two years of age but occasional cases are seen in adults and it can occur in younger animals particularly if they have not had sufficient colostrums (NADIS, 2003).

In cattle the most obvious sign is a crepitate swelling particularly in the hind and fore quarter, which crackles when rubbed with fingers as a result of large quantities of gas produced subcutaneously by the organism. The fevered animal will be lame and muscle in the affected region will show trembling and sometimes violent twitching. Death usually occurs suddenly and often within 24-48 hours of the symptoms first being observed (Wilson, 2004).

In few cases the first sign seen in tongue and throat swelling with the tongue protruding. Bloody discharge from the nose, mouth and other body opening are also seen (Goss, 1919).

In sheep an acute febrile condition develops within 1-2 days following an injury, castration, or shearing, docking (Sterne, 1981) and a typical black quarter lesion can be observed near the site of injury. In case of ewes which have recently lambed the lesion develops in region of the perineum which becomes swollen and dark red in colour.

The changes in the muscle are characteristic, but the extend of the damage can vary considerably. Some cases have black oozing muscle throughout the hindquarters, other have much smaller area of damage which may not involve the limbs at all.

Little work had been done on clinical changes that might be exerted for cattle infection with *C. chauvoei (*El Sawi, 1986).

1.4.11.1.3 Postmortem changes

Putrefaction occurs rapidly in the carcass of an animal infected with black leg and result in a typical bloated appearance of the carcass soon after death. The legs are extended stiffly and a frothy, bloody discharge is often apparent at the anus and the nostrils. The skin over the swelling is usually normal but in the centre it may have undergone dry gangrene. When cut and examined, the swelling are usually found to contain discolored serum and gas. When affected muscles are cut open, they are usually found swollen and either black or darker in color than normal with gas present. It is unwise to cut open a swelling unless necessary for a diagnosis, as this increases the contamination of the soil (Richey, 1987). A postmortem is essential to diagnose black leg. Many other disease cause sudden death such as anthrax, which must be ruled out before a postmortem is done (NADIS, 2003).

The pathology of calve died of black leg disease was summarized by Sippel (1972); Sterne and Batty (1972), and Sterne (1981). The principal lesions are always found in the skeletal muscle, most often in the large muscle masses of the shoulder, thigh or neck. The affected muscle is dark reddish-brown to black in colour and spongy in consistency. The cut surface of such muscle is dry although the muscle itself may be surrounded by oedematous fluid with numerous gas bubbles. The subcutaneous tissues and fascia overlying affected muscles are saturated with gelatinous fluid, basically yellow but copious mat may be located on the tongue. A characteristic rancid odour is present and may be almost diagnostic. Myocardial damge is a constant finding.

Necropsy of infected quinea pigs (Miles and Wilson 1975) shows a blood stained gaseous oedema at the site of inoculation, spreading over the abdominal wall with collection of gas in the groins and axillae. The thigh and abdominal muscle are soft and deep red in colour. The adrenals are congested and in the pericardial and peritoneal cavities there may be some fluid.

1.4.11.1.4 Histopathology

Smith *et al.* (1972) described the essential microscopic lesions in cattle died of black leg disease. The lesions are mainly found in the skeletal musculature. Gas bubbles in the fixed tissue are indicated by spherical spaces separating muscle bundles and fascia. There are irregular areas of necrosis and collection of neutrophils and lymphocytes along the muscle. Oedema is infrequent in the muscles. On the other hand, Jubb and Kennedy (1970) showed that the lesions in black leg started as cellulitis with copious oedema and haemorrhage. There is in any case, severe parenchymatous degeneration. There is often a fibrous haemorrhagic pleuritis, and the lungs are congested with some interstitial oedema and heamorrhage.

Purulent meningitis with polymorphnuclear leukocytes and mononuclear cell was subsequently confirmed on histopathological examination of the brain (Malone *et al*., 1986). This happened to be the first report of the lesions in menings associate with *C. chauvoei* infection in cattle.

1.4.11.1.5 Diagnosis

The diagnosis of blacklegs is very important. Because of close similarity to *C. septicum* that is important to differentiate between *C. chauvoei* and *C. septicum* infections since the latter is occasionally associated with such type of condition (Kuhnert *et al*., 1996).

A history of the disease and the symptoms may be strongly suggestive of blackleg disease but the final diagnosis must depend upon the detection of the causative organism. Films prepared from the lesion and from the oedematus fluid demonstrated *C. chauvoei* pathogens. These appeared as Gram positive rod when examined immediately following death of the animal.

It is more difficult to obtain pure culture from animals which have been dead for several hours because of contamination with other anaerobic species, and in this case the bone marrow may be a more satisfactory site from which to make a primary culture (El Sawi, 1986).

1.4.11.1.5.1 Experimental animal Inoculation:

Inoculation of pure cultures of this organism into laboratory animals results in death with the production of many characteristic symptoms of black leg. Guinea pigs, rabbits, white mice and white rat can be infected. The guinea pigs is the most susceptible and commonly used as the experimental animal for the organism (Bushra, 1986)

1.5.5 Treatment

Cases of blackleg in cattle or sheep are seldom treated. Treatment with antibiotics is successful if diagnosis is made earlier, but in practice the infection of blackleg is so rapid that treatment is generally of no practical value (Sterne, 1981.)

El Sanousi and Mekki (1978) showed that *C. chauvoei* is susceptible to various antibiotics which can be used for treatment when given promptly and inoculated into and around the site of the lesion.

Penicillin is effective in treating blackleg systematically and locally, if given in early stages of the disease. Because the pathogenesis of disease caused by histotoxic clostridia is similar, the procedure relevant to their treatment and control are similar (Quinn *et al.,* 2002).

1.5.6 Development of immunity and vaccination to BQ:

In immunity to BQ the immune system must be sensitized to eliminate both the invading organism and to neutralize the toxin and produce antibodies and bacterial antitoxic immunity. Animals that recover from an attack of BQ become immune for life while young animals under six months of age are most probably protected due to the acquired maternal immunity, provided that the mothers are immune (Seifert, 1977).

On active immunization (vaccination), immunity begins to develop at 15 day post vaccination and maximum titer of circulating Abs was reached in a period of 30 days, after which a sharp fall follows and leveled off at 60 days (El Sanousi *et al*., 1981).

Vaccines against BQ have proved to be the most widely used and effective in controlling the disease in cattle and sheep. They are usually administered as combined vaccines containing formalinzed bacteria and toxins. It has been found that inclusion of strains specific to the area is a prerequisite for effective control measure (NADIS, 2003).

In Sudan annual vaccination against the disease was regularly practiced since 1943, using a vaccine produced from an avirulent European strain. This was replaced in the same year by a locally isolated virulent strain. The later was also replaced in 1975 by the highly immunogenic Australia strain (CH_3).

In 1991 Babiker was able to produce a vaccine using *C. chauvoei*, Kadogli strain 894 also known as kad1 which was previously isolated in 1981 by El Azhary *et al*. The vaccine introduced by Babiker 1991 was produced using the Gottingen bioreactor system, on the basis of continuous system, replacing the old conventional flask system technique (El Hassan, I996).

1.5.7 Control and prevention of BQ

Application of adequate management measures is essential to control *C. chauvoei* and other clostridial infection such measures includes:

1. Avoidance of over grazing as well as adoption of rotational grazing.
2. In endemic area, restriction of animal movement from such area is advisable.
3. Cattle that die from clostridial infections should be completely burned or covered with quicklime and deeply buried. The clostridium organism is distributed throughout the carcass and will be a source of infection to other cattle (Richey, 1987).

Vaccination, usually with bacterin and toxoid components in adjuvant, is the most effective method for preventing the disease. Multicomponet vaccines which induce protection against serial pathogenic clostridia species may be required on same farms (Quinn, *et al.,* 2002). Avariety of vaccines have been used, and these include toxoids and whole culture bactrins. They are either monovalent, polyvalent mixed with or without adjuvants.

Vaccination of all cattle ages annually should be carried out just prior to the anticipated danger period. In the tropics boostering

through repeated natural infection is believed to confer life long immunity. However, animals under 6 month of age are protected by virtue of their acquired clostral Abs (Babiker, 1991).

Vaccination takes 10-14 days to become effective, so it is best to vaccinate before a problem occurs or a risk period is encountered (NADIS, 2003).

Once an outbreak started, prophylactic administration of antibiotic apparently has been of value in preventing the development of black leg in animals in which it had not yet occurred (NADIS, 2003).

1.5.8 Epidemiology

Soil borne clostridia are of particular significant in tropical and sub-tropical environments. They cause substantial losses, mainly in young animals and render animal production unprofitable. Black leg is referred to as a soil disease and its prevalence in certain countries was stated to be related to particular types of soil. Although the incidence of soil borne epidemics is linked to the infected environment, mostly inadequate hygienic measures, in under developed countries, lead to spread of infection through the field of animal production. Carcass scavengers also contributed to spread of infection. Infected areas can only be isolated from noninfected one by natural geographical barriers, mountain ranges, deserts and jungle areas which are not open for animal production and which are relatively difficult to surmount. Concentrated dissemination of an epidemic is exclusive to damp spot which is in the pasture area on the ranges extensive grazing (Richey, I987).

Moreover there are further specific factors in these climatic zones which raise the incidence of the epidemic complex with the start

of the rainy season. pH changes in the soil and sudden increase in the protein content of the food plants can lead to outbreak of black leg (Sterne, 1975). The survival of *C. chauvoei* in the soil depended among other factor, upon the soil type (Raducanescu and Bicapopii, 1967). In soils which are rich in organic matter, the organism was recoverable for up to 11 years where as in soils which are poor in organic matter it survived for two years.

A report from Kordofan state, showed that the disease was conferment in the clay soil areas of Rashad and Dilling, and evidence from Darfur and Bahr El Ghazal supported the theory (Bagadi, 1978). Black leg is referred to as soil disease.

Viljoean and Scheuber (1926) reported that, black leg in South Africa was prevalent in all parts of the country and tended to be regionally distributed; being more prevalent in low-lying areas and its occurrence was seasonal, being worst during the spring and early summer months and especially after heavy rains.

The clostridium organism is distributed throughout the carcass and will be a source of infection to or transmitted by predator scavengers or rain water to other premises (Richey, 1987).

Sako (1952) stated that the heamatophaqus arthropods such as tabanids are capable of black leg transmission.

In Sudan, the disease was first reported in 1939 in Kordofan State. Many outbreaks of black leg disease occurred early in Sudan causing great loss in livestock and hence the great economical importance of the disease. Annual vaccination has been practiced regularly (El Sawi, 1986).

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Sterilization

2.1.1 Flaming

It was used to sterilize glass slides, coverslips, needles and scalpels. Direct flame, was used for cotton plugged tubes. It was done by exposing the object to the direct flame for about half to one second.

2.1.2 Red heat:

It was used to sterilize loop wires, points and searing spatulas by holding them over a Bunsen burner flame until they became red-hot.

2.1.3 Hot air oven

It was used to sterilize glass wares (such as test tube, graduated pipettes, flask), forceps and cotton swabs. The holding period was one hour and oven temperature was 180^{o}C.

2.1.4 Steaming at 100^{o}C

Repeated steaming (Tyndallization) was used for sterilization of sugars and media that could not be autoclaved. It was carried out as described by Cruickshank *et al.* (1975).

2.1.5 Moist heat (Autoclave)

Autoclaving at 121^{o}C (15lb/inch^{2}) for 15 minutes was used for sterilization of some media.

2.2 Disinfection:

Phenolic disinfectant was used for disinfecting floor of laboratories and 70% alcohol was used for disinfecting the bench and anaerobic jar.

2.3 Radiation:

The ultraviolet (UV) irradiation was used to sterilize the media pouring room for 20 minutes.

2.4 Reagents and indicators:

2.4.1 Reagents:

2.4.1.1 Kovac's reagent

This reagent composed of 5g para-dimethyl aminobenzaldehyde, 75ml amyl alcohol and 25ml concentrated hydrochloric acid. It was prepared as described by Barrow and Feltham (1993) by dissolving the aldehyde in the alcohol by heating in water bath. It was then cooled and the acid was added. The reagent was stored at $4^{o}C$ for later use in indole test.

2.4.1.2 Nitrate reagent:

Nitrate test reagent was consisting of two solutions (A) and (B). They were prepared according to Barrow and Feltham (1993). Solution (A) composed of 0.33% sulphanilic acid dissolved by gentle heating in 5N acetic acid. Solution (B) composed of 0.6% dimethylamine alfa naphthylamine dissolved by gentle heating in 5N acetic acid.

2.4.2 Indicators:

2.4.2.1 Andrades indicator

It composed of acid fuchsin 5g, distilled water 1 liter and N-NaOH 150ml. The acid fuchsin was dissolved in distilled water. Then the alkali solution was added, mixed and was allowed to stand at room temperature for 24 hours with frequent shaking until the color changed from red to brown. This was used for sugar fermentation test.

2.4.2.2 Phenol red:

It was supplied by Hopkins and William Ltd. London. It was prepared as 0.2% aqueous solution.

2.5 Collection of blood for enriched media:

Blood for enriched media was collected aseptically into sterile flask containing glass beads by veinopuncture of jugular vein of healthy sheep kept for this purpose. The blood was defibrinated by shaking the sterile flask while and after collection.

2.6 Preparation of the media:

2.6.1 Nutrient broth

Thirteen grams of nutrient broth (Oxoid) were added to one liter of distilled water, mixed well and distributed in 3 ml amount into clean test tubes. Then sterilized by autoclaving at 121^{o}C for 15 minutes.

2.6.2 Cooked meat medium

The medium was prepared according to Barrow and Feltham (1993). One kilogram of minced meat was added to one liter of alkali solution (0.05N-NaOH), mixed well, heated to boiling, and simmered for 20 minutes with frequent stirring. The pH was adjusted to 7.5, strained through gauze and dried. It was adjusted in 5g amount into screw capped containers; sufficient nutrient broth was added and then sterilized by autoclaving at 121^{o}C for 20 minutes.

2.6.3 Blood agar

This medium composed of dehydrated blood agar base obtained from Oxoid and defibrinated sheep blood. The blood agar base contained heart infusion, tryptose, sodium chloride and agar. It was prepared according to manufacturer's instruction by dissolving 40g in one liter of distilled water by boiling, sterilized by autoclaving at

121°C for 15 minutes and cooled to about 50°C. Defibrinated sheep blood was then added aseptically to give final concentration 10%, mixed gently and 15 ml of complete medium was poured into each sterile Peteri dish. The poured plates were allowed to solidify at room temperature on flat surface.

2.6.4 Peptone water

Fifty grams of peptone water powder (Oxoid) were added to one liter of distilled water, mixed well, and distributed in 3 ml amount into clean test tube. Then sterilized by autoclaving at 121°C for 15 minutes.

2.6.5 Nutrient Agar

To one liter of nutrient broth (Oxoid), 15g of agar were added, dissolved by boiling, and sterilized by autoclaving at 121°C for 15 minutes. Then cooled to about 50°C and distributed in 15 ml amount per plate. The poured plates were left to solidify at room temperature on leveled surface.

2.6.6 Motility medium (Cragie's tube medium)

Thirteen grams of dehydrated nutrient broth (Oxoid) were added to 5g of Oxoid agar No-1 and dissolved in one liter of distilled water. The pH was adjusted to 7.4. This medium was dispensed in volumes of 5 ml into 20 ml test tube containing the appropriate Cragie's tubes, and then the medium in test tubes was sterilized by autoclaving at 121°C for 15 minutes.

2.6.7 Urea agar medium

This medium was supplied by Oxoid. It consisted of peptone (1g), dextrose (1g), Na_2HPO_4 (1.2g), KH_2PO_4 (0.8g), sodium chloride (5g), agar (15g) and phenol red (0.0120g). Dehydrated medium (2.4g) was suspended in 95ml of distilled water and brought to the boil to

dissolve completely. The pH was approximately adjusted to 6.8 and then it was sterilized by autoclaving at 115^{o}C (101b/inch^{2}) for 20 minutes. Then cooled to 50^{o}C and aseptically 5 ml of sterile 40% urea solution were added and mixed well. The medium was then distributed in 10 ml amount into sterile McCarteney bottles and allowed to set in a slope position.

2.6.8 Nutrient gelatin medium

One hundred and twenty eight grams of nutrient gelatin (Oxoid) CM132 were hydrated in a liter of distilled water, steamed to dissolve, and pH was adjusted to 6.8. The medium was then distributed in screw-capped bottles and autoclaved at 121^{o}C for 15 minutes.

2.6.9 Nitrate broth medium

This medium was prepared according to Barrow and Feltham (1993). Potassium nitrate 1 g was dissolved in nutrient broth 1 liter, and distributed into clean test tubes. Then sterilized by autoclaving at 115^{o}C for 15 minutes.

2.6.10 Peptone water sugar

Peptone water sugar medium was prepared as described by Barrow and Feltham (1993). It contained peptone water 900 ml, Andrade's indicator 10 ml, sugar 10 g and distilled water 90 ml. The pH of peptone water was adjusted to 7.1-7.3 before the addition of Andrade's indicator. The sugar was added to the mixture of peptone water and indicator and mixed well. The complete medium was distributed into portions of 2 ml into sterile test tubes containing inverted Durham's tubes, and covered with metal caps. Then sterilized by autoclaving at 115^{o}C (101b/inch^{2}) for 10 minutes and kept at 4^{o}C until used.

2.6.11 Casein agar medium

The medium was prepared according to Barrow and Feltham (1993). The skim milk was prepared by allowing a fresh whole milk to stay in a refrigerator overnight. The milk below the cream layer was collected, steamed for 1 hour, cooled and then filtrated. It was sterilized by autoclaving at 115^{o}C for 10 minutes. Then cooled to about 50^{o}C and was added to the double strength sterile cooled melted nutrient agar. Then mixed and distributed into Petri dishes in 15 ml amount and allowed to solidify on leveled surface.

2.6.12 Eggs-yolk medium

This medium was prepared as described by Sterne and Batty (1975). Fresh eggs were scribed with soap and water, immersed in alcohol and left to dry. Part of the shell was aseptically removed at the point end of the egg. The egg white was aseptically decanted and the egg-yolk was aspirated in sterile container. An equal volume of sterile normal saline was added. The mixture was shaken with a sterile glass rod, mixed well, and dissolved by gentle heating. The pH was adjusted to 7.6. The medium was distributed in medium bottle in 45 and 90ml amount, sterilized by autoclaving at 121^{o}C for 15 minutes.

2.6.I3 Reinforced Clostridial agar medium

Reinforced clostridial medium (RCM) was obtained from Oxoid. It contained peptone, yeast extract, lablemco, peptone, starch, dextrose sodium acetate and agar. The medium was prepared as described by manufacturer. The medium was dissolved in distilled water in a boiling water bath. The pH was adjusted to 6.8±0.1 and sterilized by autoclaving at 121^{o}C for 15 min. Sterile cysteine hydrochloride 0.05% and sterile glucose I% were added aseptically to

the medium, mixed and distributed into sterile Petri dishes in 20ml amount and allowed to solidify at room temperature on leveled surface.

2.6.13 Media for sensitivity test

Sheep blood agar medium (2.6.3), and reinforced clostridial medium (2.6.13) were used for performing sensitivity test.

2.7 Area of study:

The area investigated is located north of Kadougli, south Kordofan.

2.8 Clinical investigation:

Sick animals were examined clinically, and clinical signs were recorded. Postmortem examination of dead animals was performed and samples for bacteriological and histopathological examination were collected from dead animals.

2.9 Collection of samples

2.9.1 Samples for bacteriological examination:

A total of 55 tissue samples of approximately 1x3 cm thick were cut by sterile scissor and forceps from a periphery of fresh infected muscle and placed in sterile MacCarteny bottle containing cooked meat medium.

2.9.2 Sample for histopathological examination:

Tissue samples approximately 10x15cm thick were cut from muscles lesion by sterile scissors and forceps and placed into 10% formalin.

2.9.3 Transport of samples:

All samples collected were labeled. The samples for bacteriological and histopathological examination were then taken to laboratory.

2.10 Bacteriological examination

2.10.1 Primary isolation

Isolation attempts were made at El Obeid Regional Veterinary Laboratory. In the laboratory, samples for bacteriological examination were warmed in water bath at 42^{o}C for 2 hours. Then a loopful of the inoculum, maintained in cooked meat medium, was streaked on blood agar. The inoculated plates were incubated anaerobically at 37^{o}C for 48 hours in Bair and Talock anaerobic jar (BTC) and the Gas pack system (BBL, Oxoid) was used to provide anaerobic conditions.

After incubation period the colonies characteristic were observed, and smears were made immediately after jar was opened. The fixed smears were stained and examined microscopically as described later (2.13).

Further incubation was continued for 48 hour and if no growth was evident, then the plates were discarded as negative. All positive culture on blood agar was examined with naked eye for blood heamolysis.

2.11 Purification:

All bacteria isolated were purified by several subculturing from single well-separated colony on separate blood agar plates and then examined for purity by microscopic examination as described later (2.13).

2.12 Preservation:

Each of purified isolates was preserved by inoculation into cooked meat medium. All isolates were stored in cooked meat medium at 4^{o}C. The preserved cultures were brought to the laboratory of the department of Microbiology, Faculty Veterinary Medicine,

University of Khartoum for further studies to determine their cultural and biochemical characteristics.

2.13 Microscopic examination

Smears were made from colonies of primary culture, purified colonies, and broth culture, fixed by heating and stained by Gram stain method of Barrow and Feltham (1993). Then examined microscopically for cell morphology, arrangement, and staining reaction. Gram stain was used to recognize the cellular characteristics of primary growth and also to check the purity.

2.14 Biochemical tests:

2.14.1 Sugar fermentation test

The test was carried out as described by Barrow and Feltham (1993). The peptone water sugar was inoculated with organism under the test, incubated at 37^{o}C and then examined daily for several days. Acid production was indicated by appearance of reddish color, while gas production was indicated by presence of an empty space in the inverted Durhams tubes.

2.14.2 Indole production test

Indole production test was carried out as described by Barrow and Feltham (1993). The tested organism was inoculated into peptone water and incubated at 37^{o}C for 48 h. One milliliter of the Kovac's reagent was run down along side of test tube. Appearance of pink color within a minute indicated positive reaction.

2.14.3 Nitrate reduction

The nitrate test was carried out as described by Barrow and Feltham (1993). The tested culture was lightly inoculated into nitrate broth and incubated at 37^{o}C for two days. Then 1 ml of solution A followed 1 ml of solution B of nitrates test reagents were added. Red

color indicated positive reaction that showed nitrate in the medium had been reduced. If red color did not develop, powdered zinc was added to see whether there was residual nitrate or not. Red color development indicated that nitrate in the medium had been reduced to nitrite by zinc but not by organism, whereas unchanged color indicated nitrate in original medium had been reduced completely and nitrite was further broken down by the organism.

2.14.4 Unease activity tests

The test was carried out as described by Barrow and Feltham (1993). The tested organism was inoculated heavily onto slope of urea agar medium, and then incubated anaerobically at 37^{o}C for two days. Appearance of red color indicated positive reaction.

2.14.5 Gelatin hydrolysis

Gelatin hydrolysis test was carried out as described by Barrow and Feltham (1993). The tested culture was stabbed into nutrient gelatin and was incubated at 37^{o}C for up to 14 days. The inoculated tube was placeed in refrigerator for 2 hours every 2-3 days and was examined. The liquefaction of gelatin indicated positive test.

2.14.6 Motility test

The Craigi tube in semi-solid nutrient agar prepared as described by Cruickshank *et al*. (1975) was inoculated. A small piece of the colony of the bacterium under test was picked by the end of the straight wire and stabbed in the center of semi solid agar in the Craigi tube and then incubated at 37^{o}C overnight. The organism was considered motile if there was turbidity in the medium in/outside the Craigi tube.

2.14.7 Casein test

The casein test was carried out as described by Barrow and Feltham (1993). The plates of casein agar were inoculated with the organisms under test, the plates were incubated anaerobically at 37^{o}C for 4 days and checked for clearing of the medium around the bacterial growth.

2.14.8 Lecithinase test

Dry egg-yolk agar plates were cultured with the organism under test, and the plates were incubated anaerobically at 37^{o}C for 24 hours. The presence of opacity around the growing culture indicated that they were lecithinase positive, while absence of opacity indicated they were negative.

2.14.10 Sensitivity test

The test was performed by the standard disc diffusion method (Buxton and Fraser, 1977). The test was carried out by evenly sowing sheep blood agar plates with Reinforced Clostridial medium cultures and the excess fluid was aspirated by sterile Pasteur pipette. The plates were dried at room temperature for I5-30 min. Sterile forcep was used to apply the disc over the surface of the medium and the plates were incubated anaerobically in Baird and Tatlock anaerobic jar for 48 h. The growth inhibition zone was measured.

The antimicrobial agent examined were; Ampcillin, Tetracycline, Streptomycin, Gentamycin, Erythromycin, Cloxacillin and Chloroamphnicol.

2.15 Pathogenicity test

Two guinea pigs were used for this purpose, and they were housed and maintained in plastic cages and given feed and water adlib. The pathogencity of one isolate was tested by administering

pure culture intramuscularly. The inoculum was prepared by mixing equal volume of the overnight culture with 5% CaCL/normal saline and dose of 0.5 ml was inoculated intramuscularly. The guinea pigs were observed and postmortem was conducted when animal died. Tissues from muscle were collected and fixed in 10% formalin for histopathological studies; specimens were also removed aseptically for bacteriological examination. Smears were prepared from muscle and stained with Grams stain and microscopically examined.

2.16 Preparation of samples for histological examination

All preparations were carried out as described by Drury *et al.* (1980) and Manual of Veterinary Investigation Laboratory Techniques (1981).

2.16.1.I Fixation

Formalin (10%) was used as fixative. Samples were fixed for 48 hours or more.

2.16.I.2 Dehydration:

The tissues were cut (trimmed) into small pieces about one cubic cm, and labeled with a pencil, then washed in running tap water for 15 min to remove fixing agent. The dehydration was carried out by passing the samples through increasing concentration of alcohol 60%, 70%, 80%, 90% and 100%.

2.16.I.3 Clearing:

Clearing was carried out by chloroform, zylene, benzene, and cedar wood oil.

2.16.I.4 Impregnation

Melted paraffin wax (two changes) was used to remove the clearing agent from the tissue and penetrate the tissue to fill the intercellular spaces.

2.16.I.5 Blocking

Tissues were blocked in melted paraffin wax and quickly cooled.

2.16.I.6 Section cutting

Sections 5-6 microns thick were cut with rotary microtome.

2.16.2. fixing section to slide

The sections were transferred and floated in warm water bath (50-60^{o}C) containing an amount of gelatin powder. Then fixed to glass slide and the fixed sections were incubated for 30 min at 60^{o}C to dry.

2.16.3 Staining

Routine stain, haematoxylin and eosin were used. Sections were stained in heamatoxylin for 10 min, washed, differentiated in 1% acid alcohol, and washed in running tap water for 10 min. Then counterstained with eosin for 2-3 min. then, rinsed quickly in water and dehydrated in 70%, 90% and absolute alcohol. Sections were cleared in zylene. Sections of skeletal muscles were also stained by Grams stain for demonstration of aetiological agent in the muscles of infected cattle and inoculated ginunea pigs.

2.16.4 Mounting

The section was covered with cover glass using a suitable mounting medium, Canada balsam. After overnight drying at room temperature, section was examined microscopically.

CHAPTER THREE

3. RESULTS

3.1 Black Quarter outbreak:

The clinical symptoms noticed were fever (101-102^{o}F), swelling and crepitation of the infected quarters, anorexia and depression. The infected cattle died within 1-2 days. The infected cattle were mostly young 2-2.5 years old (Fig 3).

The infected cattle were nomadic cattle. The outbreak occurred in June 2006. The number of affected cattle and the number of herd involved are shown in Table 3.

3.2 Isolation and identification of bacteria

Bacteriological findings in this study were based on isolation and identification of the causative agent from suspected black quarters outbreak that occurred in Kadougli locality.

Samples were collected from dead calves. The collected samples were cultured onto blood agar and incubated anaerobically in anaerobic jar and Gas Pack system was used to maintain anaerobic condition. Samples collected from two calves showed positive growth.

3.2.1 Growth on solid medium

On blood agar medium, colonies were 1-2 mm in diameter, translucent and a clear zone of heamolysis was seen.

3.2.2 Microscopic and cultural characteristics of the isolates:-

Microscopic examination of smears from colonies, broth culture and infected muscles, stained by Grams stain, revealed Gram positive bacilli with subterminal spore which was spindle shaped (Fig. 4,6,7).

3.2.3 Growth in liquid medium

a. A heavy turbidity was produced by all isolates in peptone water following overnight incubation at 37^{o}C.

b. **Cooked meat medium**

In cooked meat medium, the isolated organisms grew between meat particles.

3.2.4 Biochemical characteristics of the isolates:

3.2.4.1 Sugar fermentation test

In peptone water sugar medium, the two isolates fermented glucose and lactose with production of acid and gas. Acid was indicated by change in colour of the medium to pink. Gas production was indicated by appearance of air bubbles in Durhams tube.

The isolates did not ferment sucrose, nor salicin (Table, 4).

3.2.4.2 Nitrate reduction test

The two isolates did not reduce nitrate, when grown in nitrate medium (Table, 4).

3.2.4.3 Indole test

The isolates examined did not produce indole when cultured in peptone water (Table, 4).

3.2.4.4 Gelatin liquefaction test

The two isolates liquefied gelatin (Table, 4).

3.2.4.5 Casein test

The isolates examined did not hydrolysed casein (Table, 4).

3.2.4.6 Urease test

The two isolates were urease positive and changed the colour of the medium to the pink (Table, 4).

3.2.4.7 Lecithinase and lipase test:

The isolates examined did not produce Lecithinase, nor Lipase when cultured on egg-yolk medium (Table, 4).

Hence, these two isolates were tentively identified as *C. chauveoi*.

3.2.4.8 Sensitivity test

The antibiotic sensitivity of the isolates of this study is shown in Table 5; Fig 7. The isolates were sensitive to penicillin, tetracyclene, streptomycin, gentamicin and chloromphenicol, but they were resistant to ampicillin and cloxacillin.

3.3 Postmortem examination:

On postmortem examination, the dead calves showed haemorrhage, congestion, swollen and black coloration of affected muscles. The legs are stiffy and had rancid odor. Both fore and hind quarters were involved.

3.4 Histopathology examination of cattle muscles:

Histologically, the haemotoxylin and eosin (H & E) muscle revealed necrosis manifested by deeply eosinophilic staining of muscle fiber with disappearance of many of sarcolema nuclei. In some areas the necrotic muscle fibers were separated by oedema, accumulation of extravagated erythrocytes and some inflammatory cells mainly neutrophil. These inflammatory exudates were, also seen in the interstitial tissue. In severely affected area the normal muscle tissue structure was fragmented obliterated and replaced by necrotic tissue debris, haemonhage, inflammatory cells and air bubbles (Fig. 8).

3.5 Experimental animal pathogencity test

The inoculated guinea pigs died within 48 hours after inoculation. The inoculated guinea pigs showed the characteristic signs of blackleg disease. They became lame, and lost appetite. On the postmortem, the site of inoculation showed swelling, congestion, gas and oedema. Heamorrhages were seen in lungs, heart, and spleen and had rancid odor (Fig, 9).

Smear from the lesion showed the presence of Gram positive rods. Cell morphology, staining reaction, cultural characteristic and biochemical tests proved the reisolated Gram-positive rod from inoculated Guinea pig was *C. chouvoei*.

3.6 Histopathological examination experimental guinea pigs:

Histological examination of the muscle revealed necrosis manifested by deeply eosinophilic staining of muscle fibres with disappearance of sarcolema nuclei, haemorrhages and inflammatory cell (fig. 10).

Table 3: Cattle involved in black quarter outbreak occur ed in Kadougli Locality during June 2006

Herd	Herd size	Number of infected cattle	Morbidity rate	No. of dead cattle	Mortality rate
First herd	1000	8	0.8%	8	0.8%
Second herd	700	17	2.4%	17	2.4%
Third herd	800	6	0.75%	6	0.75%

Table 4: Characters and biochemical prosperities of isolates

Character	**Result**
Staining reaction	Gram +ve
Shape	Rod
Spore	terminal
Growth temperature	37^{o}C
Motility	+ve
Glucose	+ve
Lactose	+ve
Sucrose	-ve
Salicin	-ve
Nitrate	-ve
Indole	-ve
Gelatin	+ve
Casein	-ve
Urea	+ve
Egg-yolk Lecithinase Lipase	 -ve -ve

+ve: Positive, -ve: negative

Table 5; Antibiotic sensitivity of the isolates;

Antibiotic	**Sensitivity (diameter zone of inhibition)**	
	Isolates 1	**Isolates** 2
Ampicillin AM-I0mg	R (-)	R (-)
Penicillin PEN I0mg	S (0.6cm)	S (0.6cm)
Tetracycline TE- 30mg	S (1.4cm)	S (1.4cm)
Streptomycin STR-I0mg	S (1.8cm)	S (1.8cm)
Gentamycin GEN-I0mg	S (2.0cm)	S (2.0cm)
Erythromycin ER-I0mg	S (1.4cm)	S (1.4cm)
Chloroamphnicol CH- 30mg	S (I.5cm)	S (I.5cm)
Cloxacillin CXC 10mg	R (-)	R (-)

S: Sensitive R : resistant

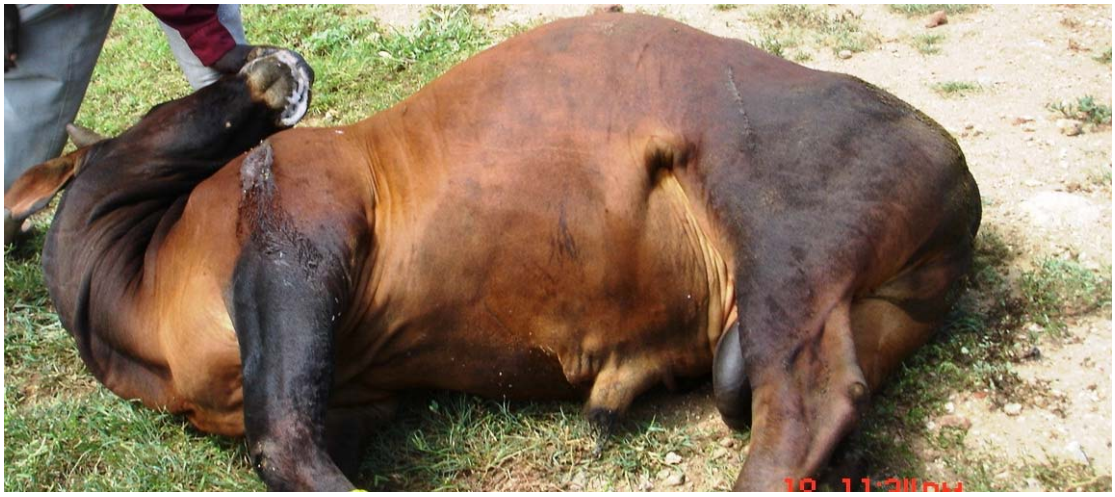


Fig. 3: Infected calf, both fore and hind limbs were affected

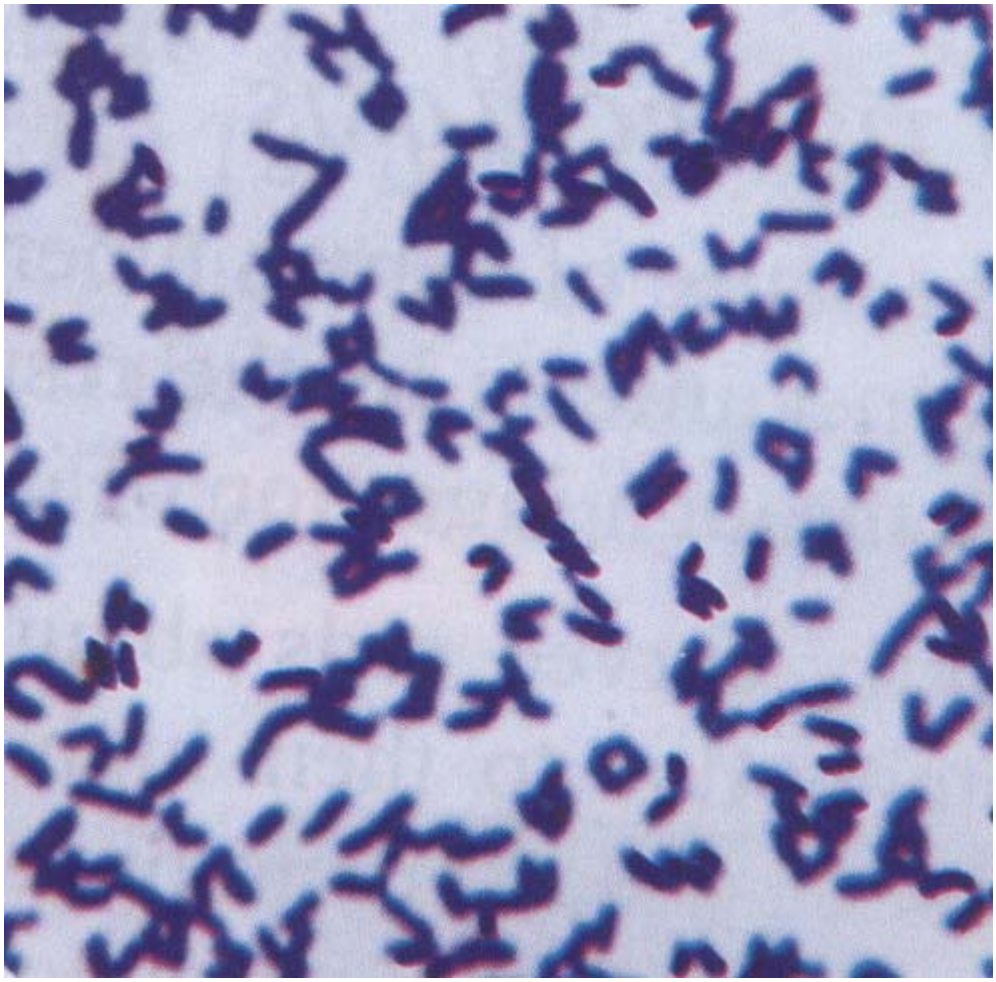


Fig. 4: Gram stained smear from broth cultures note, Gram positive rods

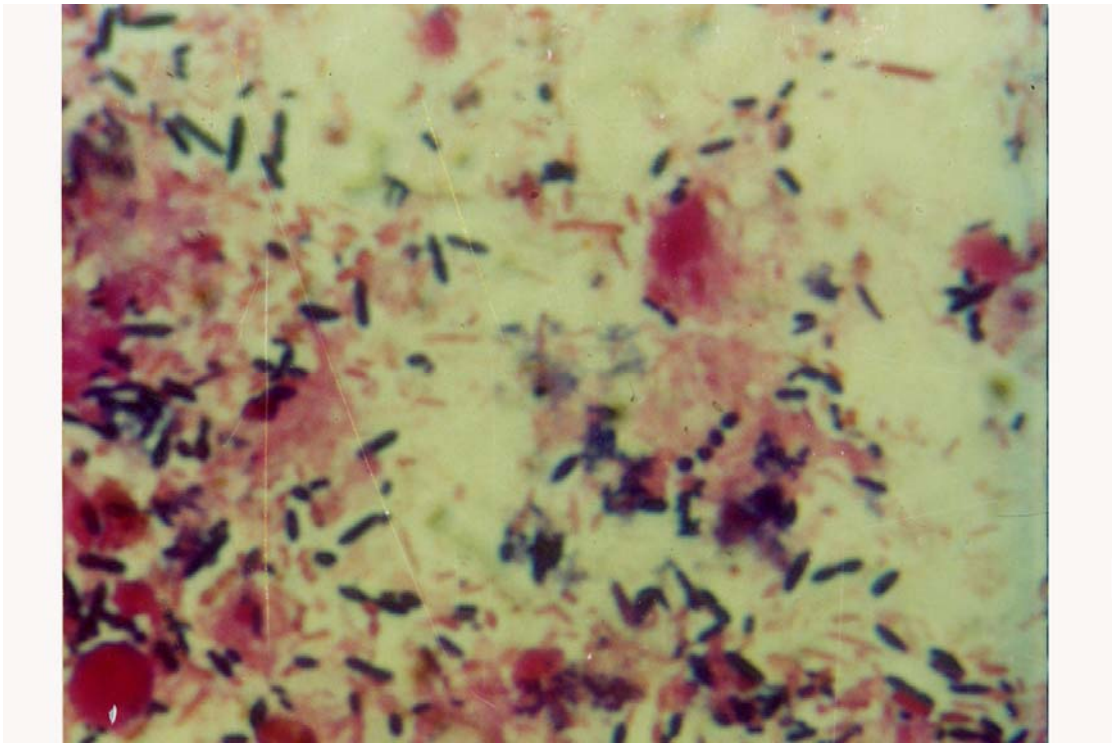


Fig 5 : Muscle section of calf stained by Gram stained method showing Gram-positive bacilli x10

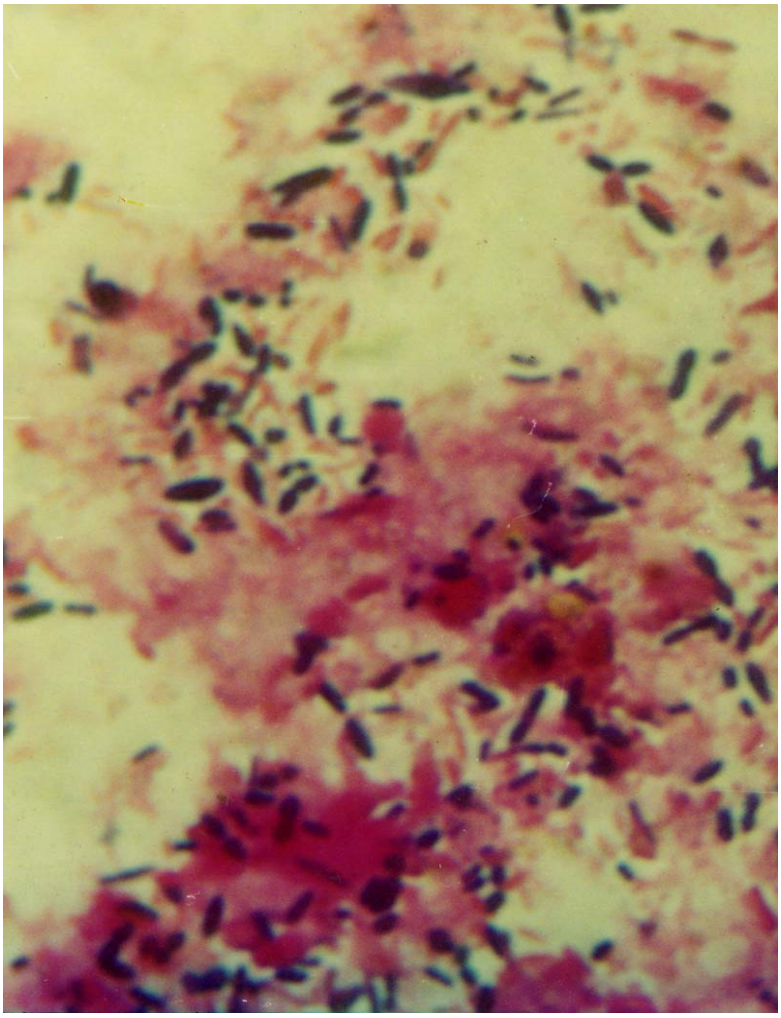
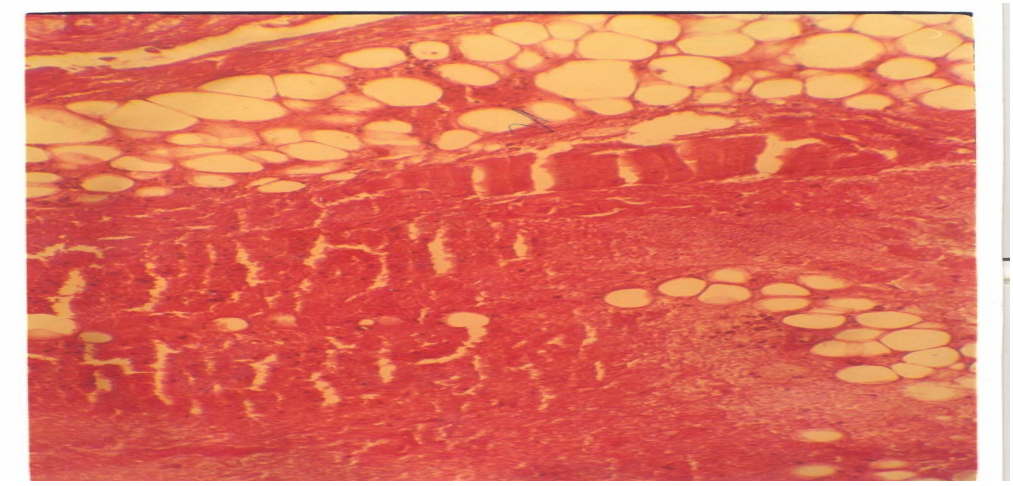


Fig. 6: Muscle section of Guina pig stained by Gram staining method showing Gram-positive bacilli x10

Fig. 7: *C. chauvoei* isolate 1, showed sensitivity to penicillin, tetracycline, gentamycin erythromycin and chloromphenicol



Fig, 8: Section of calf muscle, note necrosis, and tissue debris, inflammatory exudates and air bubbles (H & E) x10

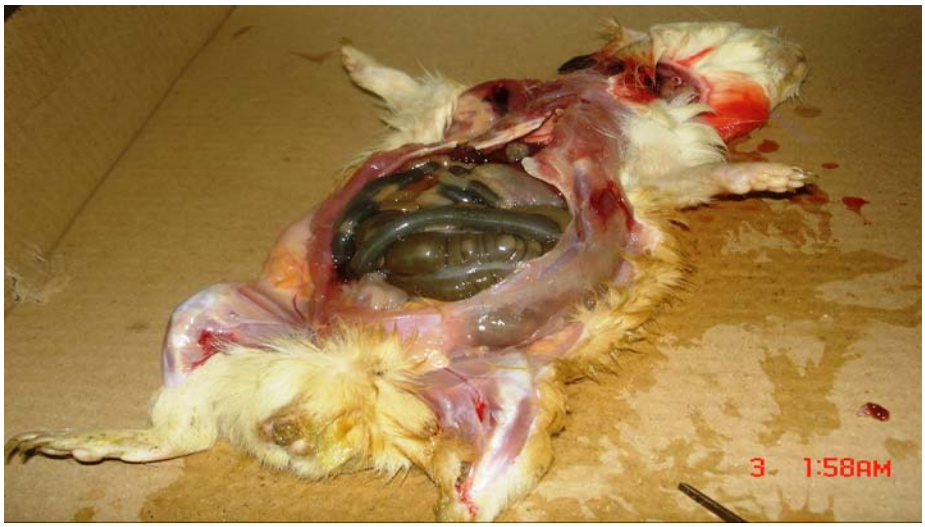


Fig 9: Congestion and haemorrhage of hind limb muscle and internal organs of Guinea pig inoculated with the isolate 1

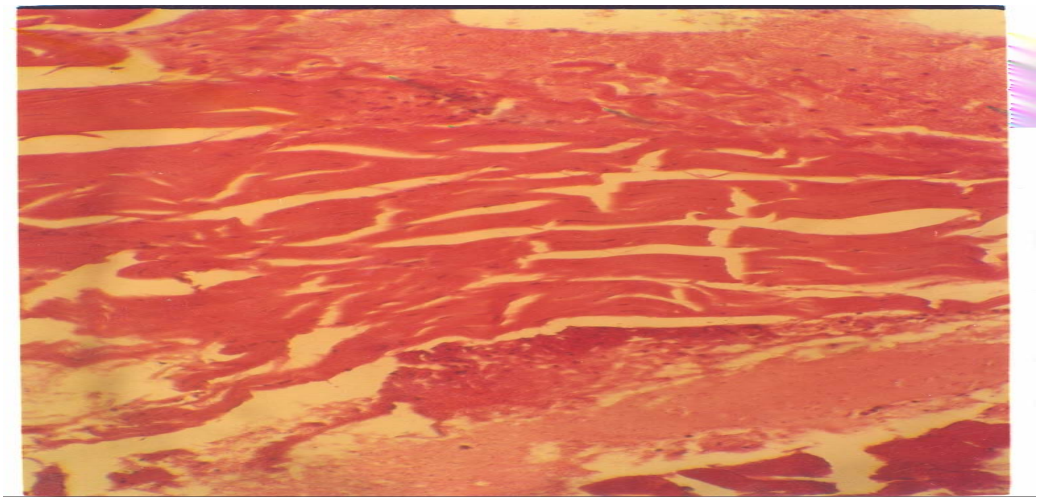


Fig. 10: Muscle section of inoculated guinea pig, note necrosis, haemorrhage, inflammatory cells (H & E)x 10

CHAPTER FOUR

4. DISCUSSION

Black leg is an acute disease of cattle and sheep caused by *C. chauvoei* and occurs worldwide. In Sudan, the first resport of the disease was in 1939 (El Sawi, 1986). In Kordofan region, outbreaks of blackleg were first report in 1942 (Annual Report, Ministry of Animal Resources, 1902 – 1980). El Azhari *et al.* (1981) isolated *C. chauvoei* from an outbreak occurred in Kadougli area, South Kordofan state. The disease continued to be reported from Kadougali area (El Azhari *et al*., 1981). Recently, blackleg outbreaks occurred in 2006, in the same area, Kadougli locality. This investigation was aimed to study the epidemiology and pathology of this blackleg outbreak. Also it was carried out to isolate and identify the causative agent.

Blackleg disease in cattle was occurring in different areas in the Sudan (Annual Reports, Ministry of Animal Resources 1902-1980). Since 1981, blackleg outbreaks were reported from Kadougli, South Kordofan state (El Azahari *et a*l., 1981), Kuku, Khartoum state, El Doeim, White Nile State, Elties and Buram, South Darfur state (Musa *et al*., 1998) and from Bahr El Arab area, South Darfur state (Musa *et al*., 1998). Diagnosis of above mentioned outbreaks were confirmed by isolation and identification of the causative agent, *C. chauvoei*.

This reveal, blackleg disease is endemic in the Sudan affecting mainly cattle. Blackleg also seems to be endemic in Kadougali area as the epidemiological information collected from previous reports indicated.

Blackleg outbreak, investigated here was encountered in calves 2-2.5 years old and the outbreak involved three herds. Involvement of smaller group of age was reported by Musa *et al* (1998) who observed, the cattle infected were mostly young 1-2 years and occionally adults. Quninn *et al*. (2002) stated that the disease is most often encountered in young thriving animal from 3 month to 2 year old. This reported blackleg outbreak occurred in late summer in June 2006. This agrees with Musa *et al.* (1998) who reported that the disease occurred in all season of the year, but notably in dry hot summer and autumn. Also agrees with Musa *et al.* (1998) who reported the disease in summer in Kuku area, Khartoum, State. However Musa *et al*. (1998) reported disease outbreak that occured in winter in Doeim area, White Nile state. Bagadi (1978) reported relationship between the annual rainfall and outbreak of black quarter of cattle.

Over million heads of livestock cattle, sheep and goats passed through Kadougli locality when moved from summer to Autumn residence in less humid areas during June-July each year. This result in overcrowding in contaminated area. *C. chauvoei* can exist in soil through contamination by animal dropping and infected carcasses. The overcrowding in contaminated land when the environmental temperature rose to higher degrees and when cattle were driven for long distance for water, predisposed cattle to blackleg infection. This supported the assumption of Smith (1975) who reported that at

stagnant water pools or wells, many animal were gathered, intermixed and subjected to starvation before watered, such practices could have had predisposed cattle to blackleg infection.

The cattle involved in reported outbreak showed classical symptoms and signs of blackleg disease, which were described by NADIS (2003) and Wilson (2004). These symptoms were also similar to those previously observed by Musa *et al.* (1998).

The findings of postmortem examination of dead calves revealed classical lesions of blackleg. However, the lesions observed in present investigation involved both fore and hind quarter. The postmortem findings of this study agree with findings described by Musa *et al*. (1998), except the lesions described by the later authors involved sternal region, neck and tongue.

The microscopical examination of skeletal muscles tissue revealed haemorrhage, neutrophils and lymphocytes infiltration and gas bubbles. The histopathological changes noticed in skeletal muscle are typical lesions of blackleg disease (Simth, 1975) and are similar to those described by Musa *et al*. (1998).

The results of this investigation revealed the isolation of *C. chauvoei* from two dead claves showing typical symptoms and postmortem lesions of blackleg. *C. chauvoei* was isolated from outbreak that occurred in same Kadougli, locality (El Azhari *et al.* (1981) and from outbreaks occurred in other part of Sudan, Khartoum State, White Nile State and South Darfur State (Musa *et al.* 1998). The biochemical properties of this study isolates are the same as biochemical properties of previouly by reported isolates (El Sawi, 1986; Bushra, 1986; Musa *et al*. 1998).

Experimental guinea pigs inoculation, indicated sketal muscles were primarily involved organs. Lung, liver and kidney were also affected. The inoculated organism was reisolated from dead experimental guinea pigs. These findings are in agreement with other guinea pigs inoculation findings (El Sawi, 1986, Bushra, 1986; Musa et al., 1998).

The cell morphology, cultural characteristics biochemical properties of the isolated bacteria and guinea pigs inoculation proved the isolates were *C. chauvoei*. The clinical signs, postmortem lesions histopathological changes and isolation and identification of *C. chauvoei* confirmed the outbreaks that occurred in Kadougli locality in 2006 were black leg outbreaks.

In 1943, cattle in the infected area were vaccinated early in the years and no further death occurred in vaccinated cattle (El Sawi, 1986). Although annual vaccination has brought the disease under control, outbreak is frequently reported from Kadougli locality. Thus, it highly recommended, the young cattle (1 – 3 years) should be vaccinated before entering the contaminated area, the Kadougali locality.

The *C. chauvoei* isolates reported here, were sensitive to penicillin, tetracycline, streptomycin, gentamycin and chloramphenicol. These antibiotics could be used to treat cases of blackleg if diagnosis is made earlier. Penicillin was reported to be effective in treating systemically and locally if given in early stages of the disease (Quinn *et al.,* 2002). However, Sterne (1981) concluded that in practice the infection of blackleg is so rapid that treatment is generally of no practical value.

CONCLUSION AND RECOMMENDATION

CONCLUSIONS

From the finding of the present study it can be concluded that:

1/ The infected cattle showed clinical signs of blackleg disease and both fore and hind limbs were involved.

2/ The died calves at postmortem examination showed congestion, haemorrhage and black colouration of infected muscle. The histopathological examination revealed muscle necrosis and cellular infiltration.

3/ The cellular morphology, cultural characteristics and biochemical properties suggest the organism isolated is *C. chauvoei* .

4/ The inoculation of guinea pig by isolate 1 caused death within 24 h. The muscle of inoculated guinea pig showed haemorrhage congestion and necrosis. The inoculated isolate was reisolated.

5/ All the above confirmed the suspected outbreak was blackleg outbreak and the causative agent was *C. chauvoei*

RECOMMENDATION

From the results and discussion of this work it is recommended that:

- Cattle should be vaccinated at young age (3 to 6 months) and annually thereafter.
- The vaccination should be carried out 2-4 weeks before passing the contaminated Kadougli area while nomads moving north.
- During an outbreak, it is recommended all cattle in the herd to be vaccinated and if possible given long acting penicillin.
- The pasture of contaminated area is advisable to be avoided.
- Further research should be carried out on these local isolates, hopefully more potent vaccine will be developed.

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