

**IN VITRO AND IN VIVO STUDIES ON *CLOSTRIDIUM CHAUVOEI* (JAKARI STRAIN) TOXIN**

***BY***

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## DECLARATION

I declare that the work reported in this thesis was carried out by me in the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria, under the supervision of Dr. H.M. Kazeem, Prof. L.B. Tekdek and Prof. I Ajogi. All information derived from the literature is acknowledged and referred to accordingly. I declare that no part of this thesis has been submitted elsewhere for a degree or diploma.

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## CERTIFICATION

This thesis entitled *In vitro* and *in vivo* studies on *Clostridium chauvoei* (Jakari strain) toxin by **Manasa Yohana Sugun** meets the regulations governing the award of the degree of Master of Science (VETERINARY MICROBIOLOGY) of the Ahmadu Bello University, Zaria, and is approved for the scholarly contribution to knowledge and literary presentation.

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## **DEDICATION**

This work is dedicated to my late mother, Mrs. Alfa Yohana Sugun and the entire family.

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## ABSTRACT

It has been documented that toxins from *Clostridium chauvoei* (Jakari strain) play a pathogenic role in blackleg. These toxins have not been characterized. It has therefore become necessary to characterize the toxins to enhance our understanding of the pathogenesis of blackleg. The production and properties of *Clostridium chauvoei* toxins was investigated *in vitro* and *in vivo* in mice given a minimum lethal dose of 0.2 ml intravenously (IV) after incubation at 37°C anaerobically in toxin production medium for 24 and 48 hours. *Clostridium chauvoei* (Jakari strain) toxin was stable at 45 °C and 70 °C temperature for 5 minutes, while hemolytic activity was completely destroyed at 100 °C for 5 minutes. Chemical agents, like ether, and hydrochloric acid did not alter the hemolytic activity of the toxin. Phenol and methanol had partial effect and some reduction in hemolytic effect was observed at 624 and 128 end-points for phenol and methanol respectively. Formalin and sodium hydroxide rapidly and completely destroyed hemolytic activity of the toxins. Hemolytic activity of the toxin was stable at 4 °C of storage for two weeks. A decline was observed when the toxins were stored at 4 °C for more than two weeks. Gross and histopathologic lesions were observed in tissues of mice which died following intravenous administration of the toxins. Hemorrhages and congestion were observed in the internal organs (livers and lungs). Focal and diffused areas of necrosis and infiltration of mononuclear cells were lesions observed in kidney, liver and pancreas of inoculated mice.

**INTRODUCTION**

*Clostridium* species form spores which survive boiling, but they can not form spores in the presence of oxygen. Although most *Clostridia* are aero-tolerant (they survive exposure to air), but may not resume growth if oxygen is present. A common disease of livestock caused by clostridium species is blackleg. Blackleg is an acute disease of ruminants caused by *Clostridium chauvoei*. The syndrome and postmortem changes are suggestive of the condition being a true toxemia. So far, attempts at obtaining a potent toxin from clostridia have been accompanied by very little success. Kerrin (1934) was the first to study in some details, the ability of authentic strain of *Clostridium chauvoei* to form a soluble toxin. *Clostridium chauvoei* is an anaerobic, Gram-positive spore-forming, rod-shaped 0.6 to 1  $\mu\text{m}$  in diameter by 3 to 8  $\mu\text{m}$  in length with a round end, occurring singly or in short chains, though the long filamentous forms are common. Their culture may or may not be surrounded by narrow zone of haemolysis, depending upon the characteristic of the strain being examined and the source of the red blood cell in the media (Louis and Hobbs, 1975). The spores are highly resistant to environmental changes and disinfectants and persist in soil for many years (Blood and Radostits, 2000). Mason (1936) studied four strains of *Clostridium chauvoei* which were different in origin from those of Kerrin (1934) and different media were also employed in the culture of the organism. Nevertheless, both workers were in agreement that culture filtrates of all the strains of *Clostridium chauvoei* studied resulted in production of a toxin which was antigenic. The generic name Chauvoei was derived from the name of Professor J.A.B Chauveau, a French bacteriologist of the

nineteenth century (Cato *et al.*, 1986). The surface colonies on blood agar are usually circular with entire margins, translucent, greyish white, matt to glossy surface. There is evidence of slight haemolysis on blood agar, but clear zones are not formed around surface colonies. Moderate growth is obtained in nutrient broth or cooked meat medium with or without fermentable carbohydrate. On culture, a toxin is produced which is pathogenic for mice and guinea pigs. Optimum temperature for growth is 37 °C. It does not grow at a temperature above 42 °C (Louis *et al.*, 1975). Many names such as blackleg, quarter evil, symptomatic anthrax and gangrene emphysematous have been used for the disease caused by *Clostridium chauvoei* (Wilson and Miles, 1975; Haagma, 1979). However, the disease must be distinguished and differentiated from malignant oedema caused by *Clostridium novyi* and from anthrax caused by *Bacillus anthracis* due to the similar course of the disease. Symptoms resembling black quarter can also be caused by *Clostridium septicum*, *Clostridium novyi*, *Clostridium perfringens* and *Clostridium sordelli* (Kurnert *et al.*, 1996). A recent study on the phylogenic positions of *Clostridium chauvoei* and *Clostridium septicum* based on their rRNA sequences revealed similarity of 99.3% between *Clostridium chauvoei* and *Clostridium septicum*. The long filament observed in *Clostridium septicum* are characteristic and they are helpful in differentiating the organism from *Clostridium chauvoei* which never form such filament. There has been a long speculation that they might represent two types of the same species rather than two different species (Moussa, 1959, Kuhnert *et al.*, 1996). Black quarter has been recognized in Nigeria since 1929, and since then it has remained a major economic problem to cattle owners. The peak incidence of black quarter in Nigeria is during the wet season, when the spores are exposed from the ground by the action of rain and worms, and therefore become accessible to animals during grazing on contaminated area. In a study on 144 black quarter cases in Nigerian cattle, the

clostridial bacteria isolated included *Clostridium welchii* 16%, *Clostridium chauvoei* 21.5% and *Clostridium septicum* 20%). Mixed infection was found in 9.3% (Osiyemi, 1975). A regime of preventive vaccination measure carried out twice annually at the beginning of rainy season with an interval of ten days before heavy rain begins is suggested for adoption as a step towards effective control of blackleg disease in cattle in Nigeria (Osiyemi, 1975)

Bacterial toxins are defined as soluble substances that alter the normal metabolism of host cell with deleterious effects on the host. Toxins and other factors associated with pathogenicity of *Clostridium chauvoei* or with immunity against it, have been of considerable interest because of the economic importance of the disease caused by this organism in cattle and sheep. The microorganism produces at least five soluble immunizing components. These include oxygen – stable hemolysin (alpha), a DNase (beta), a hyaluronidase (gamma), oxygen – liable hemolysin (delta) and neuraminidase. These toxins enhance the ability of *Clostridium chauvoei* to invade and multiply in tissues of susceptible host and exhibit immunological activity (Louis and Geoffrey, 1975).

*Clostridium chauvoei* infection in sheep differs from those in cattle in that they tend to be associated with wound infections. Most commonly, they follow shearing, castration, tail docking or parturition (Louis and Geoffrey, 1975). It has been found experimentally with sheep that a wound must be deep enough to extend through the subcutis or submucosa if an infection with *Clostridium chauvoei* is to be induced (Louis and Geoffrey, 1975). Sheep of all ages seem to be susceptible to black quarter. The disease has been found in lambs a few days after birth, in which the stump of the umbilical cord was the apparent route/site of infection.

Although, Agba and Princewill (1986) put the economic losses in cattle due to blackleg at four million naira (#4 M) annually, current losses to the disease may approximate six hundred million naira (#600 M) annually (Useh *et al.*, 2006).

*Clostridium chauvoei* possesses certain unusual characteristics that distinguish them from other species (Rossetto *et al.*, 2001) three principal ones are:

1. The ability to multiply only in the absence of oxygen (anaerobic environment)
2. The ability to survive adverse condition by transforming into highly resistant form (spores)
3. The release of potent exotoxins during the process of multiplication.

*Clostridium chauvoei* (jakari strain) toxins, the etiologic agent of blackleg disease in Nigeria, have not been characterized. It has become necessary to characterize the toxins for clearer understanding of the disease.

#### **The aims and objectives of this study**

- To investigate the production of toxins by *Clostridium chauvoei* (Jakari strain) *in vitro*.
- To examine the gross and histopathological effects of the toxins in mice
- To study the physical and chemical properties of the toxins

Many works on *Clostridium chauvoei* (Jakari strain) has been done, though the microorganisms have never been characterized. This work is being undertaken to characterize the organism as it is the aetiologic agent of blackleg in Nigeria.

## **Chapter 2.0**

## **LITERATURE REVIEW**

### **2.1**

### **Anaerobic Bacteria**

Anaerobes play an important role in nutrition, health and disease process in animals. Nutrition has a considerable effect on the microbial fermentation that occurs in the gastrointestinal tract of ruminants (Fuller, 1992). The conditions of the gastrointestinal tract (GIT) are highly suitable for the development of vast and diverse microbial populations. The pH and temperature are highly favourable for microorganisms. Oxygen is inimical to anaerobic bacteria. It is speculated that oxygen toxicity in anaerobes is due to absence of the protective enzymes: catalase, peroxidase and super oxide dismutase found in aerobes. Anaerobic bacteria have been divided into obligate anaerobes that do not form colonies on agar surface exposed to 0.55 % or more of oxygen, and moderate anaerobes that are capable of growth at oxygen levels ranging from 2 to 8 %. Strict obligates anaerobes are typically members that do not grow at all in the presence of oxygen (Fuller, 1992)

#### **2.1.1 *Anaerobes in Diseases***

Many species of pathogenic bacteria are involved in disease processes of animals and man. Anaerobic bacteria cause specific as well as non-specific diseases. These diseases may be grouped into two based on the characteristics of the causal agents. The first group is spore-forming anaerobes (Clostridial group) responsible for causing specific disease in animals and originate exogenously. The second group consists of non spore-forming aerobes, which cause non-specific diseases and originates endogenously (Hathway 1990, Fuller 1992)

### **2.1.2 *Spore-forming anaerobes***

Clostridia are widely distributed in nature and have their main habitats in soil and also occur as common inhabitants of gastrointestinal tract (GIT) of man and animals. They are from the soil or from vegetation contaminated with the organisms that the infection originates. Most of the clostridial infections are acute and fatal and the predisposing factors such as tissue/muscles damage, overfeeding, liver fluke infection etc play a dominant role in outbreak of the disease.

### **2.1.3 *Non spore-forming anaerobes***

Most of the infections caused by non spore forming anaerobes are endogenous in origin. Since they are part of the normal bacteria flora of the mucosal surface of the alimentary/respiratory tract, the conditions necessary for the development of many of these endogenous infections are not clearly understood (Carlton *et al.*, 1993)

## **2.2 Names of Major Pathogenic *Clostridia***

Bacteria have always been characterised with the tools available at the moment of their isolation. First the bacteriologists used the morphological characters of the bacteria to classify them into genera and their names refer to those characteristics (“Clostridium” means a small spindle). For the species description, the biological and biochemical characters are used such as enzymatic processes (proteolytic, saccharolytic) that enable the bacteriologists to improve the identification of the microorganisms. As the criteria change, the classification and the names are sometimes modified (Stackebrandt, 2002)

Quite recently, taxonomists developed new tools such as 16S DNA gene analysis and DNA homology studies to define bacterial species, to better characterise the bacterial isolates and to understand the path of evolution of cellular and genomic traits. This was extensively presented in the first proceedings (Stackebrandt, 2002) and scientific booklet of the European Concerted Action “Genus *Clostridium*”.

The genus *Clostridium* was proposed by Prazmowski in 1880 when he described *Clostridium butyricum*, the type species (Prazmowski, 1880). *Clostridia* are Gram-positive endospore-forming rod-shaped bacteria. The oxygen requirement for culture was not a character recognised at the early stages of the classification, and therefore most of the *Clostridia* were first classified as *Bacilli*. When their anaerobic character was recognised, they were reclassified in the genus *Clostridium*. Conversely, some species, first described as *Clostridia*, were later classified in other and sometimes new genera such as *Eubacterium*, *Filifactor*, *Moorella*, *Finegoldia*, *Caloramator* and others (Stackebrandt, 2002)

Many names have also been slightly modified by Latinists who matched the epithets with Latin grammar to improve the quality of translation (Euzéby, 2003). The nomenclature will probably still be modified in the future as new techniques are developed. However, tradition survives and it is still necessary to consult literature where the old names are used. Therefore, this review will attempt to gather the most famous names, listed in the various editions of the Bergey’s manual of determinative bacteriology (editions 1 to 8 from 1923 to 1974) and of systematic bacteriology (edition 1, 1986) of the well-known major pathogen clostridial species.

### **2.3 Description of the Genus *Clostridium***

The bacterium *Clostridium* is a spore – forming, catalase – negative, anaerobic bacillus with Gram-positive cell wall component. The vegetative cells of most species are rod shaped, straight or curved, but cells vary from short rods to long filamentous forms. Rod-shaped cells may be rounded, tapered or blunt ended. Cells may occur singly, in pairs or in chains of various lengths. Most species stain Gram-positive during the early stage of growth; however some species such as *Clostridium vamosum* and *Clostridium clostrideforme*, almost always appear Gram negative after overnight culture (Cato *et al.*, 1986). The spores of *Clostridium* are ovoid to spherical and distend the vegetative cells. Certain species such as *Clostridium perfringens*, produce spores only under special culture conditions. Majority of *Clostridium* species are obligate anaerobes, there is considerable species variation with respect to oxygen toxicity (Cato *et al.*, 1986).

In contrast some species, example, *Clostridium hemolyticum*, *Clostridium novyi* type B and *Clostridium chauvoei*, are strict obligate anaerobes and will not grow when exposed to even trace amount of oxygen. A few aero-tolerant strains (*Clostridium tertium*, *Clostridium carnis*, *Clostridium histolyticum*) and occasionally strains of *Clostridium perfringens* show scant growth on plating medium incubated in 5 to 10% CO<sub>2</sub>, in air or in a candle jar. Since aero-tolerant clostridia may grow on the surface of fresh agar medium under anaerobic conditions, it is possible to confuse these species with certain facultative anaerobic bacillus species (Brak and Madigan, 1988). However, members of the genus *Clostridium* usually form spores only under anaerobic conditions and almost never produce catalase. Also, aerotolerant clostridia show much better growth (i.e. they form larger colonies) under anaerobic condition than in air; whereas *Bacillus* species often form larger colonies on

aerobically incubated medium than on medium incubated anaerobically (Brack and Madigan, 1988).

### **2.3.1 *Guidelines to Clostridia Identification***

After growth in anaerobic conditions, *Clostridia* are suspected on the basis of colonial morphology, haemolysis, cell morphology at Gram stain, and spore morphology at Ziehl-Nelsen stain (Table 2.1). They are classically identified using biochemical tests. A very reliable identification method is the gas chromatography of structural and metabolic microbial organic acids in the culture media, although not easy to use in routine diagnostic laboratories.

**Table 2.1: General morphology and growth criteria of major *pathogenic Clostridia***

<b><i>Clostridium</i></b>	<b>Morphology</b>	<b>Spores</b>	<b>Culture features</b>	<b>Colonies/Haemolysis</b>
Argentinense Toxintype G (= <i>botulinum</i> Group IV)	1.5 to 2.0 x 1.5 to 10.0um Motile Singly or in pairs Straight rods	Oval, subterminal, Bulging, rare	Very strick anaerobe Up to five days	Circular to irregular, raised, ytransluscent, smooth, shinny Haemolysis zone
<i>Botulinum</i> Group I Toxintypes A/B/F Proteolytic	0.5 to 1.5 x 3.0 to 20.0um, Motile, Singly or in pairs Straight to slightly curved rods	Oval, subterminal, Bulging	Very strick anaerobe Up to five days	Circular to irregular, flat to raised, transluscent to semi-opaque, grey, Haemolysis zone
<i>Botulinum</i> Group II Toxintypes B/E/F Non proteolytic	0.8 to 1.5 x 2.0 to 16.0um Very Motile Singly or in pairs Straight rods	Oval, subterminal, Bulging (usually)	Very strick anaerobe Up to five days	Irregular, raised, translucent to opaque, grey-white Haemolysis zone
<i>Botulinum</i> Group III Toxintype C/D	0.5 to 2.5 x 3.0 to 22.0um Very Motile Singly or in pairs Straight rods	Oval, subterminal, Bulging	Very strick anaerobe Up to five days	Circular to slightly irregular, flat tol raised, translucent, grey-white Haemolysis zone
<i>Chauvoei</i>	0.6 to 1.5 x 3.0 to 8.0um Pleimorphic, motile, Singly or in short chains, strait sides, rounded ends.	Oval,subterminal, Bulging	Very strick anaerobe Need blood, cysteine Slow growth	Compact, spherical, smooth, greysih Slightly hemolytic
<i>Colinum</i>	1.0 x 3.0 to 4.0um	Oval, subterminal, Bulging, rare	Fatsidious, primary growth in broth	Growth on blood agar only after several passages
<i>Difficile</i>	0.5 to 2.0 x 3.0 to 17.0um Motile Singly or in short chains , slender rods	Oval, subterminal, Slightly Bulging	Fastidious, (CCFA agar) <sup>1</sup>	Rhizoid, flat or low convex, opaque, grey-white.

(Cato *et al* 1988)

<b><i>Clostridium</i></b>	<b>Morphology</b>	<b>Spores</b>	<b>Culture features</b>	<b>Colonies/Haemolysis</b>
<i>Hemolyticum</i>	0.5 to 1.5 x 3.0 to 6.0 Motile Mainly singly, Straight sides rounded rods	Oval, subterminal, Bulging, abundant	Very strick anaerobe Slow growth	Lenticular, then woolly Haemolysis zone
<i>Histolyticum</i>	0.5 to 1.0 x 1.5 to 90.0um, Motile, Singly or in short chains, Slightly rods	Oval, subterminal, Slightly bulging	Can grow aerobically no spore formation during aerobic growth	Circular to irregular, flat to low, convex translucent to semi-opaque, grey, Haemolysis zone
<i>Novyi</i>	0.5 to 1.5 x 3.0 to 10.0um Motile, mainly Singly Straight sided, rounded rods	Oval, central or subterminal, Bulging abundant	Very strick anaerobe Slow growth	Haemolysis zone grey-white Haemolysis zone
<i>Perfringes</i>	1.0 to 1.5 x 4.0 to 10.0um Singly or in pairs, encapsulated Thick, straight sides (boxcar shaped)	Oval, subterminal, bulging, rare	Microaero-tolerant	Round, smooth, opaque, glistening ochre Haemolysis zone(s)
<i>Piliforme</i>	0.5 to 8.0 x 10.0 motile, intracellular		Only on cell-containing media	Not relevant
<i>Septicum</i>	0.6 to 1.0 x 3.0 to 8.0um, motile, singly or in pairs, straight or slightly curved In-vivo: long chains and filamentous curved forms (10 – 30um)	Oval, subterminal, Bulging	Strict anaerobe Rapid growth	Swarming, cottony (veil-llike or lawn-like) Haemolysis zone
<i>SordelliiDifficile</i>	0.5 to 1.5 x 1.5 to 10.0um, Motile, Singly or in pairs, straight	Oval, central or subterminal, not or weakly bulging	Strick anaerobe	Round to irregular, translucent to grayish
<i>Spiroforme</i>	0.3 to 0.5 x x 2.0 to 10.0um, non motile, coiling, U-shaped, long rods	Round, terminal (subterminal), slightly bulging	Strick anaerobe	Circular, convex, shiny, semi-opaque, grey-white to grey-brown
<i>Tetani</i>	0.5 to 1.0 x 2.0 to 18.0um, motile, mainly singly, straight, slender	Rolund, terminal bulging, (drumsticks)	Strick anaerobe Slow growth	Circular or rhizoid,flat, translucent, grey (swarming) Narrow haemolysis zone

(Cato *et al* 1988)

## 2.4

## *Clostridia in Health and Disease*

According to Stakebrand and Rainey (1997), the genus *Clostridium* comprises of between 120 and 160 officially or not yet accepted species names. Between 70 and 80% of these species are saprophytic bacteria which are harmless to animals. Nevertheless, up to 25 species represents minor pathogens and 13 species can be regarded as major pathogens.

### 2.4.1 *Non-Pathogenic Clostridia*

Almost more than 100 harmless saprophytic species live in nature, in soil and mud of some countries and continents, in the depth of sediments of lakes and oceans and in sewage sludge. They also live in contact with animals as members of their commensal flora mostly in the digestive track of mammals, bird, reptile, fish, insect etc. and sometimes in more exotic places like snake venom. Several species are also associated with healthy or rotten plants, and several others are regularly isolated from food stuff, meat, fish, vegetables and dairy products of various origins and status (Songer, 1996).

### 2.4.2 *Minor Pathogens*

The species classified as minor pathogens live essentially in nature and in contact with animals just like the saprophytic species do. Some are also associated with plants and several have been isolated from different food stuffs, including meat, fish, vegetable, dairy products and even canned food (Songer, 1996).

***The minor pathogens include:***

*Clostridium absonum, Clostridium argentinense, Clostridium baratti, Clostridium beijerinckii, Clostridium bifermentans, Clostridium butyricum, Clostridium cadavers, Clostridium carnis. Clostridium clostridioforme, Clostridium fallax, Clostridium ghonii, Clostridium glycolicum, Clostridium hastiforme, Clostridium indolis, Clostridium innocuum, Clostridium irregulare, Clostridium limosum, Clostridium malenominatum, Clostridium paraputrificum and Clostridium ramosum*

Being pathogens, they are also isolated from clinical specimens and cause clinical conditions although usually not as severe or not as frequent as those caused by the major pathogens. Many of these clinical conditions are superficial and external: infected wounds, exudations, gangrene, abscess of the skin and subcutaneous tissues (Brandt and Rainey; 1997). The targets can also be the different mucosa. Clostridia isolated from faeces, intestinal content, genital tract and urine are less frequent (Popoff, *et al.*, 1996). The target host species are mammals, especially man and herbivores, more rarely other mammals or birds (Brandt and Rainey; 1997)

### **2.4.3 Major Pathogens**

Of all clostridia species 13 are considered as major pathogens in man and animals (Hathway, 1990):

*Clostridium botulinum* (Group ii and iii), *Clostridium chauvoei*, *Clostridium colinum*, *Clostridium difficile*, *Clostridium haemolyticum*, *Clostridium histolyticum*, *Clostridium novyi*, *Clostridium perfringens*, *Clostridium piliforme*, *Clostridium septicum*, *Clostridium sordelli*, *Clostridium spiroforme*, *Clostridium tetani*.

Most of these species live in nature in contact with animals, mainly in the digestive tract. They most probably also participate in various fermentation and putrefaction processes. A few species like *Clostridium chauvoei*, *Clostridium colinum*, *Clostridium piliforme*, and some strains like toxins types B, C, D, E, of *Clostridium perfringens*, have not been reported to live in nature normally. These thirteen species can be isolated from wide range of clinical specimens, but if we consider one species at a time, it is usually isolated from relatively specific clinical specimens. For instance *Clostridium tetani* is classically isolated from wound, *Clostridium colinum*, *Clostridium deficiile* and *Clostridium spiroforme* are

classically isolated from the intestine, and *Clostridium chauvoei* is classically isolated from necrotic and haemorrhagic myositis (Brandt and Rainey, 1997).

Another important feature of clostridial infections is that pathogenicity is purely dependent on the production of toxin which act locally or generally and are often coded by genes located on extra- chromosomal and/or mobile genetic structures. Here again exceptions so far are described for clostridial like *Clostridium piliforme*, and *Clostridium colinum* (Hathway, 1990).

## **2.5 Natural Habitat**

Member of the genus *Clostridium* are widely distributed in nature and are found in soil as well as in fresh water and marine sediments throughout the world (Kim, *et al.*, 1981). Some species are psychrophilic or haemophilic, but most are mesophilic. *Clostridium* is the only genus of spore - forming anaerobes associated with human, either as non pathogens at a variety of anatomical location or at infected site. Several *Clostridium* species reside in the lower intestinal tract of human and other animals as part of the normal flora. With certain notable exceptions, most species are considered harmless saprophytes. Among the pathogenic species, exogenous and endogenous sources exist (Kim *et al.*, 1981). Strictly toxigenic diseases such as food-borne illness, gas gangrene associated with traumatic wound infections, are associated with infections with *Clostridium botulinum* and *Clostridium perfringens* and tetanus are well known as arising from exogenous source. Endogenous infection involving clostridia that are part of the host's own microflora are much more common. Common predisposing factors include operative procedures, immunosuppressive agents, chemotherapeutic agents etc (Kim *et al.*, 1981, Mulligan *et al.*, 1988). Under the right conditions, clostridia can invade and multiply in essentially any

tissue of the body. The genus *Clostridium* contains many different species. Thirty six are listed in a highly regarded reference and of these eleven (world wide basis) have been shown to be pathogenic for livestock and of economic importance. They are *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium sordelli*, *Clostridium novyi* type A, *Clostridium noryi* type B, *Clostridium novyi* type D, *Clostridium hemolyticum*, *Clostridium perfringens* type A, *Clostridium perfringens* type B, *Clostridium perfringens* type C, *Clostridium perfringens* type D. and *Clostridium tetani* (Smith, 1975). The genus species vary from one another in tolerance to oxygen, in nutritional requirements, and in optimum and limiting temperature. Three species *Clostridium carnis*, *Clostridium histolyticum*, and *Clostridium tertium* are aero-tolerant and will form colonies on freshly prepared blood agar medium incubated aerobically. Other species of the genus possess certain unusual characteristics which distinguish them from another type. Three such principal characteristics are:

1. The ability to multiply only in the absence of oxygen (anaerobic environment).
2. The ability to survive adverse conditions by transforming into highly resistant form through formation of spores.
3. The release of potent toxin during the process of multiplying in their spore form (Smith, 1975).

The clostridium species are able to exist in the presence of oxygen in soil, on body surface and within the healthy animal.

The spores are highly resistant to the forces of nature, drying, temperature change and prolonged separation from energy supplies. Meanwhile, they retain the ability to multiply when favourable conditions occur. In many respects they are similar to seed produced by

plants that remain in the soil without growing until conditions favourable for germination occurs. When suitable anaerobic environment develops in the immediate vicinity of a clostridia spores, it can activate (germinate) and transform into vegetative bacterial cell which then begins to multiply. During this multiplication, potent toxins are released. A number of different toxins are released during multiplication. The actual types of toxin vary by clostridial species. The greatest among the toxin are those that destroy tissue cells (necrotizing) and red blood cells (haemolysing), and those that interrupt nerve impulse (neuron toxins) [Smith, 1975].

## **2.6 Cultivation of Clostridia**

Life probably evolved in an environment which lacked oxygen; however this fact was not appreciated until 1861 when Pasteur declared that some organism could exist without oxygen and seemed to die in its presence. *Clostridium chauvoei* is one of the anaerobes that have been recognized for its fastidious nature, therefore precaution should be observed when cultivating this anaerobe. Anaerobic organisms have been classified into the following groups according to Baker *et al.*, (2001):

1. Obligate anaerobes – Will not grow in the presence of free oxygen.
2. Obligate aerobes – will not grow in the absence of free oxygen
3. Facultative anaerobes – will grow with or without free oxygen
4. Microaerophiles – will only grow in the presence of carbon dioxide.

In the media preparation, method of excluding oxygen from liquid or semi-solid media is quite important and this can be achieved through either of the following procedures:

- 1 Boiling: Boiling drives oxygen out of liquids

- 2 Glucose: If a culture medium such as glucose broth is placed in a long thin tube, the reducing power of the glucose in the medium is sufficient to exclude oxygen for a short time from the depth of the medium.
  - 3 Reducing agent: a number of different reducing agents are used in culture media e.g. Cystein hydrochloride or thioglycolate.
  - 4 Agar: agar (0.2%) is sometimes added to culture media for anaerobic cultivation. The agar cuts down convection currents in the medium, helping to maintain the anaerobic status.
  - 5 Meat granules: meat granules in the medium act as oxygen scavengers, maintaining the anaerobic status in the bottom of the tube.
  - 6 Storage of medium in the dark at room temperature in tightly sealed tubes
- Some media have also been used for isolation and characterization of anaerobic bacteria (Dowell and Hawkins, 1981), these media have been categorized into three namely:
- a) Plating media which include: blood agar, Mc Clung and Toab egg yolk agar, Phenyl-ethyl alcohol blood agar, and Kanamycin- Vancomycin–menadione blood agar.
  - b) Differential media – They include: Thioglycollate medium, fermentation base (thioglycolate medium without dextrose or indicator), glucose, arabinose, glycerol, lactose, maltose, manitol, rhamnose, sucrose, trehalose, xylose, salicin, indole-nitrite, chopped meat- dextrose, chopped meat agar slant for spores iron, milk, hydrogen sulphide (H<sub>2</sub>S), thiogel, urea, lactose – mortality medium, esculin broth, 20% bile broth, peptone –yeast extract medium, infusion agar slant, infusion agar-glucose slants and flagella broth.

- c) Storage media – they include brain storage medium, maintenance medium for actinomyces broth.

Reinforced clostridia medium (RCM) has also been used for cultivation of *Clostridium* spp (Princewill *et al.*, 1985). This medium was designed primarily for the cultivation of *Clostridium butyricum* and a subculture of this organism gave visible growth after 2 – 3 hours incubation and also proved to be suitable medium for many other clostridia (Hirsch and Grinsted, 1954).

*Clostridium* spp is first cultured in (RCM) before sub-culturing into blood agar and later to cooked meat medium. This was aimed at obtaining pure culture from field sample that might have been contaminated. The composition of RCM have been described by Hirsch and Grinsted, (1954) and comprise the following materials: yeast extract (Yeastrel) 0.3%, peptone 1%, meat extract (Lablemco) 1%, glucose 0.5%, sodium acetate 0.5% cystein 0.5%, soluble starch -0.1% and agar 0.05%. Sheep blood agar is also prepared using the following composition as described by Dowell and Hawkins (1981)

- i. Add 0.5 grams of yeast extract to 100 ml tryptose soy agar base.
- ii. Adjust pH to 7.3-7.5
- iii. Autoclave at 121 °C for 15 minutes
- iv. Cool to 48 °C
- v. Add 1 ml sterile vitamin K- helmin solution
- vi. Add 5.0 ml defibrinated sheep blood per 100 ml. Mix and pour plates.

Cooked meat medium has also been prepared as described by Bridson, (1995)

	Gram/litre of phosphate buffer saline
Heart muscle	454.0
Peptone	10.0
Lablemco powder	10.0
Sodium Chloride	5.0
Glucose	2.0
pH	7.2 $\pm$ 0.2

Cooked meat medium (CMM) prepared from heart tissue has been found to be the best medium for the cultivation of anaerobic and aerobic organisms. The medium has the good ability to initiate growth of bacteria from very small inoculate and to maintain the viability of cultures over long periods of time. Mixture of cultures of bacteria survives in cooked meat medium without displacing the slower growing organism. The products of growth do not rapidly destroy the inoculated organism and therefore it is an excellent medium for storage of aerobic and anaerobic bacteria (Dowell and Hawkin, 1981). Glucose added to the formulation allows rapid heavy growth of anaerobic bacteria in short time and leads to a more rapid identification of important anaerobes. For proper utilization, ideally, the specimen should be cultured as soon as possible after collection and every effort should be made to prevent specimen from exposure to free oxygen. Fresh specimen should be plated on freshly prepared media under anaerobic condition. The plating media should be placed in anaerobic jar or in an air tight cabinet containing an oxygen carbon dioxide atmosphere. Cooked meat medium is the best media for culturing clostridia. There are four important considerations in the cultivation of clostridium species (Dowell and Hawkin, 1981) and these are:

1. Proper collection and transport of material to be examined.
2. Culture of the material as soon as possible after collection
3. Use of freshly prepared and properly oxygen reduced media.
4. Proper anaerobic condition

The collection and transport of specimen is of primary importance in recovery of anaerobes. The samples should be collected from the active site of infection of the diseases and strict precaution should be made to exclude surface contamination and coming in contact with free oxygen materials collected on swabs and should never be allowed to dry out. The whole specimen should be placed under anaerobic conditions immediately after collection for transport to the laboratory since the organism after collection will die rapidly in aerobic environment .So many anaerobic systems have been described for the cultivation of anaerobic bacteria (Dowell and Hawkins; 1981). Such systems are anaerobic jar, the Hungate roll-tube or roll-streak tubes and anaerobic glove boxes. Many anaerobic culture jars have been used (Brewer, Torbal, Gaspak etc) (Colle, *et al.*, 1977). These anaerobic jars rely on the same, general principle for removal of oxygen. Each jar uses a catalyst that accelerates the rate of oxygen reduction. The lid of anaerobic jars should be kept clean when not in use to prevent inactivation of catalyst. The catalyst (Palladium-coated aluminium pellets) used with Gaspak system is known to be inactivated (poisoned) by hydrogen sulphide, chlorine and sulphur dioxide gases. Therefore, the pellets must be replaced at frequent intervals (preferably each time the jar is used). Gaspak system probably give more reliable and more reproducible result in a laboratory in which experience with strict anaerobes is limited or sporadic (Colle *et al.*, 1972). Three types are recognized and available (Dowell and Hawkins, 1981). Gaspak anaerobic system (polycarbonate jar),

Gaspak anaerobic system vented (polycarbonate jar) and Gaspak disposable anaerobic system. Each of these is a self contained anaerobic system in which hydrogen and carbon dioxide are supplied from disposable Gaspak hydrogen plus carbon dioxide generator envelope. A room temperature catalyst is used to accelerate reduction of oxygen. A polycarbonate Gaspak system (vented or unvented) is used as follows (Dowell and Hawkin, 1981).

- i) Remove used catalyst from the lid of the jar and replace with an equal quantity of new or rejuvenated pellets.
- ii) Place material to be incubated and the methylene blue indicator in the jar.
- iii) Cut the corner of the gaspak hydrogen + carbon dioxide generator envelope, spread foil to an opening, and place in the jar in an upright position.
- iv) Pipette 10 ml of water through the opening into the envelop. Do not insert pipette.
- v) Quickly place Gaspak in lid on the jar, apply the clamp and secure it until hand tight.

Cortinas *et al* (1994) obtained the anaerobic conditions required for growth of *Clostridium chauvoei* by the addition of 0.75 g L of cysteine chlorohydrate to a clostridial medium described by Guzman *et al.* (1988) and the inoculation of media immediately after they were sterilized and cooled. It was observed that after growth started, no additional measures were necessary. The optimum temperature for the growth of *Clostridium chauvoei* is 37 °C and the duration of culture varies depending on media used and purpose of cultivation.

## 2.7

### **BlackLeg (Blackquarter - BQ)**

Blackleg is an acute, sporadic, enzootic, infectious disease of primarily cattle caused by *Costrdium chauvoei* characterized by inflammation of heavy muscles, severe toxæmia and high mortality (Abdulkadir, 1989). Other synonyms of this disease are blackquarter, emphysematous gangrene, and gangrenous myositis of ruminants (Jubb and Kennedy, 1970). The disease develops in affected animals and often death occurs in per acute cases before the owners notice any sickness in the herd or it can also be acute where some clinical signs are noticed before death (Singn *et al.*, 1993). Stable blackleg or false blackleg caused by *Clostridium septicum* can also mimic blackleg (Jubb and Kennedy, 1970). This syndrome has also been referred to as pseudoblackleg. An attempt had also been made to distinguish gas gangrene and malignant edema from blackleg. Malignant edema will occur wherever animals are kept and it is usually caused by *Clostridium septicum*. It is sporadic and being a wound infection, it is susceptible to surgical and sanitary control. Blackleg on the other hand is not a wound infection and inspite of worldwide distribution and its peculiarity in localization to region and within regions to farms within these locales, it is persistently enzootic, but rather selective of its host, and controllable only by biological means (Jubb and Kennedy, 1970). Among the spore-forming anaerobes that cause diseases of veterinary importance in Nigeria, *Clostridium chauvoei*, the causative agent of blackleg in ruminants has been given more attention. The peak incidence of the disease has been observed during the wet season (Osiyemi 1975, Bagadi, 1977).

#### **2.7.1 *Clostridium chauvoei***

First described by Arloing *et al.* (1887) as *Bacterium chauvoei*, it later received other names like *Bacillus chauvaei*, *Bacillus carbonis* and *Bacillus anthracis symptomatici*.

It was called *Clostridium feseri* by Trevisan in (1885). This name only appears in the 6<sup>th</sup> edition of the Bergey's Manual (1948). It was later named after Chauveau, a French bacteriologist.

### **2.7.2 *Clostridium chauvoei* as causative agent of blackleg**

True blackleg, the clostridial myositis of skeletal muscles is caused by *Clostridium chauvoei*, a Gram-positive spore-forming rod bacterium. The spores are highly resistant to environmental changes and disinfectants and persist in soil for many years. False blackleg may be caused by *Costridium septicunm*, and *Costridium novyi*, but this disease is more accurately classified as malignant edema (Blood and Radostists, 2000). Mixed infections of *Clostridium chauvoei* and *Clostridium septicum* are not uncommon, but the important significance of *Clostridium septicum* as a cause of clostridial myositis in cattle and *Clostridium chauvoei* either alone or with *Clostridium septicum* has been speculated (Blood and Radostists, 2000). This indicates that maximum protection to cattle can be provided only when a multivalent vaccine which contains the antigens of *Clostridium chauvoei*, *Clostridium novyi* and *Clostridium septicum* has been used. When the disease occurs, it is usual for a number of animals to be affected within a space of few days. Blackleg has been known to be enzootic in particular areas, especially when they are subjected to flooding; such an area may vary in size from a group of farms to an individual field. The case fatality rate in blackleg infection approaches 100 % (Blood and Radostits, 2000).

### **2.7.3 *Enviromental and Animal Risk Factors***

A typical blackleg of cattle has a seasonal incidence with most cases occurring in the warm months of the year. The highest incidence of blackleg disease in Nigeria occurs during the

wet season when the temperature is low, depending probably on when calves reach the susceptible age group. Some outbreaks of blackleg in cattle have occurred following excavation of soil which suggests that the disturbances in soil may expose and activate latent spores. Blackleg usually thought to be a disease of cattle and occasionally sheep but outbreaks of the disease have been recorded in deer and in one case in the horse (Osiyemi, 1975). In cattle, the disease is largely confined to young stock between the ages of 8 months and 2 years. In the field the disease appears to occur most frequently in rapidly growing cattle on a high plane of nutrition. Elevation of nutritional status in sheep by increased protein feeding increases their susceptibility to blackleg. In sheep there is no restriction to age group (Osiyemi, 1975).

#### **2.7.4 Toxin Typing**

Toxins can be identified not only in lesions but also in culture supernatants. The historical and classical test to detect clostridial toxins is the mouse lethality test (Cato *et al.*, 1988). The mice are inoculated intra-peritoneally or intravenously and death within 24 hours is recorded. Clostridial toxins killing mice have been called “lethal or major toxins” in the past and those which do not, “minor toxins”, though some of the latter become lethal after concentration. The identity of the toxin is confirmed by neutralisation with specific immune sera. When a clostridium species produces more than one major toxin, combinations of immune sera must be tested. The mouse lethality test is still the reference and most sensitive test for the botulinum neurotoxins (Brack and Madigan, 1988). Other biological assays are based on the activity of the toxins. Examples are the dermonecrosis assay in rabbits or guinea pigs (*Clostridium perfringens* major toxins) and cytotoxicity assays (*Clostridium difficile* A and B toxins are active on various cell lines, which *Clostridium perfringens*

enterotoxin is active on Vero cells). Besides biological assays, clostridial toxins can be detected by immunological methods. ELISA assays are commercially available for the detection of *Clostridium difficile* A and B toxins and *Clostridium perfringens* major toxins and enterotoxin. ELISA assays for other clostridial toxins have also been developed (for botulinum toxins), but are not yet commercially available. Several other immunological assays have been used. The most reliable one from a diagnostic point is the reverse latex agglutination assay for the *Clostridium perfringens* enterotoxin. Conversely, the latex agglutination assay for *Clostridium difficile* toxins is neither sensitive, nor specific (Cato *et al.*, 1986).

Clostridial toxins can also be detected by *in vitro* assays based on their biological activity or affinity for a specific substrate (Cato *et al.*, 1986). Two examples are the *Clostridium perfringens*  $\alpha$  toxin and its phospholipase C activity on phosphatidylcholine. As for any bacterial species, *in vitro* toxin production can be poor. An alternative is therefore to genetically type the clostridia isolated either by DNA colony hybridisation using specific gene probes or by PCR using specific primers (Cato *et al.*, 1986). When different toxin variants have been described, the primers can be toxin or variant specific. Toxins typing positive results of genetic assays do not however imply that the toxin-encoding genes are actually expressed *in vitro* or *in vivo* i.e. *botulinum* toxin type A isolates can harbour silent copy of toxin type B-encoding genes.

### **2.7.5 Pathogenesis of clostridial Infection**

The pathogenesis of most clostridial infections and disease follow the same cascade of events, for all the bacterial or the host species. These involve contamination or colonisation of site by spore or living bacterial cells, occurrence of anaerobiosis and low oxidation-

reduction potential allowing spore germination and bacterial multiplication. There is toxin production, toxin absorption, toxin distribution, and toxin activity, either locally or systemically (Hathway, 1990). The problem of the pathogenesis of blackleg has exercised the imagination of many researchers, nevertheless, toxin and neuraminidase produced by *Clostridium chauvoei* are believed to play a significant role in the pathogenesis of the disease (Useh *et al.*, 2003).

#### **2.7.5.1 The Role of toxin in *Clostridium chauvoei* infection**

The production of lethal toxin by *Clostridium chauvoei* was first demonstrated by Duenschmann (1894) and it had since been known that the organism produces hyaluronidase (gamma toxin), deoxyribonuclease (beta-toxin) and oxygen-labile hemolysin (Moussa, 1958). The role of *Clostridium chauvoei* toxin in the pathogenesis and pathology of blackleg infection has been investigated after experimentally infecting guinea pigs (Singn *et al.*, 1992) and hill bull (Singn *et a.*, 1993). It has been reported that toxin plays a significant role in the pathogenic mechanism of blackleg infection. Microscopically, the muscles of the infected animals became hyalised with mild necrosis and neutrophilic infiltration of the affected muscles. The blood vessels of the toxin-injected hill bulls degenerated via alteration of vascular endothelium (Singn *et al.*, 1992). The popliteal lymph nodes of the bulls showed pinkish homogenous material in the centre of the lymphoid follicles, with necrosis and infiltration by neutrophilic leucocytes. There was hyperplasia of the Malphigian corpuscle in the spleen and this was attributed to the initiation of humoral immune response by the toxin. The congestion, haemorrhage and digestive foci in the liver and kidney were also attributed to toxin. There was noticeable decrease in leucocytes and thrombocytes when whole bacteria were injected in the hill bulls, 1 to 3 days post infection.

It was argued that the leucopenia and thrombocytopenia observed was as a result of destruction of leucocytes and platelets in the peripheral blood by the toxin produced by the bacteria *in vivo* (Singn *et al.*, 1993).

#### **2.7.5.2 The role of neuraminidase in *Clostridium chauvoei* infection**

This enzyme has been detected in a variety of microorganisms, such as virus, bacteria and protozoa (Oladele *et al.*, 2002). *Clostridium chauvoei* (Jakari strain) which causes blackleg infection in indigenous Nigerian cattle is known to produce neuraminidase (Useh *et al.*, 2003). The role of pH and temperature in the pathogenesis of blackleg infection in relation to neuraminidase activity has been investigated *in vitro* and found that the optimum pH for *Clostridium chauvoei* (Jakari strain) neuraminidase activity was 4.5. Neuraminidase therefore plays a significant role in the pathogenesis of infectious diseases, whose aetiological agents produces the enzyme cleaving sialic acid in infected tissues to facilitate the spread of the disease in the tissues of infected animals (Useh, 2001).

#### **2.7.6 Disease Production**

In living animals, anaerobic conditions are established when the normal blood supply to a particular tissue site is interrupted or when change occurs in the digestive process of the intestinal tract (Osiyemi, 1975). The interruption of blood supply occurs as a result of tissue damage, the origin of which varies considerably if a Clostridia spore is present in the damaged tissue. When present, clostridium species germinates and transforms into the vegetative form. Multiplication and toxin production follows. As the process advances, and more toxins are released, these are then carried by blood stream to the other parts of the body resulting in wide spread destruction of cells, disruption of organ functions and rapid death of the animal (Osiyemi, 1975).

### ***2.7.7 Clinical Signs of Blackleg***

The incubation period of blackleg could be 2-5 days in cattle (Abdukadir, 1989). In many clinical cases no signs are observed, because the affected animal is found dead. The acute form of the disease is followed by death after short duration of onset. Often, when infrequently inspected, animals may be found dead without signs or illness of disease. Infected animals usually presents high fever of up to 45 °C and depression. When limbs are affected the characteristic blackleg lesion can be seen in striated muscles in the site of infection. The swelling of the limbs in the area of lesion results from edema which initially is hot and painful, but latter becomes cold and painless. Palpation reveals crepitus and emphysematous state due to gas bubble formation that accompanies multiplication of the bacterium. Skin is discolored, becomes dry and cracks, complete anoxia and ruminal stasis are all noticed on closer examination of infected animal (Abdukadir, 1989). Sudden death may also occur in cases of visceral blackleg in sheep and cattle when the heart is affected. Jubb and Kenned (1970) reported that blackleg in sheep presents as declination to move due to severe lameness in one, or more commonly in several limbs.

The lameness may be severe enough to prevent walking in some animals, but may be moderate in others (Radostists *et al.*, 2000). In sheep, subcutaneous edema is not common and gaseous crepitation can not be felt before death. Discolorations of skin may be evident, but skin necrosis and gangrene do not occur (Radostists *et al.*, 2000). Neurological signs of the disease have not been noticed (Melone *et al.*, 1986). *Clostridium chauvoei* has been isolated from meninges of cattle that displayed neurological signs. Blackleg is not a disease of horses, but there has been a report of pectoral edema, stiff gait and incoordination in a horse infected with the disease (Hagemoser, 1980).

### **2.7.8 Gross Pathological Findings**

Bloating and putrefaction occurs quickly and blood-stained froth exudes from the nostrils and anus. Clotting of blood occurs rapidly (Blood and Rodostits, 2000) Incision of the affected muscle mass reveals dark red to black, swollen tissue with a rancid odor and thin sanguineous fluid containing bubbles of gas. A circumscribed swelling may be visible on superficial inspection and creptitation detectable on palpation. The overlying skin is taut and resonant but otherwise it is usually normal, dark-coloured with patchments (Blood and Radosstis, 2000).

The affected muscles present slightly different appearance at different distance from the primary focus. Towards the periphery of the lesion, the muscle is dark-red in color and slightly moist with edema fluid. Towards the center it is dark, occasionally, with grey or patchy colored islands, and the tissue is dry, friable and porous as gas bubbles separate the small bundles of the fibers. If the tissue is squeezed, it crepitates and small amount of thin fluid oozes out. When the tissue is left exposed to air it takes on a light-red color and pools of the exudates seep and drips from the affected portions of the carcass. The odor of the tissue resembles that of rancid butter (Jubb *et al.* 1985). As indicated by Jubb and Kennedy (1970), if there is fibrinohemorrhagic and pleuritis without pneumonia, blackleg should be suspected. The parietal pleura are hemorrhagic and blood- stained cloth of fibrin overlie the ventral mediastanum and epicardium. Pneumonia is not part of the intrathoracic lesion, but the lungs are congested with some interstitial edema and hemorrhage. The myocardium may be pale and friable or dark-red, in some cases there are foci of emphysematous myositis and necrosis, and in these there is no overlying fibrin hemorrhagic pericarditis. It appears that the younger the animal, the greater is the likelihood of endocarditis. The endocarditic

lesions are hemorrhagic, ulcerative and thrombotic and they affect the right heart, the arterial endocardium, the atrioventricular valves, and the outer wall of the right ventricle. The spleen may be normal or enlarged with congested mushy pulp. When there is terminal invasion of the blood stream, pale round foci may be found in the liver and renal cortex without reaction (Jubb and Kennedy, 1970).

As reported by Blood and Radostits (2000), cattle that die of blackleg are often seen in characteristic position lying on the side with the affected hind limb stuck out stiffly. The solid organs show some degree of degeneration and post mortem decomposition with the production of gas in the liver occurring rapidly. William, (1977) reported the following gross lesions of blackleg in cattle namely: dryness, blackened muscles, and crepitations with spongy appearance and rancid odors. The gross pathologic picture of sheep that suffered from blackleg is similar to the findings in cattle, except that in sheep the muscle lesions are localized and deeper, and subcutaneous edema is not so marked, although it may be marked around the head (Radostis *et al.*, 2000).

When the disease has resulted from infection of skin wounds as found in sheep shearing, the lesions are more obvious superficially, with subcutaneous edema, swelling and involvement of underlying musculature. Lesions have also been reported in guinea pigs experimentally infected with clostridium at 6 hours post infection (Singn *et al.*, 1992) these are swelling and edematous subcutaneous tissues and moist muscles with considerable amount of serous hemorrhagic fluid in the intramuscular space. By 12 h post infection, post mortem lesions were rough hair coat which sloughed off by its self and the subcutaneous edema and emphysema had extended to the abdomen and shoulder area. By 24 h, the muscular lesions had become markedly pronounced and blackish-grey and discolorations had become

intensified. In the center, the muscles were necrosed, blackish and less moist. Except for the liver which becomes soft with few pale areas and the lungs with patchy congestion, the internal organs remained apparently normal. The small intestine is congested and has yellow contents in dead guinea-pigs. There was no crepitation when clostridium toxin alone was injected into hill bulls (Singn *et al.*, 1993).

### **2.7.9 Histopathological Findings**

The affected musculatures of animals usually have extensive necrosis and gangrenous changes with fibrinohaemorrhagic exudates and infiltrated leucocytes, sparsely in the centre and more profusely in adjoining region (Singn, *et al.*, 1993). The peri and endomyocardial connective tissues are thickened due to proliferation edema and fibrinous exudation containing predominantly neutrophils. At the same places hemorrhages are noticed between the hyalinized muscle bundles. In the sacrificed animals the lesions are similar but milder. In the absence of living organism in the toxin injected hill bulls, the muscles are mostly hyalinized. Necrosis is mild and the gangrenous changes may be absent. The neutrophilic reaction was also less intense. These findings emphasize the roles of both bacteria and toxin in the pathogenesis of blackleg (Singn *et al.*, 1993).

For the toxin- injected hill bulls, the blood vessel wall are often degenerated, necrosed and infiltrated with few neutrophills and even thrombosed, indicating the action of toxin on vascular endothelium. In the popliteal lymph node, pinkish homogenous material is seen in centers of few lymphoid follicles. In few cases, few lymphoid follicles are necrosed and infiltrated with neutrophils and leucocytes. At some places, large gas bubbles are usually observed in necrotic follicles. Large extravasted blood mass is observed in subendocardial region, splitting the underlying myocardial bundles. In some few cases hemorrhages are

also observed along the muscle fibers deep in the myocardium. Hyperplasia of the Malpighian corpuscles is seen in the spleen perhaps due to initiation of humoral immune responses which is not uncommon following bacterial toxin/antigen inoculation (Singh, *et al.*, 1993).

#### **2.7.10 Epidemiology of Blackleg**

Ruminants contract clostridial infection from grazing grounds and this is widely recognised in veterinary practice. Farmers are quite familiar with endemic areas and carefully avoid taking their herds to such places for grazing (Roberts and McEwen, 1931). Except where there is clear history of wound infection, it is not certain whether the animals pick up the infection by ingestion with herbage or with soil in the course of grazing. The quick disposal of rapidly putrefied infected carcasses has been blamed for the difficulty in isolating *Clostridium* species from pathological specimens in Nigeria (Princewill and Agba, 1982). Results of bacteriological examination on 140 specimens revealed that 30 of them yielded *Clostridium chauvoei*, the definitive cause of bovine blackleg disease in Nigeria. Other clostridia causing other diseases like gas gangrene, malignant edema and tetanus have also been isolated in various percentages (Osiyemi, 1975). Blackleg is a dreaded disease because of its epizootic nature and the economic loss from death and sickness of affected cattle. Blackleg occurs on all continents, although there are some local areas in various countries which are not affected. Blackleg is one of the five reportable diseases in cattle, buffaloes caused by *Clostridium chauvoei*, and is a non contagious soil-borne disease. The pathogenesis is obscure, and no evidence of lateral spread as in the case of hemorrhagic septicemia (Ramaroa and Rao, 1990).

### 2.7.11 *Diagnosis of Blackleg*

Livestock owners should familiarize themselves with the signs of this dangerous and costly disease, so that cases are not ignored or passed off as bloat. It is often difficult to make an exact diagnosis in the field because of the similarity of signs of blackleg to certain other diseases. Out of 144 specimens obtained from clinical blackleg disease in Nigerian cattle, *Clostridium welchii* accounted for 61%, *Clostridium chauvoei* for 21.5% (Osiyemi, 1975). As a result of the possibility of mixed infections by clostridium species in blackleg in Nigeria, Osiyemi (1975) advocated the production of multivalent vaccine that contains *Clostridium chauvoei*, *Clostridium perfringes*, and *Clostridium septicum* antigens.

In an attempt to develop a more sensitive and reliable diagnostic technique for blackleg, Kuhnert *et al.* (1996) studied the phylogenic positions of *Costridium chauvoei* and *Costridium septicum* based on their 16s rRNA (rrs) gene sequence using polymerase chain reaction (PCR). A sequence similarity analysis of the genes of both bacteria revealed a close phylogenetic relationship of *Costridium chauvoei* and *Costridium septicum*. This identity could be responsible for the difficulty in distinguishing these bacterial species either on culture or in biochemical tests. Cato *et al.*, (1986) showed that one quick distinguishing colonial morphology between these organisms is that *Costridium septicum* may swarm on culture, while *Clostridium chauvoei* does not. Kuhnert *et al.* (1997) identified *Costridium chauvoei* in cultures and clinical material from blackleg using polymerase chain reactions using specific oligonucleotide primers based on the 16s rRNA (rrs) gene sequences of the bacterium.

In establishing a diagnosis when a number of animals are found dead in a group not kept under close observation, one must depend on the knowledge of local disease incidence,

season of the year, the age group affected, pasture conditions, and close inspection of the environment in which the animals have been maintained Radostits, (2000).

#### **2.7.12 Differential Diagnosis of Blackleg**

According to Blood and Radostits (2000), diagnosis of blackleg may be difficult because of failure to find extensive lesions. Such cases may be confused with other causes of sudden unexpected death. Disease condition like malignant edema, anthrax, lightning strike and bacillary hemoglobinuria should be distinguished from blackleg. Lactation tetanii and acute lead poisoning may also cause sudden death in a number of cattle but typical lesions of blackleg are not present. Definite isolation of *Clostridium chauvoei* from a fresh cadaver or sick animals available for examination will establish a definite diagnosis..

#### **2.7.13 Management of Blackleg**

Management of already affected animals with penicillin is logical if the animal is not moribund, but results are generally only fair because of the extensive nature of the lesions (Blood and Radostits, 2000). Large doses (10,000 units/kg body weight of penicillin) could be administered, commencing with crystalline penicillin intravenously and followed by long acting preparations, some of which should be given into the affected tissues if they are accessible. Blackleg antiserum is unlikely to be of much value in treatment, unless very large dose is given (Blood and Radostits, 2000).

In a recovered case, the animal may be stiff in leg, shoulder etc, due to shrinking or thickening of the muscles (Blood and Radostits, 2000). Some extracts, of medicinal plants, *Tamarindus indicus* and *Combretum fragrans* at 100 -1000 µg/ml could significantly reduce

the activity of neuraminidase, there by reducing the severity of blackleg infection in the affected animal (Useh *et al.*, 2004).

#### **2.7.14      *Antibiotic Resistance Mechanisms in Clostridia***

As with most anaerobes, clostridia are constitutively resistant to aminoglycosides, aztreonam and tigemonam. Antibiotic resistance mechanisms of *Clostridium perfringens* and *Clostridium difficile* are relatively well documented (more than 600 references in 20 years) in opposition to the other clostridia species (from 6 to 60 scientific papers) (Dubreuil and Neut, 2004).

The susceptibility data of *clostridia*, like any other bacterial group, can vary locally, from region to region, and nationally, because, among other factors of the specific epidemiology of the resistance mechanism genetic determinants play the major role. Every Medical and Veterinary Institutions should generate local epidemiological data to follow the changing patterns of resistance, in relation to the common usage of antibiotics. One example is the evolution pattern of clindamycin resistance. An increase in the minimum inhibitory concentration (MIC) values and of the numbers of *Clostridium difficile*- resistant isolates have been observed during multicentric surveys among strains isolated for over five years from hospitalised patients, although the therapeutic use of clindamycin had significantly decreased. A cross-resistance with other macrolides that are widely used outside the hospitals has been postulated (Menozzi, and Mastrantonio, 2004). These data are comparable to others recently published, but different from data published more than ten years ago.

Antibiotic treatment of *clostridia*-associated diseases is not frequent in animals. If decided,  $\beta$ -lactams, macrolides, tetracyclines are the drugs of choice, taking into account the existence of resistant strains. Chloramphenicol (florfenicol may be an alternative) and metronidazole are not allowed in farm animals anymore. Treatment with ampicillin is often associated with clavulanic acid and fluroquinolone (Hirsh, 2000).As in aerobic species, quinolone resistance in anaerobic bacteria is mainly attributed to mutations in hot-spot positions of the *gyrA* gene and to active efflux of the drug. To what extent other mechanisms, such as mutational events in other target genes or alterations in outer-membrane proteins, contribute to resistance among anaerobes needs to be further investigated.

## 2.8

### *Clostridial Toxins*

The role of the alpha- toxin in disease appears to be in two-fold (Rosseto, *et al.*, 2001). The toxin is produced at the focus of infection and diffuses into surrounding tissues. Here it causes the up-regulation of cell adherence markers in endothelial cells lining blood vessels. The consequence is mistrafficking of neutrophils: They accumulate within the blood vessels rather than becoming trafficked to the site of infection. Secondly the toxin causes extensive host cell damage by activating the arrachidonic acid cascade (with the resultant production of thromboxanes) and by the activation of protein kinase C that results in muscle contraction. Together these changes might promote vasoconstriction enhancing the anoxic conditions required for bacterial growth.

The major and minor pathogenic clostridia that cause disease are almost exclusive due to production of wide range of the toxins including enterotoxin, neurotoxin, histotoxin, and

lethal toxins (Herrerros *et al.*, 1999). This classification is based on the main clinical signs and tissue lesions. The origin of the word ‘toxin’ (French), that was use for the first time by Roux (1888) in their publication on production of soluble toxics factor present in culture supernatants of *Corynebacterium diphteria*, is uncertain, but is most probably a Greek word *toxique, poison*, (Rossetto *et al.*, 2001). Clostridia toxins numbered not only several among most potent of toxins of microbial world, but also several among the first identified and recognised as virulence factors of bacterial toxins. For instance *Clostridium tetani* was first observed in smears from a wound in 1884 by Kitasato (Herrerros *et al.*, 1999). One year later tetanus toxin was discovered in culture supernatant of *Clostridium tetani*. The toxinogenic species listed below and their toxins were successfully identified over a period of about 100 years, (Rossetto *et al.*, 2001 Hathway, 1990, Songer, 1996, Popoff, *et al.*, 1996).

Species	No. of toxins
<i>Clostridium septicum</i> (1877)	4
<i>Clostridium chauvoei</i> (1887)	4
<i>Clostridium tetani</i> (1889)	2
<i>Clostridium perfringens</i> (1892)	14
<i>Clostridium novyi / oedematiens</i> (1894)	8
<i>Clostridium botulinum</i> (1895)	3
<i>Clostridium histoluticum</i> (1916)	5
<i>Clostridium bifermentans</i> (1919)	3
<i>Clostridium sordelli</i> (1922)	4
<i>Clostridium hemolyticum</i> (1929)	3
<i>Clostridium difficile</i> (1935)	3

<i>Clostridium spiroforme</i> (1965)	2
<i>Clostridium butyricum</i> (1977)	1
<i>Clostridium baratii</i> (1985)	2
<i>Clostridium argentinense</i> (1985)	<u>1</u>
<b>Total</b>	<b><u>59</u></b>

Bacterial toxins have been defined as “soluble substances that alter the normal metabolism of host cells with deleterious effects on the host (Hathway, 1990).

*Clostridium perfringens* alpha-toxin is the first bacterial toxin for which a biochemical mode of action was described at the molecular level (Tibball, 1999). Progress in the knowledge and understanding of the mode of action, structure and genetic determinism of several clostridia toxin has been tremendous at the end of the 20<sup>th</sup> century, but many of them are still poorly characterized. It has been evident from the report of Sign *et al.* (1992) that toxin was equally responsible for the ability of the bacteria to cause degeneration and necrosis of skeletal muscles, and to a lesser extent in the supporting connective tissue and the vascular endothelium. The organism produced not only toxin in the tissue, but in addition generated gas which also gives rise to air bubble formation rendering muscles porous (Sign, *et al.*, 1992). It also has become evident that calcium chloride produced local necrosis which provided suitable environment for the germination and proliferation of the organism, thus it enhances the pathogenesis of the organism (Caruthers and Stubbs, 1987). Toxins are the virulence factors of clostridia; human being has been able throughout the year to counter the deleterious effects of the bacteria and utilize them to their advantage. The most remarkable utilization is certainly the development of vaccine against several clostridial diseases in man and animals. Also in some cases, man has used the toxin in biological warfare.



<i>Novyilysin</i>	<i>Clostridium novyi</i>
<i>Perfringolysin</i>	<i>Clostridium perfringens</i>
<i>Septicolysin</i>	<i>Clostridium septicum</i>
<i>Sordellilysin</i>	<i>Clostridium sordelli</i>
<i>Tetanolysin</i>	<i>Clostridium tetani</i>

***Aerolysin*- like pore- forming toxin**

Alpha toxin	<i>Clostridium septicum</i>
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**Hiar-like pore forming toxin**

Beta toxin	<i>Clostridium perfringens</i> B and C
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Source: (Bryant *et al.*, 1993)

After binding of Q–toxin to membrane cholesterol 40 -50 molecules of Q – toxin oligomerise within target cell membrane (RossJohn *et al.*, 1997) forming large pores with diameter up to 150A which result in cell lysis. It seems likely that the other clostridia thiol-activated toxins have structures and mode of action which are related to those of Q –toxin. The role of Q – toxin in disease caused by *Clostridium perfringens* is not fully clarified. The toxin has been reported to enhance vascular permeability (Stevens and Bryant, 1993) and cause degranulation and priming (Bryant *et al.*, 1993).

**2.8.4 *Aerolysin-like Pore-forming Toxins***

The *Clostridium septicum* alpha toxin is a polypeptide with a molecular weight of 40,450 and show significant sequence homology over its length (27% identity, 72% similarity) with aerolysin from *Aeromonas hydrophila* (Ballard *et al.*, 1995). The toxin is formed as a protoxin that is activated by proteolytic cleavage of the *Clostridium terininus* (Ballard *et al.*, 1995). The hemolytic activity of *Clostridium septicum* alpha toxin is approximately 100-

fold lower than that of aerolysin (Diep *et al.*, 2002). Also like aerolysin, the toxin is known to bind to GPI (glycosylphosphatidylinositol) anchored proteins (overlapping subset) with aerolysin receptor (Hong *et al.*, 2002). The toxin is able to form oligomeric pores in target cell membrane with an estimated pore size of 1.6 nm (Ballard *et al.*, 1995).

### **2.8.5 Haemolysin $\alpha$ -like Pore-forming Toxins**

The beta-toxin produced by types B and C strains of *Clostridium perfringens* is non-hemolytic and has a predicted molecular weight of 34,861 DG (Hunter *et al.*, 1993). The mode of action is still not fully understood. The deduced amino acid sequence of the toxin shows significant sequences of the toxins homology with the *Staphylococcus aureus* alpha toxin (Hunter *et al.*, 1993). Since *Staphylococcus aureus* alpha toxin is known to oligomerise and form pores in the target cell membrane (Menestrina *et al.*, 2001) it has been suggested that the beta toxin might also be a pore- toxin (Hunter *et al.*, 1993). In support of this suggestion, beta-toxin has been shown to form cation- selective channels in lipid bilayers (Shatursky *et al.*, 2000). Beta toxin plays major roles in the pathogenesis of necrotic enteritis in domesticated livestock such as calves, lambs and piglets and in humans (Leary and Titball, 1997). Differential centrifugation and cesium chloride-equilibrium centrifugation were used to purify the flagella from the strain Okinawa of the formalin-fixed *Clostridium chauvoei*. SDS PAGE profile of purified flagella showed that a major protein band with a molecular mass of 46 KDa, corresponding to the flagellin monomer, and at least two minor protein bands with molecular masses of approximately 73 and 100 KDa were found. The amino acid composition of *Clostridium chauvoei* flagellin was similar to the flagellin of *Salmonella typhimurium* and *Bacillus subtilis*. In addition, *Clostridium chauvoei* flagellin monomer shared limited sequence homology with the N-terminal amino acid

sequence reported for other bacterial flagellins. N-terminal sequence of two minor bands corresponding to the flagellin monomer, indicating that higher molecular mass bands were polymeric forms of the flagellin monomer (Kojima *et al.*, 1999).

### **2.8.6 Effect of Heat on Hemolytic Activity of *Clostridium chauvoei* toxin**

Studies on sample of haemolysin given heat treatment at different temperatures and for different periods of time revealed that haemolysin are not easily destroyed by heat. Haemolysin could be stable at 4 °C and 70 °C even for a period of 30 minute. At 100 °C (boiling water bath), the hemolytic activity is unaffected up to 5 minutes, but completely lost at 100 °C for 10 minutes (Jain *et al.*, 1990).

## **2.9 Human Use of Clostridial Toxin**

The concept of using microbial toxin for treatments of some physiological disorders in human arose progressively as a result of many research works and collaboration between physicians, physiologist, pharmacologist, and toxicologist. So far, the most successful story concerns the use of the *botulinum* A toxin produced by several strains of *Clostridium botulinum*, *Clostridium butyricum* and *Clostridium baratii*, in the treatment of permanent muscle contraction (Tsui, 1996., Johnson, 1999., Quiun and Mintor 2001). The specific site of action of the *botulinum* toxin at the neuromuscular junction was demonstrated in 1940s and the concept of its therapeutic use arose progressively during 1950s (Scott *et al.*, 1973). It was however not before ten years later that the first trials were in humans in the U.S.A. after receiving approval from the National Institute of Health. During the late 1970s, the *Clostridium botulinum* A toxin was successfully utilized for correction of strabismus (Scott, 1981). In 1979, the U.S Food and Drug Administration approved the production of toxin

batch for local treatment of strabismus, blepharospasm (Scott *et al.*, 1985) and hemifacial spasm (Mauriello *et al.*, 1987).

Early scientist noted that the botulinum A toxin have

1. Showed no known focal or symptomatic effect apart from muscle paralysis.
2. Did not apparently elicit immune response at the doses used.
3. Diffuse slowly out of the injected muscles into adjacent muscles.
4. Acted for several weeks to months
5. Showed a dose – effect relationship (Quinn and Minton, 2001)

However, some problem has arisen such as antibody production and diffusion into other muscles. Another problem is the need for repeated injection in the case of chronic disorders as this treatment is only systematic and not etiological (Quinn and Minton, 2001).

## **2.10 Sporulation and germination**

### **2.10.1 Sporulation**

Spore-forming bacteria initiate sporulation under conditions of nutrient limitation and cell crowding, reacting to the disappearance of key nutrients that are necessary for growth, and probably often also to the rise in concentration of species-specific signal compounds produced during growth ('quorum sensing'; Sonenshein, 2000). Intracellularly, a fall in GTP levels and a rise in ppGpp, or both, are probable common triggers (Lopez *et al.*, 1981). The sequence of events following the cessation of multiplication has been much studied, and the genetic control of seven distinct stages of the process, thought to be common to all spore-formers, has been described in detail (Ellner, 1956). Sporulation genes are switched on in a temporal cascade, primarily regulated by successive replacements of RNA polymerase sigma factors, leading to the formation of the unique structures and components

that are specific to spores. The resultant dormant and resistant spore is protected from environmental insult by multiple disulphide-rich protein coats and a wide underlying peptidoglycan cortex, which in turn surrounds the relatively dehydrated cytoplasm in the central protoplast. The condensed cytoplasm contains spore-specific components, most importantly calcium dipicolinate and small acid soluble DNA-binding proteins (Lopez, *et al* 1981). Many *Bacillus* species sporulate readily in complex or defined media following depletion of a limiting nutrient, commonly glucose or some other carbon and energy source. In contrast, clostridia commonly require complex sporulation media and the presence of a slowly fermentable carbon source at the end of the log phase of growth, to allow continuation of energy supply during the early stages of the sporulation process. Furthermore, even within a single species, there is often a problem that different strains vary widely in the ease with which they can be induced to sporulate. As a consequence, there is some bias in the selection of strains for study, with 'difficult sporulators' being neglected. The difficulties are illustrated by the plethora of media that have been developed to encourage optimal clostridial sporulation. For example, for *Clostridium perfringens*, while the older Ellner's medium (Ellner, 1956) and DS medium (Duncan and Strong, 1968) perform well, and the latter is still widely used, many attempted improvements have been reported during the intervening years. More recently, an alternative bile, peptone, theophylline medium, methylxanthines such as theophylline were originally shown to stimulate sporulation by modifications of the original DS medium, such as substitution of starch by raffinose or raising the medium pH from 7.2 to 7.8 which led to improved spore yields from some, but not all strains. Since a key activity of food poisoning strains of *Clostridium perfringens* is their sporulation in the gastrointestinal tract, attempts to mimic exposure to stomach contents and to conditions in the upper intestinal tract have been made.

Exposure of cells to low pH (Wrigley *et al.*, 1995) and the addition of bile salts have been evaluated, and shown to increase sporulation of some strains (Heredia *et al.*, 1991). Furthermore, culture filtrates have been shown to contain substances ('sporulation factors') that, when added to fresh media, encouraged spore formation from new inocula (Tseng and Labbe, 2000). A comprehensive evaluation of most of these factors was made using more than 20 outbreaks and culture collection strains and performing a variety of modifications of the original medium (Duncan and Strong, 1968). Their results emphasised the variability of strain responses. For example, sporulation of some strains was enhanced by bile salts, while that of others was inhibited; and addition of theophylline improved sporulation of some strains, but not of others; replacement of starch by raffinose likewise had a strain-specific effect; and exposure to low pH and the addition of culture supernatants gave disappointing results. While media with added bile salts and theophylline to DS medium gave the best overall results, no single modification stimulated sporulation of all the strains.

### **2.10.2 Germination**

Bacterial endospores germinate naturally when exposed to specific biochemical 'germinants'. Those that have been most studied have been shown to interact with specific receptors in the inner membrane and to initiate a sequence of changes resulting in the activation of enzymes that hydrolyse peptidoglycan in the spore cortex (Johnstone, 1994). Hydration of the enclosed protoplast results, with leakage of spore-specific components such as calcium and dipicolinic acid, and fragments of spore-specific DNA-binding proteins, accompanying loss of the spore's characteristic resistance and dormancy, and the onset of metabolism. The majority of *Bacillus* spores that have been studied respond to single germinants or simple combinations of them. These most often include an amino acid,

of which L-alanine is the most common. Ribosides such as inosine and adenosine and, less effectively, guanosine and xanthine, alone or synergistically with an amino acid, are often germinative (e.g. for *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus megaterium*).

Other species do not respond to ribosides. Requirements for sugars and cations as components of germinant mixtures are common, e.g. glucose, fructose and potassium with L-alanine or L-asparagine, in various combinations for *Bacillus subtilis* spore. Likewise, clostridial spores are generally unresponsive to ribosides, but more often respond to amino acids, sometimes with simple requirements. Some of the earliest studies showed that spores germinated in L-alanine alone, and were competitively inhibited by D-alanine, and also responded to L-cysteine, with a pattern of responses that closely resembled that of many *Bacillus* species (Uehara and Frank, 1965). Adding L-arginine and L-phenylalanine, which are not generally implicated in germination of *Bacillus* spores, are necessary for maximal germination (Gould, 1969; Waites and Wyatt, 1974). Clostridial spores are often responsive to lactate and to bicarbonate, usually as adjuncts, accelerating germination in the presence of sub-optimal concentrations of L-alanine (Holland *et al.*, 1970). Spores of proteolytic *Clostridium botulinum* were shown to be germinable by L-alanine alone or, less effectively, by L-valine, L-glycine, and L-serine, and the rate of germination was accelerated by additional lactate or bicarbonate (Alberto *et al.*, 2003). Spores of nonproteolytic *Clostridium botulinum* were germinated optimally by combinations of L-alanine and L-lactate or, less effectively, by L-cysteine plus L-lactate, or L-serine plus L-lactate, in bicarbonate-containing buffer (Plowman and Peck, 2002). As has been found for most clostridial spores, the initial stage of germination can occur aerobically or anaerobically and usually, slowly, at temperatures outside of the normal growth range (e.g. non-proteolytic *Clostridium*

*botulinum*, (Plowman and Peck, 2002). Triggering of spore germination has been used successfully to reduce spore levels in heat-sensitive media, e.g. by ‘Tyndallisation’, short periods of heating to activate the spores, with intervening periods of incubation to allow germination to proceed. However, the heterogeneous distribution of dormancy in spore populations (Gould, 1970) has prevented this procedure from becoming sufficiently effective for confident use in foods. Likewise, addition of germinants to foods in order to encourage germination prior to heating has not been found to be useful. However, interestingly, (Braconnier *et al.*, 2003) found that spores of *Clostridium botulinum* germinated more rapidly in a number of vegetable purees if L-cysteine, L-alanine and lactate germinant mixtures were added.

In addition to the ‘natural’ germinants, spores may be caused to germinate by means that probably seldom occur in nature, but some of these may be important in food preservation and safety. As with those of *Bacillus* sp., spores of clostridia have been shown to be germinable by calcium dipicolinate, which has recently been shown to probably act by activating one of the two spore cortex-lytic enzymes formed in *Bacillus subtilis* (Ragkousi *et al.*, 2003).

Some surfactants, most effectively n-dodecylamine, will cause germination of spores, in the absence of any nutrient germinant (Rode and Foster, 1961). The effect has been shown by (Setlow *et al.*, 2003) to involve the alkyl amine creating or opening channels or pores in the spores’ inner membrane, allowing dipicolinic acid to exit. Germination caused by calcium dipicolinate and by long chain alkyl amines is of no significance in foods but, for the surfactants in particular, may have some potential in enhancing the effectiveness of anti-spore disinfectant formulations. High hydrostatic pressure, as detailed above, will cause



Because it is practically impossible to prevent animals from coming into contact with the disease, the chief control method for blackleg lies in building up resistance in animals by the use of a bacterin vaccine. In Nigeria, blackleg which is a disease of cattle, sheep, and other ruminants was first reported in 1929, and remains the major problem of cattle in the country (Osiyemi, 1975). Clostridial diseases have been of concern to cattle producers for many years. Many clostridial vaccines require revaccination 4 to 6 weeks following the initial treatment (Compendium of Beef Products, 1993), but many cow – calf producers fail to revaccinate their calves at that time. A previous study of the cow-calf segment revealed that approximately 60% of producers vaccinate calves for clostridial disease before weaning. The effects of maternal antibody on active immunization and the period of time between injections of vaccines that require multiple doses are reasons for immune failure (Schultz, 1994).

Nigeria's epidemiologists classify the disease as list D disease in the country, because of the very high mortality rate associated with it at the onset of the rainy season (Abdulkadir, 1989). Although vaccination against the disease has been carried out since 1930 (Osiyemi, 1975), many sporadic outbreaks have been recorded annually. The nomadic Fulani pastoralists of rural Nigeria who own about 70-80% of the total livestock in the country prefer the use of traditional (herbal) remedies to manage blackleg (Suleiman, 1988). Most of them still have reservations about the use of conventional drugs to manage the disease (Abdulkadir, 1989). The recommended procedure for vaccination is to inoculate all young cattle between one and three months of age with bacterin. Blackleg and malignant oedema are so similar and often may both be present in an outbreak, it is recommended that the mixed bacterin containing the killed *Clostridium chauvoei* and *Clostridium septicum*

bacteria could be used. A second injection of the bacterin should be given when an animal reaches six months of age, to be on the safer side, and to ensure a permanent immunity. Where the disease has occurred before, all cattle should be revaccinated annually until they reach three years of age. Routine vaccination procedure will vary with the type of livestock operation involved. When the disease is present in a group, other measures are necessary to protect the unaffected members of the group until immunity has developed. Movement of the cattle from the affected pasture is advisable (Blood and Radostits, 2000).

Tamura *et al.* (1984) recommended the isolation of affected animals, destruction of affected carcasses and slaughtering of the affected animals should be avoided and that the use of polyvalent serum provides passive immunization (Osiyemi, 1975). In Nigeria, Osiyemi (1975) suggested a regime of preventive vaccination to be carried out twice annually at the beginning of the rains with an interval of ten days before heavy rains begins.

Vaccine and antisera have been developed against blackleg using different methods. Formalin-treated whole cultures are currently used for vaccination. In contrast to immunity against other clostridial disease, in which toxoid serve as highly efficacious vaccines, immunity against *Clostridium chauvoei* infections depends to a considerable extent on flagella antigens (Tamura *et al.*, 1984). Mboma (1985) reported that antigenic composition of the vaccines could be influenced by the composition of the medium used for their production. Cortinas *et al.*, (1994) agreed with the report of Mboma, (1985) having found that culture conditions influenced growth conditions and protective antigenicity of *Clostridium chauvoei* vaccines. Vaccines with better protection have been produced in culture media of pH 7.3 than when pH of 8.8 or 5.6 was used.

Sanitation is very important in the prevention and control of this disease. The bacteria that cause blackleg are capable of living in the soil. They have the capacity to form spores which can protect the organism from the effects of weather, and hence soil can remain infected for many years. Carcasses of animals affected by this disease are the chief source of soil infection. They harbor the germ in large numbers and liberate them from both artificial and natural body openings into the surrounding soil. For this reason, every dead animal should be promptly burned or buried (Oxer, *et al.*, 1967). The surface of the ground may be treated by burning it over with heavy layer of straw. Cultivation of known blackleg-contaminated ground (soil) could be done so that it will not be available for grazing (Oxer, *et al.*, 1967). Young animals should be kept out of known contaminated areas. In sheep, the use of alum-precipitated bacterin is also highly recommended, but good immunity does not develop in sheep vaccinated when less than a year old (Blood and Radostits, 2000). In areas where the disease is enzootic, it is recommended that pregnant ewes be vaccinated 3 weeks before lambing. This vaccination will give permanent protection. In subsequent years, the young ewes are vaccinated at six weeks intervals. This vaccination will also protect lambs against umbilical infection at birth (Oxer *et al.*, 1967) and infection of the tail wound at docking, provided the tail is docked before the lamb is 3 weeks old. Vaccination can also be carried out 2 – 3 weeks before shearing or crutching if infection is anticipated. Because of the common occurrence of the disease in young sheep, vaccination before they go on to the pasture and get exposed to infection is recommended in danger areas. The duration of immunity in these animals, vaccinated at 7 month of age, is relatively short and ewes in particular must be vaccinated before they lamb for the first time (Blood and Radostits, 2000). If an outbreak commence in a flock of ewes at lambing time, prophylactic infection of penicillin and antiserum to ewes requiring assistance has been recommended (Wat, 1960

cited by Blood and Radostits, 2000). The constitution of the vaccines is very important. A bacterin prepared from a local strain of *Clostridium chauvoei* is preferred (Cortinas, *et al.*, 1994). If death continue after an approved vaccination programme has been used, an investigation should be made of the antigenic composition of the vaccine relative to the isolates found in dead vaccinated animals. If necessary, the spectrum of the antigens in the vaccine should be expanded (Blood and Radostits, 2000). In Argentina, as in other countries in Nigeria, the most commonly used polyvalent vaccine formulations contains antigens that provide protection against *Clostridium chauvoei*, *Clostridium novyi* (black disease), *Clostridium sordelli* and *Clostridium histolyticum* (Cortinas, *et al.*, 1994). The improvement to be expected would be greater still if the toxin composition of each isolate is known rather than its identifying antigenicity. It has therefore been advisable to use a combined bacterin containing *Clostridium chauvoei*, *Clostridium septicum* and *Clostridium novyi*, if these organisms occur in an area and cause clostridial myositis. Attenuated organisms are also used in the preparation of vaccines for use in cattle and the same attenuated strain of bovine origin or a recently isolated, virulent, ovine strain may be used to prepare vaccines for use in sheep. Multiple *Clostridium* vaccines are now the vogue, in preference to separate vaccine for each disease. It is also possible to administer a polyvalent vaccine-anthelmintic combination Hogathin Scott *et al.*, (1980), cited by Blood and Radoostits (200). The polyvalent vaccine is highly recommended for the extra protection acquired at a very little extra cost.

### **2.11.1 Vaccine and Vaccination**

The most remarkable utilization of clostridia toxins is certainly the development of vaccine against several clostridia diseases in man and animals (Brown and Williamson, 1997). As

early as December, 1890, Behring and Kitasato immunized animals with tetanus toxoid preparations and discovered neutralizing antitoxin in the sera of the animals (Alouf, 2003). In the course of time following the discovery, description and the study of the toxin produced by the most pathogenic clostridia, several of them have been included in vaccines to protect the animals against one or several clostridia diseases. The most remarkable achievements have been the tetanus vaccines in humans and horses and the vaccine against several enterotoxaemia and soft tissue infections in small and large ruminants. There are still several toxins that, for various reasons are not included in the vaccines. The most remarkable are probably the two toxins of *Clostridium defficile* causing the pseudo membranous colitis in humans (Moncrief *et al.*, 1997, Just *et al.*, 2003; Delmee, 2002). Typically the vaccines contain toxins as toxoids after semi-purification of culture supernatants. Toxoids are detoxified toxins retaining their immunogenic properties. The detoxification steps are usually performed by a formaldehyde and heat treatment (Brown and Williamson, 1997). Sometimes, the whole cell bacterium is added to the vaccine after formalization. It is for instance, *Clostridium chauvoei*, because experiment have shown a protective effect of a cell surface heat-labile antigen (bacterin; toxoid vaccine) and of *Clostridium novyi* in some vaccines. Initially two injections at a six week interval are recommended. Protection induced by the clostridia vaccines vary in length according to the *clostridia toxins* targeted and to the host species. Human tetanus vaccine are long term protective needing a booster every 5 to 10 years. On the other hand in animals, annual to semi-annual booster are recommended for all Clostridia vaccines

## **Chapter 3**

## **MATERIALS AND METHODS**

### **3.0 Introduction**

### **3.1 Media**

The media that are required for the cultivation of *Clostridium chauvoei* to liberate toxin are: Reinforced Clostridial Medium (RCM), Blood Agar (BA), Cooked Meat Medium (CMM) and Toxin Production Medium.

### **3.2 Media Preparation**

#### **3.2.1 *Cooked Meat Medium***

Cooked meat medium (CMM) base was commercially obtained (Oxoid, England) and used for this study. It contained beef (45.4 g), proteose peptone (20 g), bactor- dextrose (2 g), and sodium chloride (5 g) per liter, pH (7.2) at 25 °C. The medium was reconstituted as recommended by the manufacturer as follows: 237.5 g of the pelleted CMM granules (1-25 g/10 mls) was weighed in a 2.5 liter capacity bottle that was thoroughly washed and improvised for culture of *Clostridium chauvoei* (Jakari strain). One and half litres of distilled water was added and allowed enough time to thoroughly become wet. The mixture was then sterilized by autoclaving at 15 pounds pressure and 121 °C for 15 minutes. The CMM was poured and allowed to cool to room temperature and placed in the refrigerator to be used after 24 hours.

#### **3.2.2 *Preparation of Reinforced Clostridial Medium***

The Reinforced Clostridial Medium (RCM) used in the study was prepared using a method similar to that described by Grinsted (1954) cited by Useh, (2001) with a minor modification in the composition of ingredients required. The ingredients required include:

		MODIFICATION
Bacteriological peptone	1%	1%
Meat extracts (lablemco)	1%	1%
Yeast extract	0.3%	0.5%
Sodium Chloride	0.5%	0.5%
Sodium acetate	0.8%	0.5%
Agar	0.05%	1%
Soluble starch	0.1%	0.5%
L. Cystein Hydrochloride	0.06%	1%
D- Glucose	0.6%	1%
Distilled water	100 ml	100ml

Source: USEH, 2001

These ingredients were mixed in 100 ml of distilled water and autoclaved at 121<sup>0</sup>C and 15 pounds pressure for 15 minutes. The pH was adjusted to 8.4 after cooling. Ten (10) ml of prepared RCM was dispensed in sterile bottle and kept at 4 <sup>0</sup>C to be used on the same day it was prepared.

### ***3.2.3 Preparation of Blood Agar***

The medium was prepared as described by Dowell and Hawkins, (1981) as follows: 37 grams of agar base (powder) was weighed and dispersed in 1 liter of distilled water. This was allowed to soak for 10 minutes. It was thereafter swirled to mix and then sterilized by autoclaving at 121 <sup>0</sup>C and 15 pounds pressure. It was allowed to cool to 47 <sup>0</sup>C, and sterile defibrinated sheep blood was added and mixed very well before pouring on the glass petri dish (plates).

### 3.3

### Bacterial Strain

*Clostridium chauvoei* (Jakari strain) was obtained in its lyophilized form from the National Veterinary Research Institute (N.V.R.I), Vom, Plateau State, Nigeria for this experiment. The bacterium was isolated from clinically infected zebu cattle and its pathogenicity indices fully determined (Princewill, 1965). It was kept at 4 °C until when required. The organism (packed in glass ampoules) were removed from the refrigerator and left on the bench to attain room temperature. The glass ampoules were broken using a sterile pair of scissors. A sterile bottle containing 10 ml of RCM (broth) was opened and small amount of the medium was poured into glass ampoules, rinsed and emptied back into the sterile bottle containing RCM (broth). At each stage of handling the medium and the microorganism, sterile (aseptic) procedures were maintained. The sterile bottles containing RCM and *Clostridium chauvoei* (Jakari strain) were placed in the incubator for 48 h. After this duration, growth was visible with naked eyes, as the original transparent or translucent maroon colour of RCM changed to a turbid one. The duration of incubation is a slight modification of the method of Princewill *et al* (1986) who incubated RCM broth containing *Clostridium* spp for 24 h before sub-culturing on blood agar.

### 3.4 Subculture of *Clostridium chauvoei* (Jakari strain) on Blood Agar Plates

The isolate was confirmed by the morphological and biochemical characterization as described by Cato *et al.* (1986).

Following the incubation of the inoculated RCM broth, it was removed from the incubator, and using a Pasteur's loop, the RCM containing the organisms, *Clostridium chauvoei* (Jakari strain) was transferred onto blood agar plates that were prepared a day prior to the day of use and stored at 4 °C. Before use, the blood agar was rid of any moisture by drying in the

incubator. After incubation for 24 h in an anaerobic jar using gas pack, only *Clostridium chauvoei* colonies were seen, devoid of contaminants.

### 3.5 Preparation of the Toxin Production Medium

Jayaraman *et al.* (1962) described the most suitable medium for *Clostridium chauvoei* toxin production which was used in this study and included:

Cooked meat medium	3 g
Bacteriological peptone	2.5 g
Sodium chloride	2.0 g
Dipotassium hydrogen orthophosphate (K <sub>2</sub> PO <sub>4</sub> )	2.0 g

The medium composition were mixed in 10 ml of phosphate buffer saline (PBS), and adjusted to pH 9.2 while still hot. Whole toxin production medium was allowed to boil and clarified through 0.4 µm filter paper. Two and half (2.5) g of peptone was added and the reaction adjusted to pH 8.0. Two (2.0) g of sodium chloride was added and finally the medium was buffered by addition of two (2.0) g of Dipotassium hydrogen orthophosphate (K<sub>2</sub>HPO<sub>4</sub>). This medium was then dispensed in the required capacity flask. The medium was sterilized in a running steam at 15 pound pressure and 121 °C for one hour. Finally, 2.0g of dextrose powder was added in the final concentration of 1% medium. 2% of sucrose powder was prepared and sterilized separately in a sufficient quantity to give the desired concentration. The sucrose was added just at the time of inoculation of flask with young actively growing pure culture of *Clostridium chauvoei* (Jakari strain) grown on blood agar. The medium was incubated at 37 °C for 24 and 48 hrs, respectively. The toxin was freed from the organisms and spores by centrifugation at 3000 gravity for 30 minutes and filtered through a millipore type 0.2 µ pore size filter paper, (Lacy Hulbert England) to create a

negative pressure. The toxin filtrate was tested free of microorganisms by checking its growth on blood agar incubated at 37 °C for 24 h.

### **3.6 Experimental Design for Toxicity Testing of *Clostridium chauvoei* Toxin**

#### ***Materials***

- 20 Swiss white mice were divided into 3 groups: A1, A2, and B
- Group A1 (7 mice) for toxin recovered at 24hrs of culture
- Group A2 (7 mice) for toxin recovered at 48hrs of culture
- Group B (6 mice) Control group (Administered with sterile medium only)
- Route of administration: Intravenous (iv) through the tail vein for all the groups

#### ***Methods***

Twenty Swiss white mice weighing 16 to 19 grammes were employed for testing the toxin produced. The mice were divided into groups A (14 mice) and B (6 mice). Group A was further divided into groups A1 (7 mice) and group A2 (7 mice). Group A1 and A2 were inoculated with the toxin filtrate from 24 h and 48 h of incubation, respectively. Group B (control), were inoculated with the toxin production media only. The clinical signs and symptoms were observed.

### **3.7 Gross and Histopathology**

- The dead mice were examined grossly for lesions of the disease
- Tissue sections of muscles, liver, lungs, pancreas and kidneys were made and stained with H & E.

### **3.8 Hemolytic Activity of the Toxin Filtrate at Different Titres**

Titration of the toxin filtrate was carried out to determine the hemolytic titer. *Clostridium chauvoei* toxin was used for the titration. Diluent used was potassium buffered saline. Sheep blood was collected using an anticoagulant (Ethylene Diamine Tetracetic Acid [EDTA]). Blood sample was washed with phosphate buffered saline (PBS) 3 times, by centrifuging and decanting the supernatant with a Pasteur pipette. From the packed red blood cell (RBC), 2% RBC suspension was prepared. By addition 0.2 ml of red blood cell RBC to 9.8 ml of phosphate buffer saline PBS). Twenty five (25)  $\mu$ l of the PBS was added to a well in the U-shaped titertek plate and equal volume of the toxin was added, mixed with a micro-diluter, before adding 25  $\mu$ l of the 2% prepare RBC. It was incubated at 37  $^{\circ}$ C for 2 h. The mixtures was observed for haemolysis.

### **3.9 Effect of Physical and Chemical Agents**

The toxin filtrate was given different physical and chemical treatments and tested for hemolytic activity using 2% sheep red blood cell. The sample filtrates were given heat treatment at different temperature for different periods. (The hemolytic titres were tested using 2% sheep red blood cell).

## Chapter 4

## RESULTS

### 4.1

### Bacterial isolate

The organism *Clostridium chauvoei* (Jakari strain) was found as a straight rod, motile with peritrichous flagella. The spore is subterminal, Gram +ve. The surface colonies were circular, entire margin, translucent, greyish white, matto glassy surface. Slight to moderate growth in nutrient broth, or cooked meat broth, with fermentable carbohydrate. Gas production continues after growth has ceased. Optimum temperature for growth was 37°C and does not grow above 42°C. Toxins were formed, and pathogenic for mice and guinea pig.

### 4.2 Description of the pure culture of *Clostridium chauvoei*

*Clostridium chauvoei*, grown on blood agar is umbonate, small circular, raised greenish, slightly hemolytic colonies. This was obtained after 24 h of anaerobic incubation at 37 °C (Plate 1).

The toxicity of *Clostridium chauvoei* toxin in mice after 24 h culture Table (4.1) revealed that all the mice inoculated with the toxin showed respiratory distress and three showed convulsion before death. Nevertheless all the mice inoculated died at various time intervals within 48 h. The minimum lethal effect in ml of the toxins was 0.2 ml.

The toxicity of the mice that died after intravenous inoculation of mice with *Clostridium chauvoei* toxins obtained after 48 h culture (Table 4.2) revealed that out six out of the seven mice showed respiratory distress, two of the mice showed convulsion before death. Two mice survived with their tail eventually cut off. Five died at various length of time within 48 h of inoculation.

Plate 1

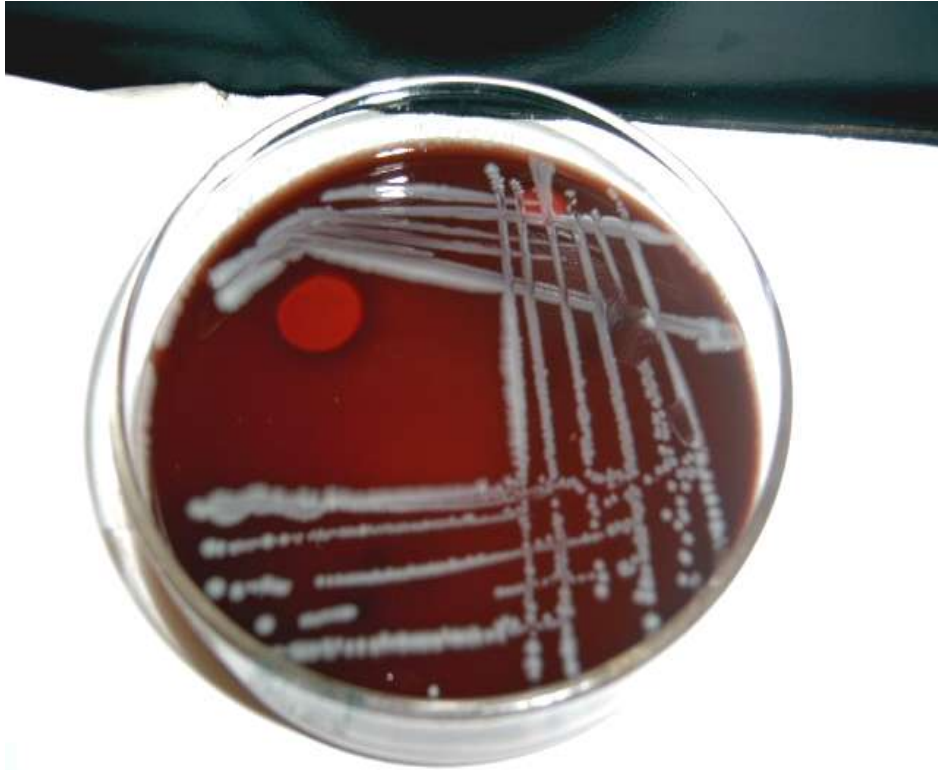


Plate 1: Pure culture of *Clostridium chauvoei* (Jakari strain) grown anaerobically on Blood agar after 24 h of incubation at 37°C

Table 4.1 Toxicity of *Clostridium chauvoei* 24 h-culture Toxin in Mice

Mouse No.	Volume of inoculum (ml)	Time of inoculation of toxin (pm)	Onset of clinical signs post inoculation (Minutes)		
			Respiratory Distress	Convulsion	Death
1	0.2	6.10	2	15	18
2	0.2	6.20	10	None	2880 (48 h)
3	0.2	6.30	10	20	360 (6 h)
4	0.4	6.05	28	30	33
5	0.4	6.00	24	None	720 (12 h)
6	0.4	6.15	2	None	1440 (24 h)
7	0.4	6.25	15	None	2880 (48 h)

Table 4.2 Toxicity of *Clostridium chauvoei* 48 h-culture Toxin in Mice

Mice No.	Volume of inoculum (ml)	Time of inoculation of toxin (pm)	Onset of clinical signs post inoculation (Minutes)		
			Respiratory Distress	Convulsion	Death
8	0.2	7.30	10	15	30
9	0.2	7.35	25	40	720 (12 h)
10	0.2	8.10	10	None	None
11	0.2	9.00	None	None	None
12	0.4	8.00	None	None	1440 (24 h)
13	0.4	8.20	10	None	2880 (48 h)
14	0.4	8.30	6	None	4320 (72 h)

### 4.3

### Gross Pathology

The hairs on the skin were sparsely raised up, subcutaneous tissues were oedematous with substantial amount of serosanguineous exudates. The skeletal muscles turned blackish grey, less moist, irregularly fragmented, emitted rancid odour and contained larger gas bubbles. Some gross pathological lesions were observed with the 24 h toxin filtrate. Gross lesions in mice that died after 3, 24, and 48 h respectively were very similar except for the mice that died within 3 minutes, where only haemorrhages were vividly observed from the lungs. The lungs and heart were haemorrhagic, the liver, kidney and pancreas were observed to be necrotic in the others.

### 4.4 Histopathology findings in Mice inoculated with *Clostridium chauvoei* Toxin produced from 24hours and 48 hours of bacterium cultures

#### **Mice that died 33 minutes post intravenous inoculation of toxin**

The mice that died thirty three minutes post intravenous injection of the *Clostridium chauvoei* toxin had focal areas of necrosis of the hepatic cells with mononuclear cells infiltration (Plate 2). The livers had congested blood vessels. Their kidneys had focal areas of glomerular and renal tubular necrosis with mononuclear cellular infiltration. The renal blood vessels were congested and there were focal haemorrhagic areas (Plate 3). The pancreas had no observable microscopic lesions. The lungs had haemorrhagic areas, and mononuclear cellular infiltration in the alveoli and the interalveoli septae. It had ejected blood vessel and haemorrhages in the bronchioles.

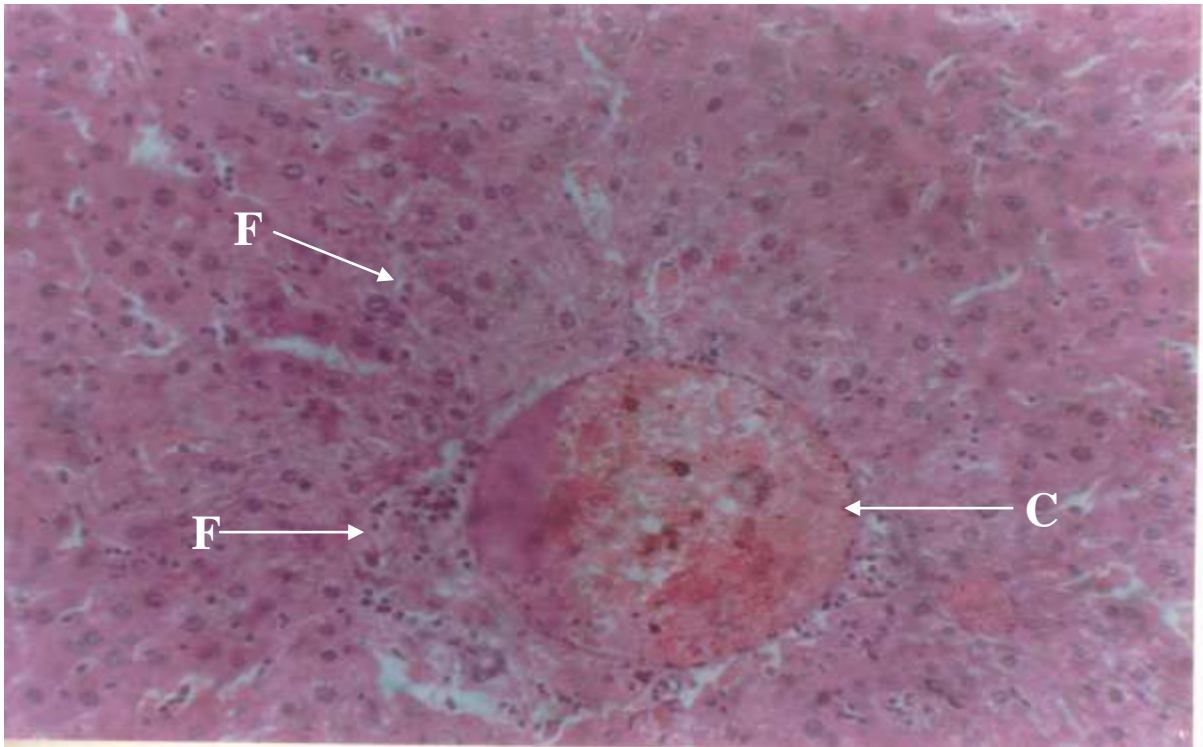


Plate 2: Liver: Congested blood vessels (C) (central vein); Focal areas of necrosis(F) in mice that died after 33minutes post intravenous administration of *Clostridium chauvoei* toxin. H & E stain; Mag x 100

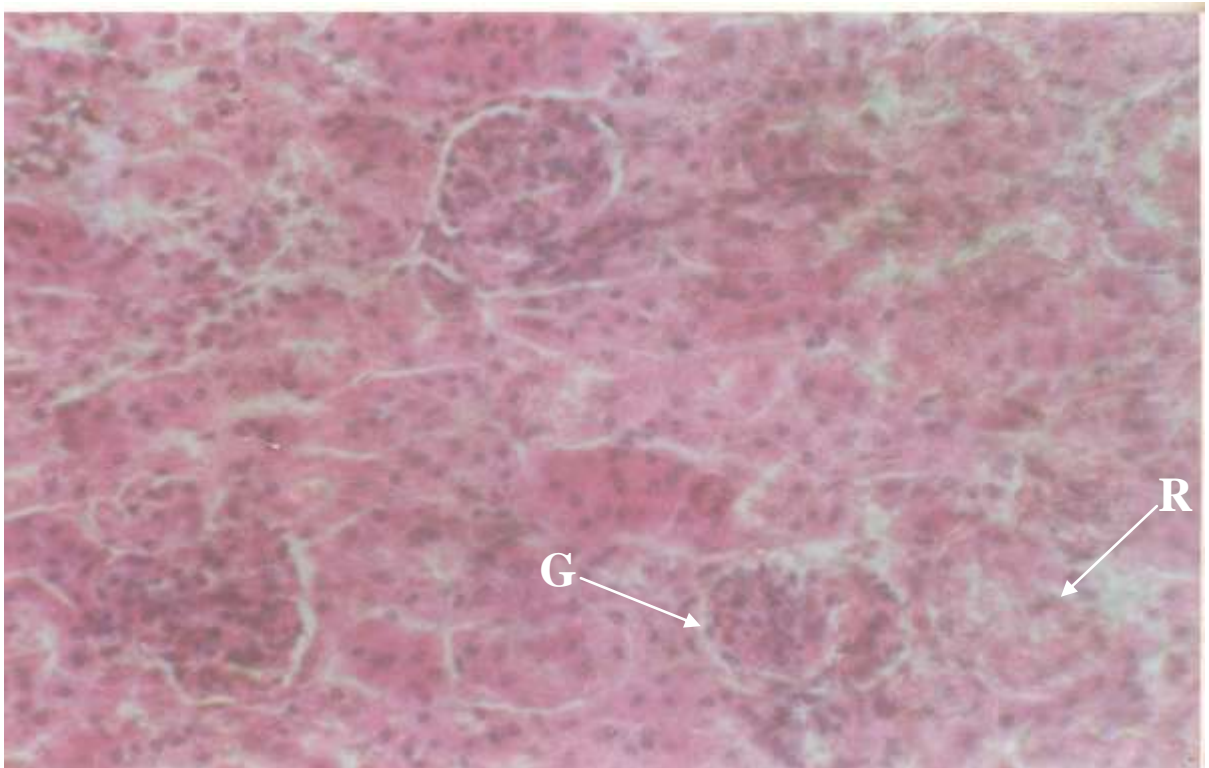


Plate 3: Kidney: Glomerular (G) and Renal tubular (R) necrosis in mice that died after 33minutes post intravenous administration of *Clostridium chauvoei* toxin. H & E stain; Mag x 100

### **Mice that died 1440 (24 h) minutes after intravenous inoculation of toxin**

The mice that died 24 h post intravenous injection of the toxin had diffuse necrosis of the pancreatic cells (Plate 4). The livers had diffuse necrosis of the hepatic cells with few mononuclear cellular infiltrations. There were haemorrhagic areas and congested blood vessels in the liver. The kidneys had glomerular and renal tubular necrosis infiltrated by mononuclear cells within the necrotized areas. There were haemorrhagic areas and congested blood vessels in the kidney (Plate 5). The spleen had focal areas of necrosis. The heart had focal areas of necrosis of the myocardial cells infiltrated by mononuclear cells. There were congested blood vessels in the heart.

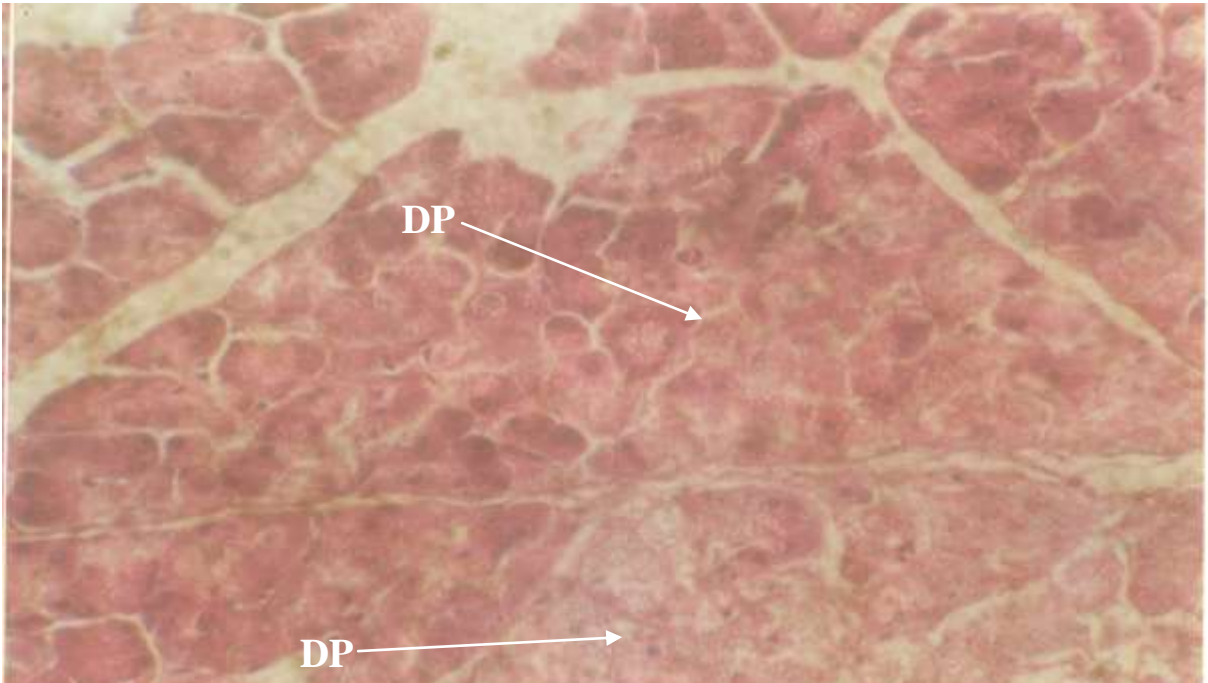


Plate 4: Pancreas; Diffuse necrosis of the pancreatic cells (DP) of mice that died after 1440 minutes of *Clostridium chauvoei* toxin administration. H & E stain; Mag x 100

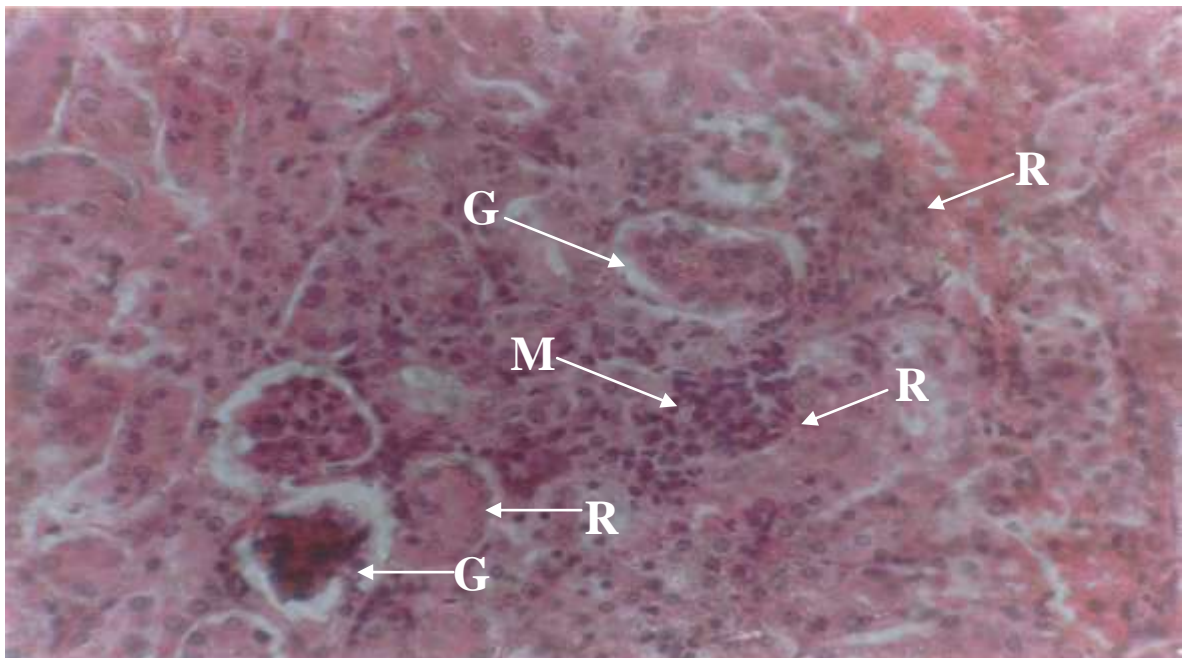


Plate 5: Kidney; Glomerular (G) and Renal (R) necrosis, Mononuclear cells (M) of mice that died after 1440 minutes of *Clostridium chauvoei* toxin administration. H & E stain; Mag x 100

### **Mice that died 2880 (48 h) minutes after intravenous injection of toxin**

Mice that died 48 hours post intravenous injection of the toxin had diffuse mononuclear cellular infiltration in the alveoli and interalveolar septae in their lungs. There was marked haemorrhages in lung parenchyma. The blood vessels of the lungs were congested. Their kidneys had atrophied glomeruli, glomerular and renal tubular necrosis with mononuclear cellular infiltration. There were haemorrhagic areas and congested blood vessels in the kidneys (Plate 6). The livers had fatty degeneration and diffuse necrosis of the hepatic cells infiltrated by mononuclear cells. There were haemorrhagic areas and congested blood vessels in the livers (Plate 7). The skeletal muscle had diffuse areas of necrosis of the skeletal muscle cells infiltrated by mononuclear cells. There were haemorrhagic areas and congested blood vessels in the skeletal muscles. The heart had focal areas of necrosis of the myocardial cells infiltrated by mononuclear cells. There were congested blood vessels in the heart.

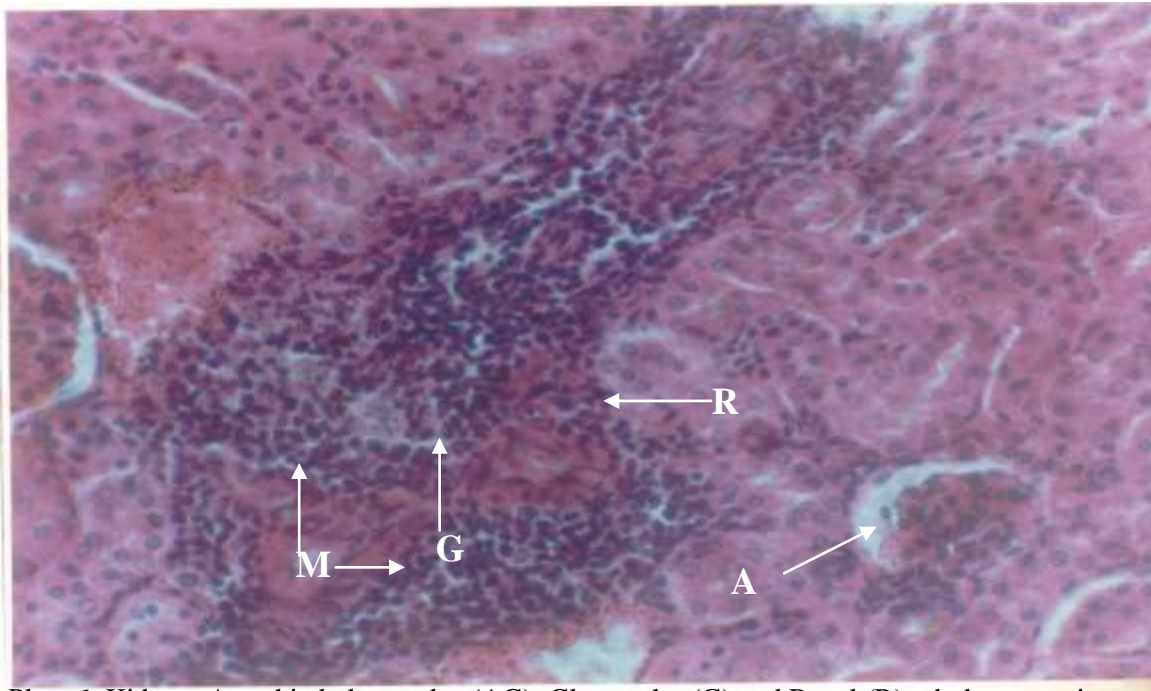


Plate 6: Kidney; Atrophied glomerulus (AG), Glomerular (G) and Renal (R) tubular necrosis, Mononuclear cells (M) of mice that died after 2880 minutes of *Clostridium chauvoei* toxin administration. H & E stain, Mag x 100

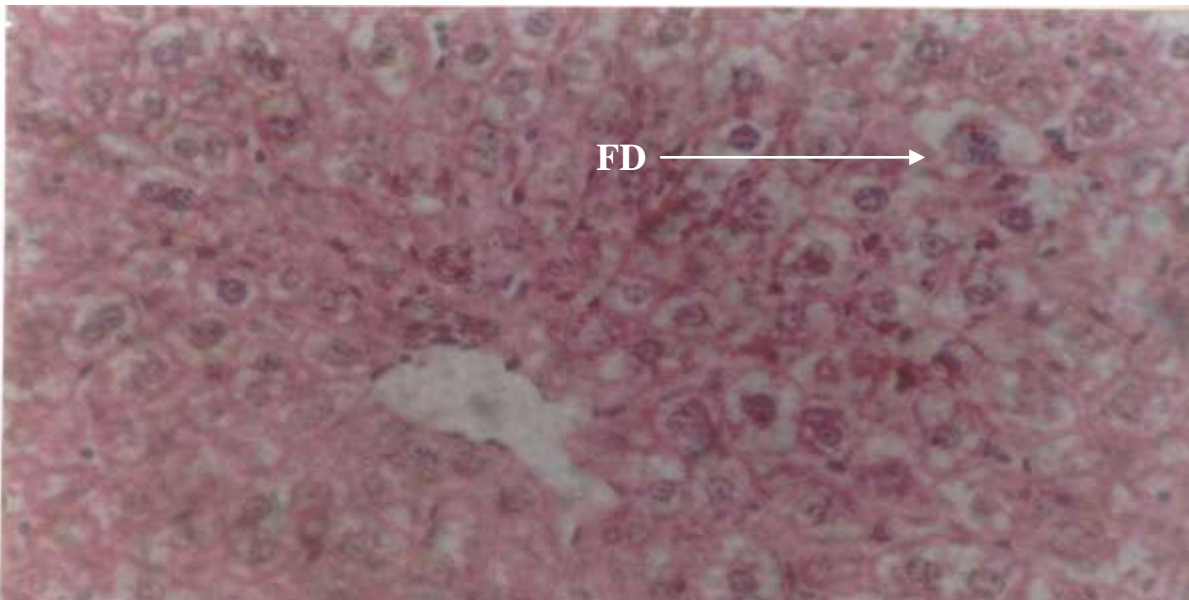


Plate 7: Liver; Fatty degeneration (FD) of mice that died after 2880 minutes of *Clostridium chauvoei* toxin administration. H & E stain, Mag x 100

#### **4.5 Effects of Temperature on Hemolytic Activity**

The samples of filtrate were given heat treatment at different temperatures and for different period and the study revealed that toxin filtrates are not easily destroyed by heat (Table 4.3). These were stable at 45 °C, 70 °C for 10 minutes. At 100 °C (boiling waterbath) the hemolytic activity was completely lost at 5 minutes.

#### **4.6 Effects of Chemical Agents on *Clostridium chauvoei* toxin Filtrate**

Ether and HCl, have no ill effect on the filtrate (Table 4.4). Phenol and methanol have partial effect and some fall in activity was observed at 312 for phenol and 156 for methanol. Formalin and NaOH had quick adverse effect and activity was completely destroyed.

#### **4.7 Effects of Storage Duration**

The filtrate was stored in a capped tube and stored at 4°C for a period of two weeks (Table 4.5). It was observed that the hemolytic effect was stable at 4 °C for two weeks. A decline was observed when sample was stored at 4 °C for more than two weeks.

Table 4.3 The Effects of Temperature on the Hemolytic Activity of *Clostridium chauvoei* toxin 24 h of culture

Temp (0C)	Time of exposure of toxin (min)	Hemolytic activity of toxin
45	5	+ve
	10	+ve
70	5	+ve
	10	+ve
100	5	-ve

Key:

+Ve positive (Haemolysis);  
-Ve Negative (No Haemolysis).

Table 4.4: The Effects of Chemical Agents on Hemolytic Activity of *Clostridium chauvoei* Toxin 48 h of culture

Chemical Agents	Concentration	No chem Activity (Control)	Hemolytic effects of toxin on sheep RBC (in serial dilution)											
			2	4	8	16	32	64	128	256	512	1024	2048	
Formalin	0.5%	+	-	-	-	-	-	-	-	-	-	-	-	-
Phenol	0.5%	+	+	+	+	+	+	+	+	+	+	+	-	-
NaOH	1N	+	-	-	-	-	-	-	-	-	-	-	-	-
Ether	0.03/0.5ml	+	+	+	+	+	+	+	+	+	+	+	+	+
Methanol	0.5%	+	+	+	+	+	+	+	-	-	-	-	-	-
HCl	1N	+	+	+	+	+	+	+	+	+	+	+	-	-

Key:

+ There is haemolysis  
- No haemolysis

Table 4.5 Effects of the Duration of Storage of *Clostridium chauvoei* at 4<sup>0</sup>C

Storage duration (days)	Hemolytic activity of toxin
0 – 14	+ve
Beyond 14	-ve

After incubation of *Clostridium chauvoei* (Jakari strain) at 37 °C in a toxin producing medium, a toxin that was lethal to mice was obtained. The toxins produced during 24 h of the culture were found to be more lethal to mice than toxin produced during the 48 h of culture. This finding may be due to a high concentration of toxins produced during the 24 h of culture than that produced during the 48 h. A similar toxin was produced by Jayaraman *et al.* (1962) when they incubated the organism in a toxin producing medium for 36 h. This may explain why all the mice treated with 24 h toxin had marked respiratory distress and 100 % mortality.

Those mice that died after 48 h of toxicity had diffused area of necrosis infiltrated with mononuclear cells. This may be due to the longer effect of the toxins in the tissues, while only focal area of necrosis was observed in those mice that died thirty three minutes post intravenous inoculation of the toxins. This observation is hereby documented for the first time.

This study revealed that one mouse had convulsion, with 24 h toxin while two mice had convulsion with 48 h toxins. A similar result has been observed by Jayaraman *et al* (1962) with 36 h toxins only. This is because of the higher concentration of the toxins inoculated to the mice which were produced during the 24 h of culture.

All the seven mice inoculated with 24 h died at intervals ranging between 30 to 2880 minutes (48 h) post intravenous inoculation of the toxins. The highest mortality recorded was among the mice inoculated with 24 h toxins, while the lowest was among mice inoculated with 48 h toxins. Similar findings of varied death percentages had been reported

by earlier workers (Jayaraman *et al*, 1962), who also attributed the cause to the concentration of the toxins.

Generally, from this study, the 24 h toxins produced are more potent than 48 h. This affirms that *Clostridium chauvoei* produces toxins that is more lethal at 24 h incubation in a toxin producing as documented by earlier reports of Jayaraman *et al.*, (1962).

Gross lesions in mice that died from 24 h and 48 h toxins were very similar except for mice that died 33 minutes post intravenous inoculation with 24 h toxins, where only haemorrhages were observed from the lungs. This observation is again being reported in this study.

At histopathology, the mice that died thirty three minutes with 24 h toxin post intravenous inoculation had focal areas of hepatic necrosis, infiltrated by mononuclear cells, while those mice that died 2880 minutes (48 h) with the 48 h culture toxin had diffuse mononuclear cellular infiltration in the alveolar septae of their lungs. This observation has not been reported. The focal area of necrosis observed with 24 h toxins, may be associated with the toxins concentration. The mice that had longer contact with the toxins, had areas of necrosis that were diffused. This may be the possible explanation of diffuse necrosis observed with mice inoculated with 48 h toxins. Again we are documenting this observation in this study.

Chemical agents like ether had no adverse effect on the toxins, therefore hemolytic effect was observed when ether and the toxins were added to the sheep red blood cell. This observation was in agreement with the finding of earlier report of Jain *et al* (1999). Chemical agents like phenol, methanol and HCl that had partial adverse effect and a decrease in hemolytic activities at various titres of 1024, 128 and 1024. Formalin and

sodium hydroxide had quick adverse effect on the toxins, and hemolytic activities were completely destroyed following the addition of these chemicals. This observation was also in line with the reports of Jain *et al.*, (1990).

The effects of heat on hemolytic activities revealed that the toxins were not easily destroyed by heating. The toxins were stable at 45°C and 70°C five and ten minutes. The hemolytic activities was unaffected at five minutes but was completely lost at the 10<sup>th</sup> minutes when subjected to 100°C. Such a high thermostability was also observed by earlier workers (Jain *et al.*, 1990). This study also observed that storage of the toxin at 4°C for two weeks, does not affect the potency of the toxins. However storage beyond two weeks, has an effect on the hemolytic effect on the toxins which was similar to the finding of Jain et al (19990).

## Chapter 6 SUMMARY, CONCLUSION AND RECOMMENDATIONS

The production of *Clostridium chauvoei* (Jakari strain) toxin was carried out in the laboratory. Gross pathology on mice inoculated with the toxin intravenously showed some lesions in the internal organs, the skeletal muscles turned blackish grey. Lungs and heart were haemorrhagic, necrosis was observed in liver, kidney and pancreas. Histopathology findings on mice that died 33 minutes post intravenous inoculation of the toxin showed some focal area of necrosis in the kidney and necrosis of the hepatic cells, with mononuclear cell infiltration. Mice that died 1440 minutes after intravenous inoculation of the toxin showed some diffuse areas of necrosis of the liver, and those died after 2880 minutes post intravenous inoculation had atrophied glomeruli.

Hemolysis was observed after storage at 4°C for duration of 14 days. Hemolytic activity was destroyed on storage beyond 14 days.

It was further observed that hemolytic activity was stable at 40°C, 70°C and 100°C for five minutes. Hemolytic activity inactivated at 100°C beyond 5 minutes. The toxins were stable at 4°C for 14 days. This study also reveals that the toxin is the major component of the organism and it could be responsible for its pathogenicity.

A characterized toxin is beneficial in further research, such as further research on the molecular weight and purification of the toxin. The hemolytic and necrotic effects of the different toxins can further be investigated.

The application of multivalent vaccine and toxoid in protecting animal against blackleg should be looked into, since the organism *Clostridium chauvoei* infection is always associated with other organism like *Clostridium novyi*, *Clostridium perfringens* and *Clostridium septicum*.

A characterized toxin can enable the production of multivalent vaccine containing the toxin antigens of *Clostridium septicum*, *Clostridium novyi* and *clostridium chauvoei*.

Based on these preliminary findings further research needs to be done on the toxin filtrate regarding the storage effect and the best form it can be stored for further research work. In addition, it is important to determine the immunogenicity of the toxin. In this study, it was observed that toxins produced following 24 h of culture were more potent than that of 48 h. Besides toxins storage should not exceed two week at 4 °C. Further research need to be done on the storage form and temperature at which it can stay for a longer period to enable further research.

## APPENDIX



Control mice inoculated with the medium lacking toxins



Survived infected mice from 48 h

## REFERENCES

- Abdu, A. A, Jagun J, A.G, Gefu J. O, Mohamed A.K, Alawa C.B.I, and Omokanye A.T. (2000) In Ethnoveterinary Practices, Research and Development. Gefu, J. O, Abdu, P.A, and Alawa, C. B. I (Eds), National Animal Production Research Institute (NAPRI), Ahmadu Bello University Zaria pp162-164.
- Abdulkadir, I (1989) Infections disease of livestock in Nigeria. An outline. Ahmadu Bello University Press First edition pp 8 – 12.
- Agba, M I and Princewill, T. J .T (1986). Combined Clostridial Vaccine for Controlling blackquarter disease in Nigeria. XI V International Congress of Microbiology 86.Abstract 70.
- Alberto, F, Broussole, V Mason, D.R, Carlin. F, and Peck, M.w. (2003). Variability in spore germination response by strains of proteolytic *Clostridium botulinum* type A, B and F. *Applied Microbiol* 36, 41-45
- Amaro, A.D. and Rao (1990) Studies on the incidence of blackquarter in Karnataka during 1979-85 *Indian Veterinary Journal*.67.795-801
- Alouf, J.E. (2003). Milestone in clostridial toxin research. In: Proceeding of the meeting of the European concerted Action QLK2-CT-2001-01267. pp17-24.
- Bagadi, H.O (1977). Production and counting of spores of *Clostridium chauvoei*. *Applied and Environmental Microbiology*. pp 1287 – 1288
- Ballard, J. Crabtree, J. Roe, B.A. Tweten, R.K. (1995) The primary structure of *Clostridium septicum* alpha toxin exhibits similarity with that of *Aeromonas hydrophila* aerolysin. *Infectious Immunology*. **63**, 340-344.
- Bejerinckii (formally *Clostridium acetobutylicum*) NCIMB8052 chromosome. *Journal Bacteriol* 177,439-448
- Bryant, A.E. Bergstong, R. Zimmermann, G.A. Saylyer, J.L. Hill, H.R. Tweten, R.K. Sato, H and Steven, D.L. (1993). *Clostridium perfringens* invasiveness is enhanced by effects of theta toxin upon PMNL structure and function. The role of Leucocytotoxicity and expression of CD11/CD18 adherence glycoprotein. *FEMS Immunology and medical Microbiology*. 7: 321-336.
- Blacha T. (1989) *Applied Veterinary Epidemiology* Gustav Fischer Press, Jena New York. Pp. 261 – 262
- Blood D.C and Radostits, O.M (2000). *Veterinary Medicine. A textbook of Disease of Cattle, Sheep, Pig's, Goat, and Horses*. 9<sup>th</sup> edition. E.L. B.S/Bailliere Tindal pp 753-758.

- Brack, T.D., and Madigan B.S (1988). Biology of microorganism 5<sup>th</sup> ed. Printies Hall inc, Englewood Cliffs, N.J Roux E, Yersin A. (1888) contribution a.l etude de la diphterie. Ann. Inst Pasteur, 2, 629-661
- Braconnnier, A., Broussolle, V, Dagaigainaratz, C, Nguyen, and Carlin, F. (2003). Growth and germination of Proteolytic *Clostridium botulinum* in vegetable based media. *Journal.food protection*.66, 833-839.
- Bridson, H.B and Hall, T.C. (1974). The oxoid manual. 7<sup>th</sup> edition. Complid by E.J. Bridson Unipath Ltd. England, pp2 – 47
- Broda, D.M., Saul, D.J., Lawson, P.A., Bell ,R.G. and Musgrave, D.R. (2000) *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum packed meats. *International Journal Systmic Evluion Microbiology*. **50**, 107-118.
- Brown, J.E, Williamson, E.D.(1997). Molecular approaches to novel vaccines. In: The Clostridia: Molecular biology and pathogenesis .Accademic press. Pp505-524
- Carminati, D. and Carni,S. (1989) Antimicrobial activity of lysozyme against *Listeria monocytogens* in milk. *Microbiology Aliment Nutrition*. 7, 49-54.
- Carruthers, J.D.A. and Stubbs, H.A. (1987) Botulinum toxin for benign essential blepharospasm, hemifacial spasm and age-related lower eyelid entropion. *Can Journal Neurological Science*. **14**, 42-45
- Cassier, M., and Sebald, M. (1969). Germination lysozyme dependent des spore de *Clostridium perfringens* ATCC 3624 apres traitement thermique. Ann.Inst Pasteur 117,312-314.
- Cato, E.R Geoge W.L and Fineggold, S.M (1986). Genus *Clostridium*. Bergey's manual of systematic bacteriology Vol. 2, the Williams and Wilkens CO Baltimore pp1141-1200.
- Claus, S and Macheak, W (1972). Characterisation of Haemolysin by *Clostridium chauvoei*. Amer. Journal vet Reseach. 33:1045.
- Clouston, J. G. and Wills, P. A. (1969). Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. *Journal Bacteriology*. **97**, 684-690.
- Cohen, S.H. (2001) Resistance to moxifloxacin in toxigenic *Clostridium difficile* Isolates is associated with mutations in *gyrA*. *Antimicrobiology Agents Chemother*. **45**, 2348-2353.

- Colle, J.G., Watt, B. Flower E.B. and Brown R. (1972). An evaluation of gaspak system in the culture of anaerobic bacteria. *Journal of applied Bacteriology* 35: 1, 71 – 82.
- Cortinas, T.I., Micalizzi, B. and Deguzman M.S (1994). Influence of cultures conditions on growth, and protective antigenicity of *Clostridium chauvoei*. *Journal of applied Bacteriology* 77: 382 – 387.
- Dainty, R. H., Edwards, R. A. and Hibbard, C. M. (1989). Spoilage of vacuum-packed beef by a *Clostridium* sp. *Journal Science Food Agriculture* **49**, 473-486.
- Delmée, M., Homel M. and Wauters G. (1985) Serogrouping of *Clostridium difficile* strains by slide agglutination. *Journal Clinical Microbiology*. **21**, 323-327.
- Delmée, M. (2002). Enteric clostridial diseases in humans: *Clostridium difficile*. In: Molecular genetics, classification, pathology and ecology of the genus *Clostridium*. Proceedings of the first meeting of the Concerted Action QLK2- CT2001-01267 (Duchesnes C. and Mainil J., Eds), pp 48-52, Liege, Belgium.
- Delmée M. (2004) Diagnosis, epidemiology and antibiotic resistance of *Clostridium difficile*. In: Diagnosis, epidemiology and antibiotic resistance of the genus *Clostridium*. Proceedings of meeting 3 of the European Concerted Action QLK2-CT2001-01267 (Duchesnes C. and Mainil J., Eds), pp 20-23, Liege, Belgium.
- Dowell, V.R. and T.M. Hawkins (1981). Laboratory methods in anaerobic bacteriology, Center for Disease Control (CDC) Laboratory manual, HHS Publication, Atlanta, Georgia pp1 – 96
- Duncan, C.L. and Strong, D.H. (1968). Improved medium for sporulation of *Clostridium Perfringens* .*Journal of Applied Microbiology* **16**, 82-89.
- Duncan, A.J, Carman, R.J, Olsen, G.J, and Wilson, K.H. (1993). Assignment of the Agent of Tyzzer's disease to *Clostridium piliforme* comb on the bases of the 16 rRNA sequence analysis. *International Journal Systemic. Bacteriology*.43, 314-318.
- Dubreuil., L and Neut, C. (2004). Clostridia: Antibiotic susceptibility, present and Near feature. Proceeding of meeting of the European concerted action QLK2-CT2001-01267 (Dunchesnes C, Mainil J, Pelkonen S. Menozzi M.G, Ed) pp 49-55, presses Fac. Med vet Ulg, Liege, Belgium.
- Duenschmann, H (1894). Etude Experimentale surle charbon sytmatique et ses relations avec l' oedema malin. *Ann. Inst.Pasteur*, 8: 403 – 434.

- Ellner P.D. (1956). A medium promoting rapid quantitative sporulation in *Clostridium perfringens*. *Journal Bacteriology* **71**, 495-496.
- Etodo, A.E. (1991). Association of *Clostridium perfringens* type D Epsilon Toxin with sudden death of sheep in and around Vom, Nigeria ISR. *Journal Veterinary Medicine* **46**: 51 – 53.
- Euzeby J. P. Le Latin dans la nomenclature bacterienne. Society of systemic and Veterinary bacteriology.<http://www.bacterio.cict.fr>. Last update: nov.2003.
- Fernandez, P.S. and Peck M.W.(1999). A predictive model that describes the effect of prolonged heating at 70 °C to 90 °C and subsequently at refrigeration temperature on growth from spores and toxigenesis by nonproteolytic *Clostridium botulinum* in the presence lysosome.*Applied Environmental Microbiology* **56**, 3449-3457.
- Felix, M and Thomas, D .F (1982) .The family Bacillaceae Medical. Microbiology Pp389-392.
- Fuller, .R. (1992). Probiotics-The scientific basis, Chapman and hall. London 34-335
- Ganum, P, E and Brynsted, S. (1999). Bacterial toxin as food poisons.In: The Comprehensive sourcebook of Bacterial protein toxin. (Alouf J.E and Freer J.H, Eds) Academic press, London. pp669-681,
- George,W.L and S.M.Fingold (1985) Clostridia in human gastrointestinal flora. In S.P Borrielo(ed), Clostridia in gastrointestinal disease.C.R.C Press. Boca Raaton fla Pp1-37
- Gould, G.W. (2003) Microbiological safety of new food preservation technologies. *Acta Aliment.* **32**, 215-217.
- Gould G.W and Hitchins A.D. (1963).Sensitization of spore to lysozyme and hydrogen peroxide with agents which rupture disulphide bonds. *Journal of General Microbiology*.**33**, 413-442.
- Gould, G.W. (1969). Germination. In: *The Bacterial spore*. (Gould G.W. and HursA., Eds), Academic Pres, London.pp 397-444,
- Gould G.W. (2003). Microbiological safety of new food preservation technologies. *Acta Aliment.* **32**, 215-217.
- Gould G.W. and Hitchins A.D. (1963) Sensitization of spores to lysozyme and hydrogen peroxide with agents which rupture disulphide bonds. *Journal General Microbiology*. **33**, 413-442.

- Gree, Z.N. and Arvay, L.H. (1982). Effects of temperature on spore germination and vegetative cell growth of *Clostridium botulinum*. *Applied Environmental Microbiology*. 43:331-337.
- Guzman, A.M.S. de Terrespagano, C.E and Micalizz, B. (1988). Identification of *Clostridium chauvoei* by coagulation test. *Revista Latinoamericana de microbiologia* 30: 73 -77.
- Haagma, J (1979). Clostridial Disease in Europe. In “CRC Handbook series in Zoonosis” Vol. 1 Editor J.H. Steele pp 225 – 276 CRC Press. Inc Boca Ration Florida.
- Hathway, C.L (1990). Toxigenic *Clostridia*. *Clinical microbiology Rev.* 3: 66-98
- Herreros, J. L, C. Montecucco, G. Schiarro. (1999), pathophysiological Properties Of Clostridia neurotoxin in the comprehensive source book of Properties of Clostridia neurotoxins. In: E, Higbe. Change in Clinical values cattle infected with *Clostridium Chauvoei* preliminary report 11 clinical Relationship during infection. *Journa Veterinary Research*. 35:1037-1044.
- Heredia, N.L. Labbe. R.G, Rodriguez, M.A, and Garcia- Alvarado. J.S (1991). Growths, sporulation and enterotoxin production by *Clostridium. perfringens* type A in the presence of human bile salt. *FEMS microbial.Lett.* 84, 15-22.
- Hirsch, A and E. Grinsted (1954). Method for growth and enumeration of anaerobic spore formers from cheese, with observation on effects of nisin. *Journal of Dairy Research*. 21; 101-110.
- Hirsh, D.C. (2000) Special considerations. Selected Bacterial Infections. Anaerobes. In: *Antimicrobial Therapy in Veterinary Medicine* (Prescott J.F., Baggot J.D. and Walker R.D., Eds), pp 458-461, Iowa State University Press, Ames, Iowa, USA.
- Hong, Y., Ohishi, K., Inoue, N., Kang, J.Y., Shime, H., Horiguchi, Y., van der Goot, F.G., Sugimoto, N., Kinoshita, T. (2002). Requirement of N-glycan on GPI anchored proteins for efficient binding of aerolysin but not *Clostridium septicum* alpha-toxin. *EMBO JOURNAL*. 21, 5047-5056.
- Holland, D Barka, A.N. and wolf, J. (1970). The effect of carbon dioxide on spore Germination in some *Clostridia*. *Journal Applied .Bacteriology*. 33, 274-284.
- Hunter, S.E.C., Brown, J.E., Oyston, P.C.F., Sakurai, J. and Titball ,R.W. (1993) Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma-toxin and leukocidin of *Staphylococcus aureus*. *Infectious Immunology*. 61, 3958-3965.

- Itodo, A.E, Adekeye A.A, Umoh J.U (1987). Isolation of *Clostridium perfringens* from sheep and padock soil in Kaduna and Kano States of Nigeria. *Tropical Veterinary*. 5: 93-96
- Itodo, A.E. and Ike, .R.O. (1990). Enterotoxaemia in sheep and cattle in Jos Plateau, Nigeria. *Topical Veterinary*. 8:120-122.
- Jain, U.C, Tanwani S, K and Moghe, M.N (1990). Studies on characterisation of Hemolysin produced by *Clostridium chauvoei* Indian Veterinary Journal 67.97-102.
- Jayaraman, M.S, R Lali, M .Dhanda (1962). Toxin Production by *Clostridium Chauvoei*. *Indian Veterinary journal* 39; 481-484
- Johnstone, K. (1994) .The triggers mechanism for spore germination-Current concept. *Journals of Applied Bacteriology* 76, S17-S24.
- Johnson, E.A (1999). Clostridia toxins as therapeutic agents; benefit of nature's Most toxic proteins. *Annual Review Microbiology*. 55: 551- 575.
- Jones D.T. and Woods D.R. (1986) Acetone-butanol fermentation revisited. *Microbiology Review*. **50**, 484-524.
- Jubb, K.V.F., Kenedy, P.C. and Palmer, N. (1985). Pathology of domestic animals, 3<sup>rd</sup> edition vol1, Academic Press Inc. London. Pp85 – 89
- Jubb, J.V.F. and Kennedy P.C.(1970). Pathology of domestic animals II. Academic prss, London. 1<sup>st</sup> edition. pp 465-472
- Just, I, Gerhard, R Genth, H, Hoffmann, F. (2003). Mode of action of toxin A and Toxin B from *Clostridium difficile*. In; Proceeding of the meeting of the European concerted action QLK2-CT 2001-01267. pp 52-61
- Kerrin, J.C. (1934). A method of producing high yield of exotoxin of *Clostridium chauvoei*. *Journal of Pathology and, Bacteriology*. 38; 219
- Kerry, J.B. (1964). A note on the occurrence of *Clostridium chauvoei* in the spleens and livers of normal cattle. *Veterinary Record* 76; 396
- Kimagasa H., Takao K., Fukumoto K and Ishihara M. (1992) Changes in tea Components during processing and preservation of tea extracts by hydrostatic pressure sterilisation. *Nippon Nog. Kaichi*. **66**, 707-712.
- Jubb, J.V.F, and Kennedy P.C (1970). Pathology of domestic animals II. Academic Press, London. 1<sup>st</sup> edition pp149-154.

- Kim, K.H, Fekety, R.F Batts, D.H Brown, D Cudmore, M Silva, J, and waters, D (1981). Isolation of *Clostridium difficile* from the environments and contacts of patients with antibiotic associated colitis. *Journal Infectious Disease*. 143:42-44
- Kojima, A Amimoto, K Ohgitani, T Tamura, Y (1999). Characterisation of flagellin from *Clostridium chauvoei*. *Veterinary Microbiology* .30; 67, 3;231- 237
- Kowalski ,E., Ludwig H. and Tauche B. (1992) Hydrostatic pressure to sterilise Foods 1. Application to pepper (*Piper nigrum*). *Deutsche Lebens. Runds.* **88**, 74-75.
- Kuhnert, P.M; Krampe, S.E Capaul, J. Frey and J. Nicolet (1997). Identification of *Cl. Chauvoei* in culture and clinical material from blackleg using PCR. *Veterinary Microbioloy* 51: 291 – 298.
- Kuhnert, P Capaul, S, Nicolet, J Frey J (1996.). Phylogenitic position of *Clostridium Chauvoei* and *Clostridium Septicum* based on rRNA gene Sequence. *Inernaional Journal of Systemi. Bacteriology*. 46, 1179 – 1176.
- Lawrence, G.W. and Cook, R. (1980) Experminetal Piglet: The Production and pathology of necrotizing enteritis due to *Clostridium welchi* type C in the Guniea pig. *British Journal of Experimental Pathology*,6:261 – 271.
- Leary, S.E.C. and Titball, R.W. (1997). The *Clostridium perfringens* b-toxin. In : The Clostridia: Molecular Biology and Pathogenesis. (Rood J.I., McClane B.A., Songer J.G. and Titball R.W., Eds.) p 243-250, Academic Press, London.
- Lopez, J.M., Dromerick, A. and Freese, E. (1981). Response of guanosine 5' - Triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. *Journal of Bacteriology*. **177**, 605-613
- Louis D. S and Geoffrey, H (1975). Family Bacillaceae. Bergey's Manual of Determinative Bacteriology. Eight Editions. Pp 563-566.
- Manso, J.H. (1936). Toxin Production by *Clostridium chauvoei* on dorstepoort *Journal Veterinary Science. and Animal Industry*. Vol 7.432 – 482.
- Mauriello, J.A, Coniarris, H. And Haupt, E.J. (1987). Use of *botulinum* toxin in the treatment of one hundred patients with facial dyskinesias. *Ophthalmology* 944,976-979
- Mason J .H (1936). Toxin production by *Clostridium chauvoei*. *Onderstepoort Journal Veterinary Science, and Animal, Industry*. 7,432-482

- Mboma, J.R.L (1985). Observation on the influence of media composition on the efficacy of vaccine derived from some *Clostridium chauvoei* strain. *Bulletin of Animal Health and Production in Africa*. 33: 117-121.
- Menestrina, G., Serra, M.D., Prevost, G (2001). Mode of action of beta-barrel poreforming toxins of the staphylococcal alpha-hemolysin family. *Toxicon* **39**: 1661-1672.
- Menzio M.G The pathogenesis of most clostridial infections and disease follow the Toxin genes and MLSB resistance determinants on *Clostridium difficile* clinical isolates. Proceedings of Meeting 3 of the European Concerted Action QLK2-CT2001-01267 (Duchesnes C., Mainil J., Pelkonen S. Menozzi M.G., Eds), p 91, Presses Fac. Méd. Vét. Ulg, Liège, Belgium.
- Merchant, I.A. and Barner (1964). An outline of the Infection diseases of domestic animals. Iowa State University Press, 1<sup>st</sup> edition pp22 – 26.
- Melone, F.E. Mcparland, P.J. and Ohagan, J. (1986). Pathological changes in the pericardium and meninges of cattle associated with *Clostridium chauvoei*. *The Veterinary Record* 118: 151-152.
- Moussa, R.S. (1958). Complexity of toxin from *Clostridium chauvoei* and *Clostridium septicum*. *Journal of Bacteriology*, 76; 38-45).
- Moussa, R. S. (1959) `Antigenic Formulae for *Clostridium Septicum* and *Clostridium Chauvoei*. *Journal Pathology and Bacteriology*. 7: 341 – 350.
- Mulligan, M.E, L.R Perterson, R.Y.Y. Kwok, C.R Clabots, and D.N Gerding (1988). Immunoblats and plasmid, finger prints compared with serotyping and polycrylamide gel Electrophoresis for typing *Clostridium difficili*. *Journal Clinical Microbiology* 26; 41-46
- Murrell W.G. and Wills P.A. (1977) Initiation of *Bacillus* spore germination by Hydrostatic pressure: effect of temperature. *Journal Bacteriology*. **129**, 1272-1280
- Moncrief, J.S, Lyerly, D.M, Wilkins T.D (1997). Molecular biology of the *Clostridium deficile* toxin In: *The Clostridia: Molecular biology and pathogenesis* .Accademic press London. pp369392
- O'Neill G.L., Ogunsola F.T., Brazier J.S. and Duerden B.I. (1996) Modification of a PCR ribotyping method for application as a routine typing scheme For *Clostridium difficile*. *Anaerobe* **2**, 205-209.

- Oladele, S.B, Abdu, P.A, Nok, A.J, Esievo, K. A. N And Useh, N. M. (2002). Effects of some inhibitors on Neuraminidase of Newcastle disease virus113 strain .*Veterinarski Arhiv*, 72:185-194.
- Osiyemi, T. I. O (1975). The aetiology and data on seasonal incidence of clinical blackquarter disease in Nigerian cattle. *Bulletin of Animal Health and Production in Africa*.pp 23:367-370
- Oxer, D.T., Minty, D.W and Lifeman, C.E. (1967). Transmission to Lambs of maternal immunity against blackleg following the use of combined *Clostridium vaccince*. *Austrialn Veterinary Journal* 43: 25 – 28.
- Palmer, M. (2001) .The family of thiol-activated, cholesterol binding cytolysins. *Toxico,n* **39**: 1681-1689.
- Peck, M.W. (1997). *Clostridium botulinum* and the safety of refrigerated Processed foods of extended durability. *Trends Food Science. Technology*. **8**: 186-192.
- Peck, M. W Fairbairn, D. A. and Lund, B.M. (1993). Heat resistance of spores of non proteolytic *Clostridium botulinum* estimated on medium containing lysozyme. *Letter. Applied Microbiology*.16: 126-131.
- Pemberton, J.R,F Bates,R. Macheak, M Geoge, W.L and S.M Finegld (1985). Clostridia in the human gastro intestinal.In S.P Borriello (ed), clostridia in gastrointestinal disease. C.R.C, press. Boca Rato, fla.pp 1-37
- Pflug, I.J. and Gould G.W. (2000) Heat treatment. In: *The Microbiological Safety and Quality of Food*. (Lund B.M., Baird-Parker A.C. and Gould G.W., Eds), pp 36-64, Aspen Publishers Inc, Gaithersburg, MD pp 23-24.
- Plowman, J. and Peck, M. W. (2002).Use of novel method to characterize the Response of spore of non proteolytic *Clostridium botulinum* types B E and F.to a wide range of germinants and conditions. *Journal Applied Microbiology*. 92: 681-694.
- Popoff, M. R., Chaves-Olarte E., Lemichez E., Von Eichel-Streiber C., Thelestam M., Chardin P., Cussac D., Antony B., Chavrier P., Flatau G., Giry M., de Gunzburg J. and Boquet P. (1996) Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *Journal Bioogical Chemistry* **271**, 10217-10224.
- Prazmowski A. (1880) .Untersuchung uber die Entwicklungsgeschichte und Fermentwirking einiger Bacterien-Arten. Inaug. Diss. Hugo voigt, Leipzig

- Premkumar, .D, Andrew, C and Chandraseharan, R. (1993). pH changes of BlacklQuarter Vaccine on cold storage. *Indian veerinaryt journal* pp283-284.
- Princewill, T.J. and Agba, M.I. (1982). Examination of bovine feaces for the isolation and identification of *Clostridium species* *Journal Bacteriology*. 52:97 – 102.
- Princewill, T. J. T(1965) .Effects of calcium chloride on germination and Pathogenicity of spores of *Clostridium chauvoei* *Journal of Comparative Pathology*.75: 343-351.
- Princewill, T.J., Agba, M.I., Jemitola S.O (1985). Isolation of *Clostridium species* from herbage *Microbies*. 44: 271 – 279
- Quinn, C.P and Minton, N.P (2001). Clostridia neurotoxins. In: Clostridia: Biotechnology and medical applications. (Bahl H. and Durre P. Eds) PP 211-251.
- Ragkousi, K Eichenberger, P, Van Ooij, C, and Setlow P, (2003). Identification of new gene essential for germination of *Bacillus subtilis* spores with ca+ dipicolinate. *Journal Bacteriology*.185, 231-2329.
- Ramaroa, D. and Rao, B.U (1990). Studies on the incidence of blackleg in Karantaka during 1979 – 1985. *Indian Veterinary. Journal* 67, 795 – 801.
- Ramachandran, S. (1969).Studies on the characterization of haemolysin roduced by *Clostridium chauvoei*. *Indian Veerinaryt Journal* 46 754.
- Reddy, N.R., Solomon, H.M., Tetzloff, R.C. and Rhodehame, I E.J. (2003). Inactivation of *Clostridium botulinum* Type A spores by high-pressure processing at elevated temperatures. *Journal Food Proectiont*. 66, 1402-1407.
- Rode, L. J. and Foster, J. W. (1961). Germination of bacterial spores with alkyl Primary amines. *Journal Bacterioogyl*. 81,768-779.
- Rossetto, O, M.Seveso, P. caaccin, G .Schiavo,C. Monruteuccio(2001). Tetanus and *botulinum* neurotoxins: turning bad guys into good by Research. *Toxicon* 39, 27-41
- Rossjohn, J., Feil, S.C., McKinstry, W.J, Tweten, R.K and Parker, M.W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a Model of its membrane form. *Cell* 89, 685-692.
- Rovere, P. (1996). Prove di sterizzazione a 15000 bar per ottenere la stabilata Microbiologica Ed enzimatica. *Industry Aliment*. 35, 1062-1065.

- Rovere, P., Gola, S., Maggi, A., Scaramuzza, N. and Miglioli, L. (1998) Studies on bacterial spores by combined high pressure-heat treatments: possibility to sterilise low acid food. In: *High Pressure Food Science, Bioscience and Chemistry*. Royal Society of Chemistry Cambridge, pp 354-363
- Roux, E Yersin (1888). Contribution a l etude de la diphterie. *Animal.Institute Pasteur*, 2 629-661
- Schalch, B., Bader, L., Schau, H.P., Bergmann, R., Rometsch, A., Maydl ,G. and Kessler, S. (2003). Molecular typing of *Clostridium perfringens* from a food-borne disease outbreak in a nursing home: Ribotyping versus pulsed-field gel electrophoresis. *Journal Clinical Microbiology*. **41**, 892-895.
- Shatursky, O. Bayles, R. Rogers, M. Jost, B.H. Songer J.G Teten R.K (2000). *Clostridium perfringens* beta toxin forms potential dependent cation selective channel in Lipid Bilayer. *Infection Immunology* 68: 5548-5551.
- Schultz, R. D.(1994). Certain factors to consider when designing a bovine vaccination program. *Bovine Proceedings*, No.26 pp12-13.
- Scott A.B. (1981).*Botulinum* toxin injection of eye muscles to correct strabismus. *Trans Animal Ophtalmology. Soc.* 79,734-770.
- Scot A.B; Kennedy, K and H.A. Stubbs (1985) *botulinum* toxin injection as a Treatment for blepharospasm. *Archo Ophtalmology*. 103, 347-350.
- Scott, A.B, Rosenbaum, A, and.Collins, C (1973). Pharmacologic weakening of Extraocular muscles. *Invest ophtalmology*. 12. 924-927.
- Sebald, M and Ionesco, H. (1972). Germination Izp-dependante des spores de *Clostridium botulinum* type E. C. R. Acad. Sci. Paris (ser.D) 275, 2175-2177.
- Setlow, B., Cowan, A.E. and Setlow, P. (2003). Germination of spores of *Bacillus Subtilis* with dodecylamine. *Journal Applied Microbiology*. **95**, 637-648.
- Singh, K.P, N.S. Parihar, and B.N Tripathi (1992). Pathology and Pathogenesis of *Clostridium chauvoei* infection in guinea pigs. *Indian Journal of Animal Sciences* 62: 7, 611 - 615
- Singh, K.P, Parihar N.S Charan, K Tripathi, B .N.(1993). Haematological and Biochemical alterations in hill bulls infected with *Clostridium chauvoei*. *Acta Veterinary Ophtalmology*, 62, 89-94
- Skinner, G.E., Gendel, S.M., Fingerhutl, G.A., Solomon, H.A. and Ulaszek, J. (2000). Differentiation between types and strains of *Clostridium botulinum* by ribotyping. *Journal Food Protection*. **63**, 1347-1352

- Smith, A B (1975) Toxin production by *Clostridium chauvoe*. *Ondersteport Veterinary Science Animal Industry*, 6, 431 – 432.
- Sonenshein, A.L. (2000). Bacterial sporulation: a response to environmental Signals. In: *Bacterial Stress Responses*. (Storz G. and Hengge-Aronis R., Eds), pp 199-215, ASM Press, Washington.
- Songer, J.G. (1996) Clostridial enteric diseases of domestic animals. *Clinical Microbiology Rev.* **9**, 216-234.
- Stackbrandt, E. (1997). Phylogenic relationship. In: *The Clostridia: Molecular biology and pathogenesis* (Rood J.I., McSonger, B.A., Clane B.A., Songer J.G., Tiball, R.W: eds). Academic Press London, U.K pp3-20.
- Stackbrandt, E. (2002) Classification of Clostridia. In: Classification, molecular genetics and pathology of clostridia. Scientific booklet 1 of the European concerted action QLK2-CT2001-01267 (Mainil J, Duchesnes C., Frey J, and Stackebrandt E, Eds ) pp 8-16, presses de la faculte de medecine veterinaire de l universite de liege.
- Steven, D.L and Bryant, A.E. (1993). Role of Q. toxin Sulphydsyl-activated Cytolysin in the pathogenesis of clostridial gas gangrene. *Clinical Infection Disease*. 16 s195-s199.
- Stumbo. C.R. (1973) *Thermobacteriology in Food Processing*, 2nd ed. Academic Press Inc, New York, pp 2-4
- Suleiman, H. (1988). Policy issues on pastoral development. In: *Pastoralism in Nigeria: past, present, and future*. Proceeding National Conference Pastoral. Lagos, Nigeria, pp23-24
- Tamura, Y and Tanaka, S. (1984). Effects of antflagella serum in the protection of mice against *Clostridium Chauvoei* Infection and immunity. 43: 2, 612 – 616.
- Tano Lazare. *Manual of main Ruminant Diseases*. Technical responsible. Merial With collaboration of Dr Albert Doufissa Younde, Cameroun pp17-18
- Tiball, R.W (1999). Membrane damaging and cytotoxic phospholipasis in; the comprehensive source book of bacterial protein toxin. Academic press, London, 310-329
- Tsai J.K.C (1996) *botulinum* toxins as therapeutic agent. *Pharmacology Therapeutics*. 72,13-24.
- Tseng, W.J and Labbe, R.G. (2000). Charactrestic of sporulation stimulating factor from *Clostridium perfringens* type A. *Leter. Applied Microbiology* .30, 254-257.

- Tsui, J.K.C (1996).Boyulinum toxin as a therapeutic agent *Pharmacol.Therapeutic*. 72: 13-24.
- Uehara, M. and Frank, H.A. (1965). Factors affecting alanine-induced germination of clostridial spores. In: *Spores*. 3rd ed. (Campbell L.L. and Halvorson H.O., Eds), pp 38-46, ASM, Ann Arbor
- Useh, N M, Ibrahim, N .D .G, Nok, A J and Esievo K.A.N (2006) Relationship between annual rainfall and outbreaks of blackleg in Zaria, Nigeria. *Veterinary Record*, 158: 100-101
- Useh N.M (2001) an Investigative study on the production of neuraminidase (sialidase) by *Clostridium chauvoei* (Jakari strain). M.Sc Thesis, Ahmadu Bello University, Zaria, Nigeria, pp 224.
- Useh N.M, Nok A .J, Esievo K .A (2003). Pathogenesis and pathology of blackleg in ruminants; the role of toxins and neuraminidase. *Veterinary Quarterly* 25:155-159
- Useh, N.M, Nok, A.J, Ambali, S.F and Esievo, K.A.N (2004). The inhibition of *Clostridium chauvoei* (Jakari strain), neuraminidase activity by methanolice extracts of the stem barks of Tarmarindus indicus and *Combretum fragrans*. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 19 339-342.
- Waites, W. M and Wyatt, L.R. (1974). The effect of pH, germinants and temperature on the germination of spores of *Clostridium bifermentans*. *Journal General Microbiology*. 80,253-258.
- Wills, P.A. (1974). Effects of hydrostatic pressure and ionising radiation on Bacterial spores. *Atomic Energy Ausata*. 17, 2-4.
- William, B.M (1977). *Clostridial myositis* in cattle. *Bacteriology and Gross pathology. Veterinary Record* 100, 90 – 91.
- Wilson, G.S. and Miles, A.A (1975).*Toplay and Wilson's Principles of Bacteriology, Virology, immunity .Edward Arnold London. Vol. 2. Six in Edition* pp 2268 – 2277.
- Wrigley, D.M, Hanwella, H.D.S.H and Thon, B.L (1995). Acid exposure enhances sporulation of certain strains of *Clostridium perfringen*. *Anaerobe1*: 263-267.
- Xexones, H. and Hutchings, I.J. (1965).Thermal resistance of *Clostridium Botulinum* (62A) spores as affected by fundamental food constituents. *Food Technology*. 19, 1003-1005.